FUNCTIONAL CHARACTERIZATION OF NOVEL THIOREDOXIN REDUCTASE AND THIOREDOXIN PEROXIDASE IN *DROSOPHILA*

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ABSTRACT

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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). In mammals, intracellular defense against ROS includes superoxide dismutase (Sod), catalase (Cat) and thiol-dependent redox systems, in which glutathione reductase (GR), thioredoxin reductase (TrxR) and the corresponding peroxidases are key enzymes. Mammalian TrxRs and glutathione peroxidases (GPxs) are selenium-containing enzymes. The fruit fly *Drosophila* possesses most ROS-detoxifying enzymes reported for mammals with the possible exception of GR and GPx activities. This thesis presents an investigation of key *Drosophila* antioxidant genes encoding TrxR-1 and thioredoxin peroxidase (TPx). I show that a single *Drosophila* gene, termed *Trxr-1*, specifies cytoplasmic and mitochondrial non-selenocysteine-containing TrxRs that arise by alternative splicing. I generated transcript-specific mutants and used *in vivo* approaches to explore the biological activities of the two splicing variants by introducing the respective individual transgenes into *Trxr-1* mutant flies. The results show that although the two respective TrxRs have similar biochemical properties, they cannot substitute for each other, *in vivo*. *Trxr-1* null

mutations result in larval death, whereas mutations causing reduced TrxR-1 activity reduce pupal eclosion and cause a severe shortening of the adult lifespan. I also provide genetic evidence for a functional interaction between TrxR-1, Sod1 and Cat, suggesting that the overall burden of ROS metabolism in *Drosophila* is shared by the two defense systems. Finally, I report the *in silico* identification of two non-selenium containing GPx-like genes in the *Drosophila* genome and present an initial biochemical characterization of one of the two gene products. The results show that one ofthe GPx-like genes encodes a TPx rather than a GPx. Transgene-dependent overexpression of the TPx gene increases the resistance of individuals to experimentally-induced oxidative stress, but does not compensate for the loss of Cat, an enzyme which, like TPx, functions to eliminate intracellular hydrogen peroxide. Furthermore, transgene-derived overexpression of TPx in mutant flies lacking Sod1, an antioxidant enzyme which protects cells from superoxide radical toxicity, is detrimental. This contrasts to transgene-derived overexpression of Cat which can partially rescue the *sod1* mutant. These observations indicate that TPx1 and Cat function in metabolically distinct pathways.

To my family

and to the Greek Community and Greek Student Association of Göttingen

PREFACE

This thesis focuses on the biochemical and genetic characterization of new antioxidant defense systems of the fruit fly, *Drosophila melanogaster*, and on how the different antioxidant systems interact *in vivo*. The thesis consists of an ABSTRACT which summarizes the entire work; this PREFACE describing the organization of the thesis; a general INTRODUCTION which develops the context and rationale of the work; three independent MANUSCRIPTS which either have been or soon will be published; a SUMMARY of the overall conclusions of the thesis; a separate list of REFERENCES cited in the INTRODUCTION; and an APPENDIX describing additional data relevant to the thesis.

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LIST OF ABREVIATIONS

Cat – Catalase

BCIP – 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt

BDGB – Berkley *Drosophila* Genome Progect

DTNB – 5,5'-dithiobis(2-nitrobenzoate)

EST – Expressed Sequence Tag

Grx – Glutaredoxin

GSSG – oxidized glutathione

GSH – reduced glutathione

GPx – Glutathione Peroxidase

GR – Glutathione Reductase

H_2O_2 – Hydrogen peroxide

NADPH – reduced nicotinamide adenine dinucleotide phosphate

NBT – nitroblue tetrazolium chloride

ROS – Reactive Oxygen Species

Sod – Superoxide dismutase

TBOOH – tert-butyl hydroperoxide

Trx – Thioredoxin

TPx – Thioredoxin Peroxidase

TrxR – Thioredoxin Reductase

INTRODUCTION

Oxidative stress arising from aerobic metabolism

Oxygen metabolism is essential for aerobic life, but concurrently imposes a potential threat to cells arising from the formation of cytotoxic byproducts, referred to as reactive oxygen species (ROS) (Raha and Robinson, 2000). Normal cellular homeostasis is a delicate balance between the accumulation of oxidants and their elimination by defense systems (Yu, 1994). Overproduction of oxidants due to limited defense is refered to as oxidative stress (Halliwell and Gutteridge, 1999).

Primary ROS include the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH). The numerous secondary and tertiary products, such as alkyl hydroperoxides and lipid peroxides, are generated by the interaction of primary ROS with cellular targets (Girotti, 1998). ROS can modify proteins, inactivate enzymes, initiate chain reactions that peroxidize lipids, and damage DNA (Stadtman and Levine, 2000; Termini, 2000).

Oxidative stress can result from diminished antioxidants, mutations affecting antioxidant defense enzymes, depletion of dietary antioxidants or by increased production of ROS as a result of an excessive activation of natural ROS-producing systems, such as xanthine and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Halliwell and Gutteridge, 1999). Transient exposure to high levels of oxygen, redox cycling of cellular

components such as quinones, flavins, thiols, haem proteins, transition metals and the action of xenobiotics such as menadione, paraquat and phorbol esters also lead to oxidative stress (Halliwell and Gutteridge, 1999). Some cells exposed to ROS can undergo apoptosis (Clutton, 1997).

Oxidative damage is implicated in a wide variety of human disorders, including Alzheimer disease (Christen, 2000), Parkinson disease (Zhang et al., 2000), ischemic heart disease (Chandra et al., 1994) and many mitochondrial diseases (Beal, 1998). Moreover, the pathogenic role of ROS has been strongly implicated in familial amyotrophic lateral sclerosis (FALS) (Rosen et al., 1993) and Friedreich ataxia (Rotig et al., 1997). Additionally, ROS contribute to the pathology of atherosclerosis (Parthasarathy et al., 1992), hypertension (Nakazono et al., 1991), inflammatory diseases (Halliwell, 1995) and cancer (Cerutti, 1994). Oxidative stress is also associated with radiation induced damage (Von Sonntag, 1987), smoking (Piperakis et al., 1998) and alcoholism (Zima et al., 2001). Although most of these human disorders are probably not initiated by ROS, understanding the role of oxidative stress in each of the pathologies may lead to better treatment strategies.

In addition, oxidative stress is tightly linked with the normal aging process (Sohal and Weindruch, 1996). The free radical theory (otherwise know as the oxidative stress theory) of aging postulates that oxygen radicals generated in metabolic pathways damage cells and increase their vulnerability to death and that the incessant accumulation of structural damage which disrupts functions at a macromolecular level is the underlying cause of aging (Harman, 1993). Even though the idea that ROS are the primary cause of aging has been challenged (Le

Bourg, 2001), it is generally accepted that the aging process leaves clear footprints of oxidative stress, such as oxidative DNA modifications, an increase in the protein carbonyl content and accumulation of age pigments (Berlett and Stadtman, 1997; Hamilton et al., 2001). The links between ROS biology and the physiology of aging are still under intense investigation.

Antioxidant defense systems

To protect themselves against ROS toxicity, cells have developed different antioxidant systems. Among these are low molecular weight antioxidant molecules, such as α-tocopherol, ascorbic acid, uric acid and glutathione (GSH) as well as antioxidant enzymes such as superoxide dismutase (Sod), catalase (Cat), glutathione peroxidase (GPx) and glutathione reductase (GR) (Michiels et al., 1994). In addition, the thioredoxin (Trx) system has recently been described as an important cellular antioxidant defense (Carmel-Harel and Storz, 2000; Mustacich and Powis, 2000).

Sods are metalloproteins that dismutate the $O2^{\bullet -}$ to H_2O_2 and molecular oxygen. Three types of Sods are found in eukaryotic cells; Cu/Zn superoxide dismutase (Sod1) is predominantly located in the cytosolic fractions, Mn superoxide dismutase (Sod2) in the mitochondria and extracellular superoxide dismutase (Sod3) in the extracellular space (Beyer et al., 1991). Cat is a heme protein located predominantly in peroxisomes. It catalyzes the conversion of H_2O_2 to H_2O (Chance et al., 1979). GPx also catalyzes the GSH-dependent reduction of H_2O_2 to H_2O. In addition to the reduction of H_2O_2, GPx can catalyze the reduction of organic hydroperoxides, thus limiting oxidative damage, particularly lipid peroxidation, to tissues.

Glutathione peroxidases comprise a family of enzymes that may contain a selenocysteine or a cysteine residue in their active center, as is true for the mammalian homologs or their invertebrate and plant counterparts, respectively (Ursini et al., 1995).

GR reduces oxidized glutathione to its reduced state, using NADPH (Carlberg and Mannervik, 1975). This reaction is necessary for maintaining a proper cellular redox environment (Schafer and Buettner, 2001) through the recycling of GSSG to GSH. It is also necessary for GPx to function and it provides a link between oxidative challenge and central metabolism, because NADPH is regenerated by glucose 6-phosphate dehydrogenase, an enzyme catalyzing the first step in the oxidative branch of the pentose phosphate pathway (Stryer, 1995).

The Trx system includes Trxs, a family of small thiol proteins of approximately 12 kDa which reduce oxidized cysteine groups on proteins. In addition this system includes thioredoxin reductases (TrxRs) which catalyze the GSH-dependent recycling of oxidized Trx. At the molecular level, the TrxR genes are homologs of GR genes and their gene products reduce oxidized Trxs through an NADPH-dependent mechanism. Thioredoxin peroxidases (TPxs), which catalyze the reduction of H_2O_2 and organic hydroperoxides using Trx as the electron donor, are also components of the Trx system (Miranda-Vizuete et al., 2000; Williams et al., 2000). Collectively, these enzymatic systems are required to remove ROS produced in cells. Their functional necessity is discussed below.

***In vivo* functions of antioxidant enzymes**

In the past, the cellular function of antioxidant enzymes has been studied in a variety of organisms and cell culture systems (Michiels et al., 1994). More recently, knock-out technology has been applied in mice for deleting the genes encoding GPx1, Sod1, Sod2 and Sod3 (Carlsson et al., 1995; Ho et al., 1997; Lebovitz et al., 1996; Reaume et al., 1996). In addition, transgenic mice that overexpress GPx1, GPx3, Sod1 and Sod2 have been generated (Epstein et al., 1987; Ibrahim et al., 2000; Mirault et al., 1994; Mirochnitchenko et al., 1995).

GPx1 knockout mice show increased susceptibility to the $O2^{\bullet -}$ generating reagent paraquat (Bus et al., 1974), H2O2 and to neurotoxins (de Haan et al., 1998). Contrary to predictions, however, such mice are viable and show no increased sensitivity to hyperoxia (Ho et al., 1997). Furthermore, GPx1 knockout mice are susceptible to myocardial ischemia injury (Yoshida et al., 1997) and show increased brain dopamine loss after infusion of malonate (Klivenyi et al., 2000). On the other hand, transgenic mice overexpressing GPx1 are not only resistant to paraquat toxicity (Cheng et al., 1998), but are also protected against focal cerebral ischemia/reperfusion (Weisbrot-Lefkowitz et al., 1998) and show improved recovery of synaptic transmission after hypoxia (Furling et al., 2000). However, they show a thermosensitive phenotype due to impaired heat shock protein 70 inducibility (Mirochnitchenko et al., 1995), develop skin tumors (Lu et al., 1997) and are more susceptible to acetaminophen toxicity (Mirochnitchenko et al., 1999). These results indicate that although GPx1 is not necessary for viability, it plays an important role in the antioxidant defense system of mice.

Analysis of Sod knock-outs lead to similar observations. Sod1 and Sod3 lack-of-function mutations do not impair viability, but the survival of such mice is diminished under different types of stress (Carlsson et al., 1995; Reaume et al., 1996). In contrast, the phenotype of the mitochondrial Sod2 lack-of-function mutant is more severe and includes perinatal death and mitochondrial injury (Li et al., 1995; Melov et al., 1999). However, other aspects of the phenotype such as neurodegeneration, myocardial injury and most importantly the onset of lethality are dependent on the genetic background of the mutant strain (Lebovitz et al., 1996). Overexpression of Sod1 does not rescue the Sod2 lack-of-function phenotype (Copin et al., 2000). Taken together, these findings suggest that the antioxidant defense systems of mammals are important health factors and that an impairment of any component of these systems has a negative impact on the individual. Furthermore, antioxidant defenses cooperate in complex ways and show redundant as well as specific functions, which have recently started to be explored in *in vivo* models.

Drosophila as a model system to study oxidative stress

Although research in mice and human cell culture lines is likely to be more directly relevant to human diseases, I have chosen to study the *in vivo* function of antioxidant defense enzymes in *Drosophila melanogaster*. The reasons for this choice are manyfold. *Drosophila* genetics is widely used and offers a variety of unique experimental manipulations. For example, one can use *Drosophila* to screen for mutations, assess genetic interactions between different genes and produce transgenics (Greenspan, 1997). In addition, flies have a relatively short life-cycle and lifespan (Demerec, 1994), which is an important advantage for aging research. Furthermore, *Drosophila* has a small genome, which has been recently sequenced (Adams et

al., 2000). Analysis of the *Drosophila* genome has revealed that 61% of ~ 300 identified human disease genes have *Drosophila* counterparts (Hodgkin, 2000). Most importantly, the fly has already been used successfully to study human disorder equivalents associated with oxidative stress, such as FALS (Elia et al, 1999), Parkinson disease (Feany and Bender, 2000), Alzheimer disease (Fossgreen et al., 1998; Wittmann et al., 2001), Huntington disease (Kazemi-Esfarjani and Benzer, 2000) and other neurodegenarative disorders (Fernandez-Funez et al., 2000). Finally, mutations of the *Drosophila sod1* (Parkes et al., 1998; Phillips et al., 1989) and *cat* genes (Griswold et al., 1993) have been isolated and are available for further analysis.

Previous work on oxidative stress in *Drosophila* focused mainly on testing the "free radical theory of aging" (Harman, 1993). This theory predicts that mutations in genes encoding components of the oxidative stress response should lead to a decreased lifespan and conversely, protecting cells by increasing the activity of these components should enhance the corresponding lifespan of an individual. There is in fact experimental evidence supporting these predictions. Common features of *sod1* and *cat* mutations include reduced viability, hypersensitivity to oxidative stress and shortened lifespan (Phillips et al., 1989; Griswold et al., 1993; Parkes et al., 1998). Conversely, flies bearing a Sod1-transgene rescue *sod1* mutants and show a moderate increase (10%) in mean lifespan when expressed in otherwise wildtype individuals (Parkes et al., 1998a). Furthermore, targeted expression of human SOD1 in motorneurons of *Drosophila* increases both the mean and maximum lifespan of the corresponding individuals by over 30% (Parkes et al., 1998b). Finally, random induction of clones overexpressing this enzyme result in as much as 48% increase in the lifespan as

compared to control flies (Sun and Tower, 1999). Further support of the notion that ROS play a major role in the aging process came through a genetic screen for mutations that live longer at a high temperature. A mutation in a gene named methuselah (*mth*) that specifies a G-coupled membrane-bound receptor, was recovered (Lin et al., 1998). The mutation causes a 35% increase in normal lifespan, and enhanced resistance to oxidative stress conferred by exposure to paraquat. Moreover, experiments involving selection for delayed reproduction over many generations produced strains of long-lived line flies (Rose, 1984). Genetic factors were therefore implied to be important in lifespan determination. Interestingly, when the level of antioxidant enzymes was monitored in these flies, Sod1, Sod2 and Cat activities were elevated as compared to the control strain (Dudas and Arking, 1995; Hari et al., 1998). Moreover, the long-lived line also showed resistance to oxidative stress (Force et al., 1995).

In contrast to these observations, a recent study on antioxidant status and stress resistance in long- and short-lived lines of *Drosophila* revealed that there was no enhancement of antioxidant defenses in the long-lived lines (Mockett et al., 2001). In this study, a different set of long-lived and control lines was used; these lines were again produced through selection strategies (Luckinbill et al., 1988). In addition, transgene-dependent overexpression of Cat or TrxR has no effect on the lifespan of flies, even though the transgene-bearing flies are found to have enhanced resistance to oxidative stress (Mockett et al., 1999). Thus, an alternative hypothesis has been put forward, namely that antioxidant defenses are directed towards resistance to specific stresses and are not associated with an enhanced lifespan (Le Bourg, 2001; Mockett et al., 2001).

Early biochemical work on several insect species led to the conclusion that insects lack GPx activity (Ahmad et al., 1988; Allen et al., 1983; Smith and Shrift, 1979). This initial observation was partially reassessed and some GPx activity was subsequently reported using cumene hydroperoxide instead of H_2O_2 as a substrate (Ahmad et al., 1989). However, the activities measured were still considerably low. Based on these results, the reduction of intracellular H2O2 has been attributed solely to Cat (Orr and Sohal, 1994; Paes et al., 2001; Parkes et al., 1993). Recently, however, an extracellular GPx active toward H_2O_2 was reported from the bug *Rhodnius prolixus* (Paes and Oliveira, 1999). This observation has re-opened the question of whether insects contain GPx-activity.

Objectives of the thesis

The overall objective of this thesis is to evaluate the hypothesis that the global antioxidant defense system of vertebrates, including humans, is both molecularly and functionally conserved in invertebrates. This hypothesis anticipates that in addition to the well-described Sod1-Cat enzyme system, the GSH and Trx systems will also be found in *Drosophila melanogaster* where they function in the context of reducing oxidative stress. The hypothesis also predicts that alterations in the levels of specific components of the two thiol antioxidant systems will lead to altered sensitivity to experimentally-applied oxidants and will affect specific life history traits of the fly.

An early objective of this study was to generate experimental tools with which to test these predictions. A molecular characterization of evolutionarily conserved genes belonging to the thiol antioxidant systems was then undertaken, followed by a biochemical analysis of the

gene products. From this information I could determine whether the vertebrate and invertebrate antioxidant defense systems are molecularly conserved and whether the corresponding proteins exhibit similar enzymatic functions. In addition, I performed an *in vivo* functional analysis of the identified genes. For this purpose, mutants lacking the corresponding gene products and transgenic animals which overexpress them were generated. Phenotypic analysis of these mutant and transgenic animals was then used to determine whether vertebrates and invertebrates share functional aspects of their antioxidant defenses. The genetic system of *Drosophila* was then used to ask whether the different antioxidant systems cooperate in functional terms to protect against ROS-induced cytotoxicity and whether they can compensate for one another *in vivo*. Finally, I initiated an analysis of how alterations in levels of the different gene products affect the lifespan of the fly.

Manuscript 1

Cooperative action of antioxidant defense systems in *Drosophila*

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Abstract

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 lifespan 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 lifespan. 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.

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 bonafide GR in *Drosophila* suggest 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 analysed 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 lifespan [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]. Fig. 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 co-expressed in the germline progenitor cells, whereas *cat* is expressed in the fat bodies and the oenocytes (Fig.

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 sytems appear to be enriched in coextensive, spatially restricted patterns which 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 (Fig. 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-element caused a reversion to wildtype, 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*$^{\Delta 1}$ (Fig. 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 as first instar, 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 (Fig. 2b) or during the process of eclosion (Fig. 2c). Only about 20% eclose into normal appearing adults, the majority of which die within 2-3 days

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]. Top rows in a-c show lateral view of embryos, anterior is left and dorsal top; stages according to [28]. (a) *dmtrxr-1* expression showing that the transcript is provided maternally (stage1); zygotic expression occurs in germ cell progenitors (stage 9; arrow), the developing midgut (stages 14, 16), hindgut and proventriculous (stage 16; arrows). (b) Sod1 transcripts are provided maternally (stage 1) and continue to accummulate 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).

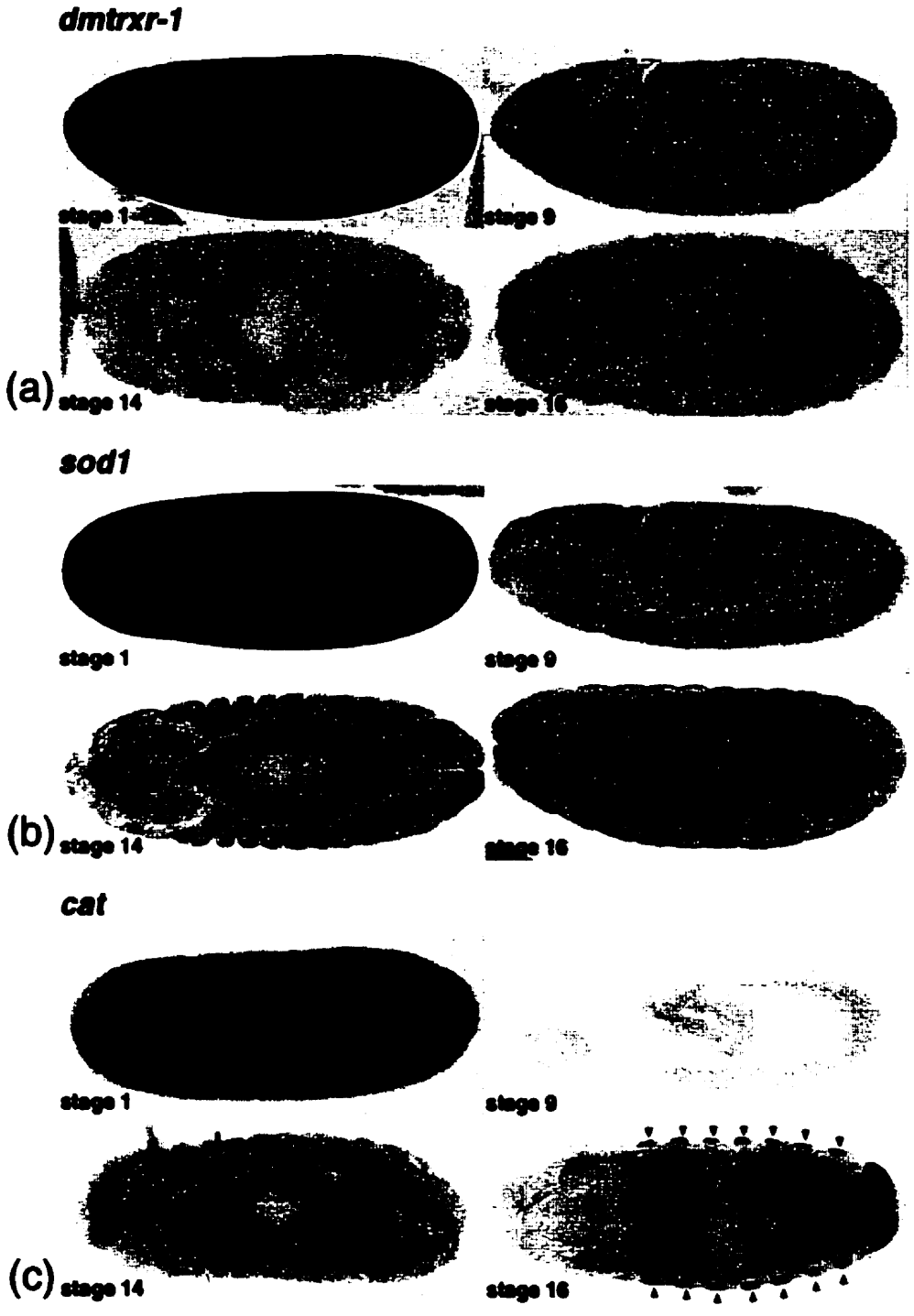

dmtrxr-1
stage 1
stage 9
(a)
stage 14
stage 16
sod1
stage 1
stage 9
(b)
stage 14
stage 16
cat
stage 1
stage 9
(c)
stage 14
stage 16

Figure 1

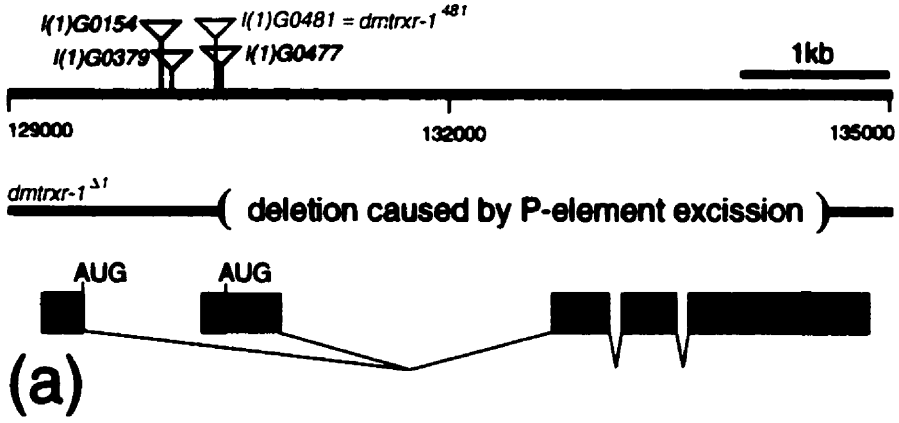
(Fig. 2d; see also below). Despite their shortened lifespan, adult mutant flies are able to mate and give progeny. No significant difference in pupal mortality and shortened adult lifespan was observed between homozygous *dmtrxr-1*^{481} females and hemizygous *dmtrxr-1*^{481} males (data not shown). 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 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 lifespan of the rescued flies is in the range of the wildtype lifespan (Fig. 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 lifespan 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 which 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 wildtype X chromosome. Table 1 shows that depending on the

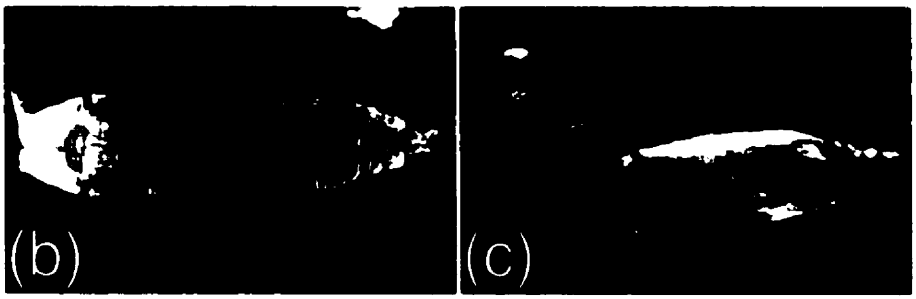
chromosomal background, the eclosion rate of hemizygous *dmtrxr-1*^{481} individuals varied between 17.2 and 25.6% as compared to *dmtrxr-1*$^{+}$ siblings. Similarly, only 27% of homozygous *sod1*^{n108} mutant individuals eclose (data not shown) and, as observed with hemizygous *dmtrxr-1*^{481} individuals, 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 normal lifespan [3]. However, only 2.4% ($\chi 2$=41.0; P<0.001; chi-squared 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% ($\chi 2$=15.2; P<0.0025; 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].

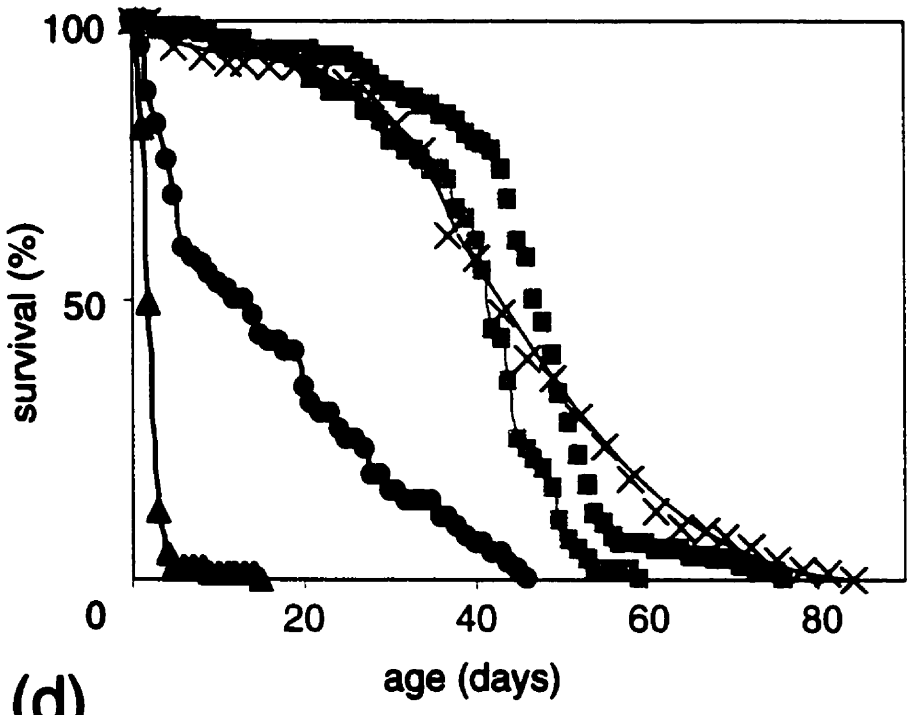
In addition to mutant analysis, we also performed gain-of-function studies to express human Sod1, previously shown to substitute for the lack of *sod1* activity in the fly [6] (own

Figure 2: The *Drosophila* gene *dmtrxr-1*, P-element insertion mutants and their effect on pupal eclosion and lifespan. (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* which 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 aminoterminal ends of the deduced protein (red and gray boxes, respectively). Remobilization of the P-elements caused reversion of the mutant phenotype to wildtype, 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) Lifespan of wildtype (red crosses) and hemizygous *dmtrxr-1*^{481} males (black triangles), hemizygous *dmtrxr-1*^{481} males ubiquitously expressing the biochemically characterized TrxR cDNA [12] (grey squares), as well as hemizygous *dmtrxr-1*^{481} males ubiquitously expressing *cat* cDNA (blue circles). Note that the lifespan of the eclosed hemizygous *dmtrxr-1*^{481} males is severely reduced (d; black triangles) and that their shortened lifespan was rescued in response to transgene-dependent TrxR (d; 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/+*).


l(1)G0154
l(1)G0379
l(1)G0481 = dmtrxr-1 481
l(1)G0477
1kb
129000
132000
135000
dmtrxr-1 Δ1
deletion caused by P-element excission
AUG
AUG

(a)


(b)
(c)


100
50
0
survival (%)
20
40
60
80
age (days)

(d)

Figure 2

observation), 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 control of the constitutively active *actin* promoter [21]. Overexpression of human Sod1 reduced the frequency of pupal eclosure from 17.2 to 2.7% ($\chi 2=12.8$; $P<0.01$; 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 wildtype 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 increased generation of 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 twofold (35.3%; $\chi 2=12.7$; $P<0.01$; 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 lifespan of the Cat-overexpressing hemizygous *dmtrxr-1* mutant flies is extended. Fig. 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 (*Δtrr1*) [27].

Table1

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

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

a: X = *white* b: X = *FM6*

Table 1: Hemizygous *dmtrxr-1*^{481} males derived from either *w*^{1},*dmtrxr-1*^{481}/*w*^{1};;***/TM3 X *w*^{1}/Y;;***/TM3 or *w*^{1},*dmtrxr-1*^{481}/*FM6*;***/*** X *y,w*^{1}/*Y;;act5C-Gal4/TM6B* parents (chromosome*** is indicated). Eclosion rate of males containing a *dmtrxr-1* wildtype 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].

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 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 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 pre-adult development and on adult lifespan.

Materials and Methods

Flystocks and generation of *Trxr-1* mutants

Flies were kept at 25°C on standard yeast-containing, cornmeal medium. In addition to the mutant and balancer chromosomes described in Lindsley and Zimm [35], *P{lacW}* insertion lines *l(1)G0477* and *l(1)G0481* of the Göttingen X chromosome collection (Peters et al., submitted) and flies containing a transposase source on the second chromosome of the genotype *w; CyO/wg^{Sp}; TM6/Sb P{ry^{+} Δ2-3}*(99B) were used. *dmtrxr-1* mutant flies were balanced with *FM7i-pAct-GFP* which allowed identification of hemizygous male mutant larvae by the absence of GFP expression.

Reversion tests and generation of null mutants for *dmtrxr-1* involved remobilisation of the *P{lacW}* element of the lines *l(1)G0477* and *l(1)G0481*.

l(1)G0477/FM7c virgin females were crossed with *FM6/Y; TM2, ry P{ry^{+} Δ2-3}*(99B)/*MKRS, Sb P{ry^{+} Δ2-3}*(99B). Female offspring with mosaic eyes were crossed with *FM6* balancer males; progeny which had lost the *P{lacW}* element were examined whether the excision event had restored wildtype function or generated a deletion corresponding to a *dmtrxr-1* null allele (see text).

Transgene construction and transformation

For construction of *UASdmtrxr-1*, *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]. For each experiment outlined in the text, results were confirmed by use of two independent transgenic lines.

Rescue experiments

Rescue experiments were performed with the *dmtrxr-1* mutants (see text) using the Gal4/UAS system [20] to drive ubiquitous expression of *UASdmtrxr-1* under the control of an *act5C-Gal4* driver [21]. Transgene-derived *dmtrxr-1* expression was monitored by *in situ* hybridization [13]. The frequency of pupal eclosion and lifespan measurements of hemizygous mutant males bearing the Gal4/UAS combination of transgenes were monitored and compared with hemizygous mutant males that contain only the *act5C-Gal4* transgene and siblings that carried the balancer chromosome *FM6* in place of the mutant X chromosome, respectively.

Lifespan measurements

Up to 10 adult males (0-24hrs old) of the genotype described in the text were kept in small food vials and transferred into fresh vials every second day. Survival of flies was monitored at 48 hr intervals. For each experiment described in Figure 2, at least 150 males were monitored.

Statistics

All genetic results presented in Table1 were compared pair-wise using chi-squared analysis as described in "Fundamentals of Biostatistics", Stanley Schor, G.P. Putnam's Sons, New York (1968). The chi-squared value for each comparison was determined by the formula $[(a*d-b*c)^2 \bullet (a+b+c+d)]/[(a+b)(c+d)(a+c)(b+d)]$. For specific calculations see text.

In situ hybridization

In situ hybridization of Dig-RNA antisense probes to whole mount preparations of embryos was performed as described in [13]. Briefly, Dig-RNA antisense probes were produced by *in vitro* transcription using the DIG RNA Labeling Kit of Boehringer (Mannheim, Germany) according to the instructions of the manufacturer. Embryos (0-22h) were collected on apple-juice containing agar plates, dechorionated in 50% sodium hypochlorite, fixed with 4% formaldehyde in PBS for 20 min, washed 4 times with PBT, and incubated overnight at 70°C with the probes in hybridization solution. The next day the embryos were washed again 4 times with PBT, incubated for 1h with preabsorbed anti-Dig antibody (purchased from Boehringer, Mannheim, Germany), followed by 4 washes and stained with AP buffer, NBT and BCIP. The embryos were dehydrated through a series of increasing ethanol concentrations, mounted on Canada Balsam (Sigma) and viewed under light microscopy.

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Manuscript 2

Alternative splicing results in mitochondrial and cytoplasmic *Drosophila* thioredoxin reductase variants with distinct cellular functions

running title: Mitochondrial and cytoplasmic TrxR-1 activities by splicing

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Abstract

Defence against oxidative stress in mammals includes the regeneration of the major thiol reductants glutathione and thioredoxin by glutathione reductase (GR) and thioredoxin reductase (TrxR), respectively. In contrast, *Drosophila*, and possibly insects in general, lacks GR and must rely solely on the TrxR system. The mammalian TrxRs described so far are selenoproteins that utilize NADPH to reduce protein as well as non-protein substrates in mitochondria and cytoplasm of cells. We show that a single *Drosophila* gene, *Trxr-1*, encodes non-selenocysteine-containing cytoplasmic and mitochondrial TrxR isoforms that arise by alternative splicing of a single primary transcript. We generated transcript-specific mutants and used *in vivo* approaches to explore the biological functions of the two splice-variants by introducing the corresponding transgenes into different *Trxr-1* mutants. The results show that although the two TrxR isoforms have similar biochemical properties, their biological functions are not interchangeable.

Key words: differential splicing / *Drosophila* thioredoxin reductase / mitochondrial enzyme / oxidative stress / transcript-specific mutations

Introduction

Molecular oxygen is key to aerobic life but is also converted into cytotoxic byproducts, collectively termed reactive oxygen species (ROS) (Halliwell and Gutteridge, 1999). In mammals, intracellular defence against ROS-induced damage includes the glutathione (GSH) and thioredoxin (Trx) redox systems (Carmel-Harel and Storz, 2000). Glutathione reductase (GR) and thioredoxin reductase (TrxR) are key enzymes which use NADPH to recycle glutathione disulfide (GSSG) and $Trx(S_2)$ to GSH and $Trx(SH)_2$, respectively (Mustacich and Powis, 2000; Williams *et al.*, 2000). GSH and $Trx(SH)_2$ act in turn as thiol-based reductants (Arner and Holmgren, 2000). GR and TrxR were found to be molecularly conserved in vertebrates, whereas the single GR homologue of *Drosophila* was shown to represent a TrxR. Thus in *Drosophila*, the Trx/TrxR system appears to shoulder the entire metabolic burden for recycling GSH (Kanzok *et al.*, 2001; Kanzok *et al.*, 2000). Recently, loss-of-function mutations of the *Drosophila* TrxR gene, *Trxr-1*, were shown to impair pupal eclosion and severely reduce adult lifespan (Missirlis *et al.*, 2001). Similarly, disruption of the thioredoxin1 gene of mice results in embryonic lethality (Matsui *et al.*, 1996). These results suggest that the Trx system is a vital component of the defences deployed against ROS-induced damage in both vertebrates and invertebrates.

In mammals, three distinct TrxR genes are responsible for maintaining Trxs in a reduced state and are capable of reducing a number of other proteins as well as non-protein redox substates in cells (Sun *et al.*, 1999). Although their activity, in particular the cytosolic TrxR1, was

known for decades, it was only recently that mammalian TrxRs were shown to contain a conserved UGA-encoded selenocysteine residue which is essential for the catalytic activity of the enzymes (Gasdaska *et al.*, 1999; Gromer *et al.*, 1998b; Lee *et al.*, 2000; Tamura and Stadtman, 1996; Zhong and Holmgren, 2000). Like other members of the disulfide oxidoreductase family, TrxRs show a number of sequence motifs that are essential for the catalytic activity in addition to the conserved selenocysteine in the C-terminal region of the proteins. These additional motifs include an aminoterminal disulfide active center, NADPH and FAD-binding domains as well as a dimer interface sequence necessary for homodimerization of the enzymes (Lee *et al.*, 1999; Sun *et al.*, 1999; Williams *et al.*, 2000). Furthermore, sequence homology analyses revealed that TrxR1 and TrxR2 are closely related, whereas TrxR3 is the evolutionarily more distant enzyme, but which still exhibits more than 50% overall sequence identity (Sun *et al.*, 1999).

TrxR3 was described as a mitochrondrial TrxR because it was shown to contain a mitochondrial signal peptide and to localize in the mitochondrial fraction of cells (Lee *et al.*, 1999; Sun *et al.*, 1999). Moreover, a tagged form of TrxR3 was found in mitochondria (Miranda-Vizuete *et al.*, 1999). A more recent report demonstrated that in addition to the products of the three different TrxR genes, both TrxR1 and TrxR3 exhibit extensive heterogeneity due to differential transcript splicing. Comparison between mouse, rat and human revealed that the multiple isoforms are conserved in mammals (Sun *et al.*, 2001). For TrxR3, alternative first exons were observed and they result in the formation of mitochondrial and cytosolic protein isoforms, respectively. These observations suggest that multiple transcription start sites within TrxR genes may be relevant to the complex regulation of

expression as reflected in the organelle- or cell type-specific location of mammalian TrxRs (Sun *et al.*, 2001).

The *Drosophila* genome (Adams *et al.*, 2000) contains two different TrxR genes, *Trxr-1* and *Trxr-2*, which were thought to encode the cytoplasmic and mitochondrial forms, respectively (Kanzok *et al.*, 2001). In the present report we describe two alternative forms of *Drosophila* TrxR-1 which code for cytoplasmic (TrxR-1^{cyto}) and mitochondrial (TrxR-1^{mito}) TrxR as observed in mammals (Sun *et al.*, 2001). The two *Drosophila Trxr-1* variants are generated by differential splicing at an evolutionarily conserved exon-intron junction, resulting in distinct N-terminal sequences. Both isoforms lack the selenocysteine residue in the C-terminus, which is replaced by a cysteine residue. We provide a biochemical characterization of TrxR-1^{mito}, showing that it has similar properties to the previously characterized TrxR-1^{cyto} (Kanzok *et al.*, 2001). Lack-of-function mutations of *Trxr-1* as well as mutations affecting either the mitochondrial or the cytoplasmic enzyme variants are lethal. Transgene-dependent rescue experiments indicate that the two forms of TrxR-1 are functionally distinct *in vivo*. Mitochondrial TrxR-1^{mito} can compensate for the lack of mitochondrial TrxR activity and partially substitutes for the cytoplasmic TrxR. In contrast, cytoplasmic TrxR-1^{cyto} is unable to compensate for the loss of the mitochondrial enzyme activity. The results show that ROS-defence is compartmentalized and that the capacity to adequately protect cells from cytotoxic damage depends on evolutionarily conserved splicing variants of a single gene.

Results and Discussion

Trxr-1 encodes cytoplasmic and mitochondrial TrxRs by differential splicing

The *Drosophila Trxr-1* locus, previously designated *dmtrxr-1* (Kanzok *et al.*, 2001), encodes two different splicing variants (Fig. 1A) (Missirlis *et al.*, 2001; Sun *et al.*, 2001). Expressed sequence tag (EST) clones (Rubin *et al.*, 2000) corresponding to each of the two transcripts were isolated from embryonic, adult head and ovarian cDNA libraries. Sequencing of ESTs (Rubin *et al.*, 2000) corresponding to the two *Trxr-1* transcripts and comparison with genomic DNA (Adams *et al.*, 2000) revealed that the two splice forms differ only with respect to their first exons, giving rise to two open reading frames with different N-terminal regions of the proteins (see below). Both transcripts are expressed during all stages of the *Drososphila* life cycle as revealed by developmental Northern blot analysis (Fig. 1B; see also Candas *et al.*, 1997) and RT-PCR with transcript-specific primers (Fig. 1C). This finding and the fact that corresponding cDNAs for each splicing variant could be isolated from staged cDNA libraries (see above) consistently argue that the two *Trxr-1* transcripts are not under differential temporal control. We therefore assume that the similar size of the transcripts reflects different polyadenylation sites within the 3'UTR regions, different lengths of the poly(A) tracks or both.

Conceptual translation of the longer transcript results in a protein which contains an N-terminal putative mitochondrial signal sequence which is absent from the other protein that recently qualified as a TrxR enzyme (Kanzok *et al.*, 2001) (Fig. 1D). For reasons detailed

Figure 1: Genomic structure of *Trxr-1*, transcript-specific mutations and expression of the spliced transcripts. A) Physical map of the *Trxr-1* locus, the *P{lacW}* insertion sites (orientation of the P elements indicated), the two alternatively spliced transcripts (4 exons each; boxes) and the location within AE003443 DNA in the region 7D18-20 of the X chromosome (Adams *et al.*, 2000). Note that $Trxr\text{-}1^{477}$ and $Trxr\text{-}1^{\Delta 2}$ represent loss-of-function and lack-of-function alleles of the $Trxr\text{-}1^{mito}$ transcript, respectively (see text) and that the two transcripts code for different 5'regions resulting in different N-terminal ends of the deduced protein (green and red boxes, respectively). Arrows represent the positions of primers described in Materials and Methods. B) Developmental Northern blot analysis, using a probe common to sequences of both *Trxr-1* transcripts. E1 is 0-2 h embryos, E2 is 0-24h embryos, L1-3 are the three larval stages, P is the pupal stage, A1 is adult males and A2 is adult females. Note a single band in all developmental stages, indicating that the mRNA length of both transcripts (see below) is approximately 2 Kb. C) Developmental RT-PCR analysis using trancript-specific primers; the lower band corresponds to $Trxr\text{-}1^{cyto}$, the upper band to $Trxr\text{-}1^{mito}$ (see Materials and Methods). Stages are as described in (B). D) Amino acid sequences of N-terminal regions of the two alternative *Drosophila* TrxR-1 proteins, and the corresponding protein portions of human and mouse TrxR3. The *Drosophila* putative mitochondrial signal peptide is depicted in red. Percentages show sequence identity to the human sequence, the vertical line represents intron-exon junctions.

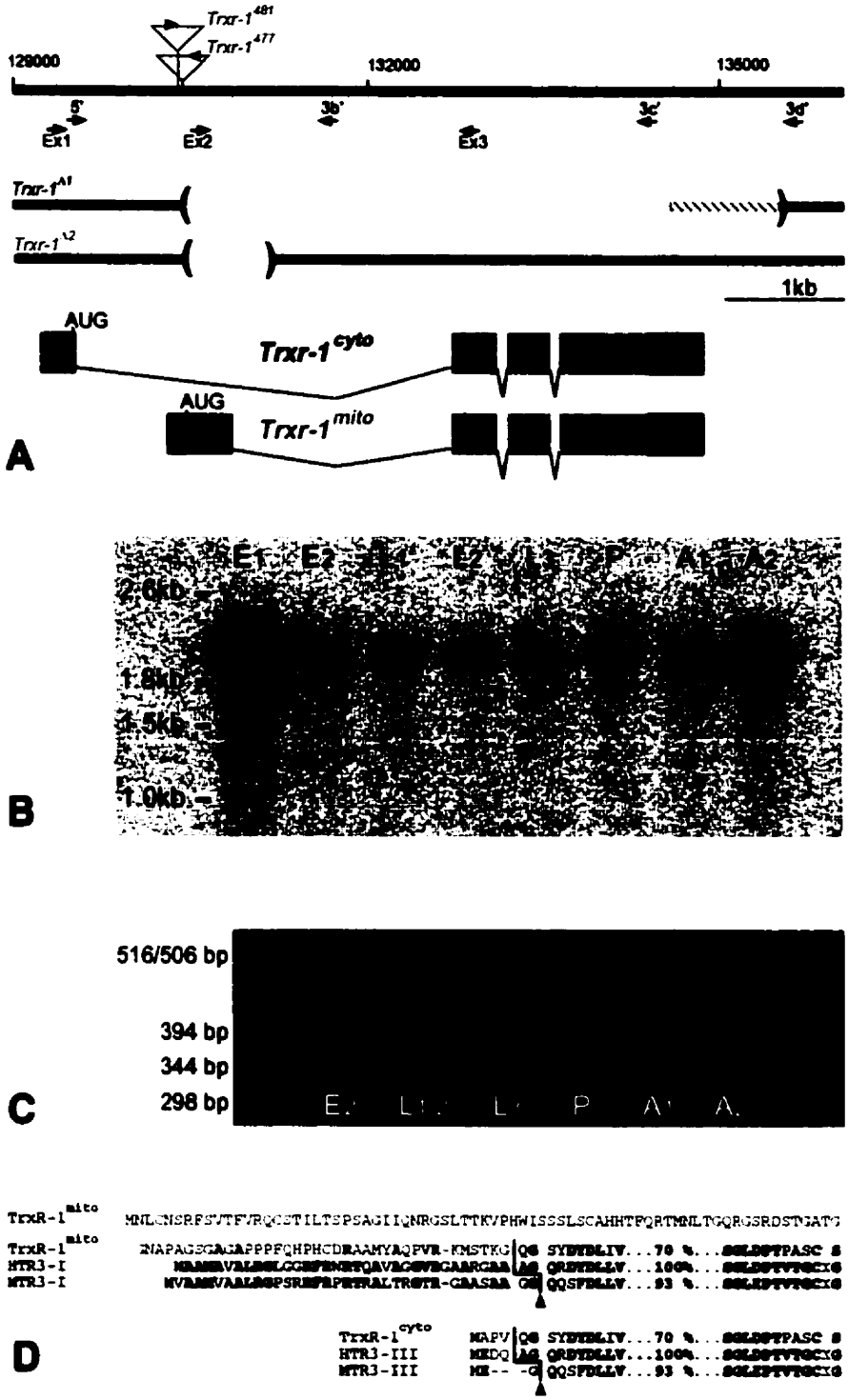
Trxr-1^481
Trxr-1^477
129000
132000
136000
5'
3b'
3c'
3d'
Ex1
Ex2
Ex3
Trxr-1^Δ1
Trxr-1^Δ2
1kb
AUG
Trxr-1^cyto
AUG
Trxr-1^mito
A
B
2.6kb
1.8kb
1.5kb
1.0kb
C
516/506 bp
394 bp
344 bp
298 bp
D
TrxR-1^mito
TrxR-1^mito
HTR3-I
HTR3-I
TrxR-1^cyto
HTR3-III
HTR3-III

Figure 1

below, we refer to this enzyme isoform as cytoplasmic TrxR-1 (TrxR-1^{cyto}). TrxR-1^{cyto} is encoded by exons 1 and 3-5. The alternative protein isoform, designated mitochondrial TrxR-1 (TrxR-1^{mito}, see below), is encoded by exons 2-5. Splicing therefore results in an extention of the N-terminus by 105 amino acids which contain a putative mitochondrial signal (Fig. 1D). These findings suggest that TrxR-1^{cyto} is the cytoplasmic enzyme isoform, whereas the longer TrxR-1^{mito} isoform is likely to be the mitochondrial counterpart (see also Sun *et al.*, 2001). Comparison of the two differently spliced transcripts with mammalian TrxR3 transcripts indicates that only the N-terminal regions of the corresponding proteins differ in sequence and that both the mammalian TrxR3 and the *Drosophila Trxr-1* transcripts involve a conserved splicing site (Fig. 1D). Notably, however, both *Drosophila* enzyme isoforms have a cysteine residue in place of the mammalian UGA-encoded selenocysteine at their third redox center (Gasdaska *et al.*, 1999; Lee *et al.*, 2000; Tamura and Stadtman, 1996; Zhong and Holmgren, 2000) (Fig. 1D).

In order to show that the two protein isoforms are indeed localized in different cellular compartments, we generated green fluorescent protein (GFP) tagged TrxR-1^{mito} and TrxR-1^{cyto} fusion proteins and monitored their cellular distribution and localization in transfected tissue culture cells. The results shown in Figure 2 indicate that GFP and the TrxR-1^{cyto}/GFP fusion proteins are distributed throughout the cell (Fig. 2A) and in the cytoplasm (Fig. 2B), respectively. In contrast, the TrxR-1^{mito}/GFP fusion protein accummulates in an organelle-specific manner in the mitochondria only (Fig. 2C). These observations demonstrate that like mammalian TrxR genes (Lee *et al.*, 1999; Sun *et al.*, 1999), *Trxr-1* encodes two isoforms that arise by alternative splicing of a single primary transcript and the proteins become localized

in different cellular compartments due to the presence or absence of the N-terminal mitochondrial localization sequence.

Properties of TrxR-1^{mito}

Since TrxR-1^{mito} and TrxR-1^{cyto} are localized in different cellular compartments, we next examined whether the two isoforms exhibit different enzymatic and kinetic properties. In order to compare TrxR-1^{cyto} (Kanzok *et al.*, 2001) with the newly identified mitochondrial isoform, we produced N-terminally hexahistidyl- (His-) tagged recombinant *TrxR-1^{mito}* protein in freshly transformed *E. coli* BL-21 cells (yield of 2 mg TrxR-1^{mito}/l cell culture). We purified the protein over a Ni-NTA agarose column, as had been done with TrxR-1^{cyto} (Kanzok *et al.*, 2001). In silver stained SDS-gels, the *TrxR-1^{mito}* protein appeared in a band of 69 kDa apparent molecular weight which corresponds well to the calculated molecular mass of the His-tagged protein of 68.17 kDa. In most aliquots tested, however, we noted a second protein band of about 65 kDa, which may represent a second conformational state of the protein, a proposal that will be addressed by future studies. Most importantly, however, the two bands were clearly distinct from TrxR-1^{cyto} which has a molecular mass of 53.2 kDa (Kanzok *et al.*, 2001; own observation).

The key enzymatic properties as well as kinetic parameters of TrxR-1^{mito} and TrxR-1^{cyto} were examined in parallel and repeated with several different protein preparations. The results summarized in Table 1 indicate that TrxR-1^{cyto} (Kanzok *et al.*, 2001) and TrxR-1^{mito} are both NADPH-dependent and are able to accept the low molecular weight oxidizing substrate 5,5`-dithiobis(2-nitrobenzoate) (DTNB). The K_M value for NADPH was determined to be approximately 1 μM in the presence of 3 mM DTNB for both enzymes. The respective K_M

Figure 2: Fluorescence microscopy images of transfected mouse NIH/3T3 cells showing the subcelllular localization of GFP and TrxR-1/GFP fusion proteins. The position of the nucleus is visualized by DAPI staining (blue) and the distibution of GFP (green) in fixed cells (A-C). Localization of GFP (green) and mitochondria (red) is shown in living cells (D-F). A) Transgenic GFP expression (control) in both the nucleus and cytoplasm. B) Transgenic TrxR-1^{cyto}/GFP expression in cytoplasm only. C) Transgenic TrxR-1^{mito}/GFP expression in mitochondria (see D-E). Transgene-expressed TrxR-1^{mito}/GFP (D) and Mitotracker (E) and co-localization (yellow) of both as seen in the merged figures D, E (F, yellow). Note that GFP is throughout the cells, the TrxR-1^{cyto}/GFP fusion protein is excluded from the nucleus and the TrxR-1^{mito}/GFP fusion protein, which contains the N-terminal putative mitochondrial signal sequence (see Fig. 1C), is restricted to mitochondria.

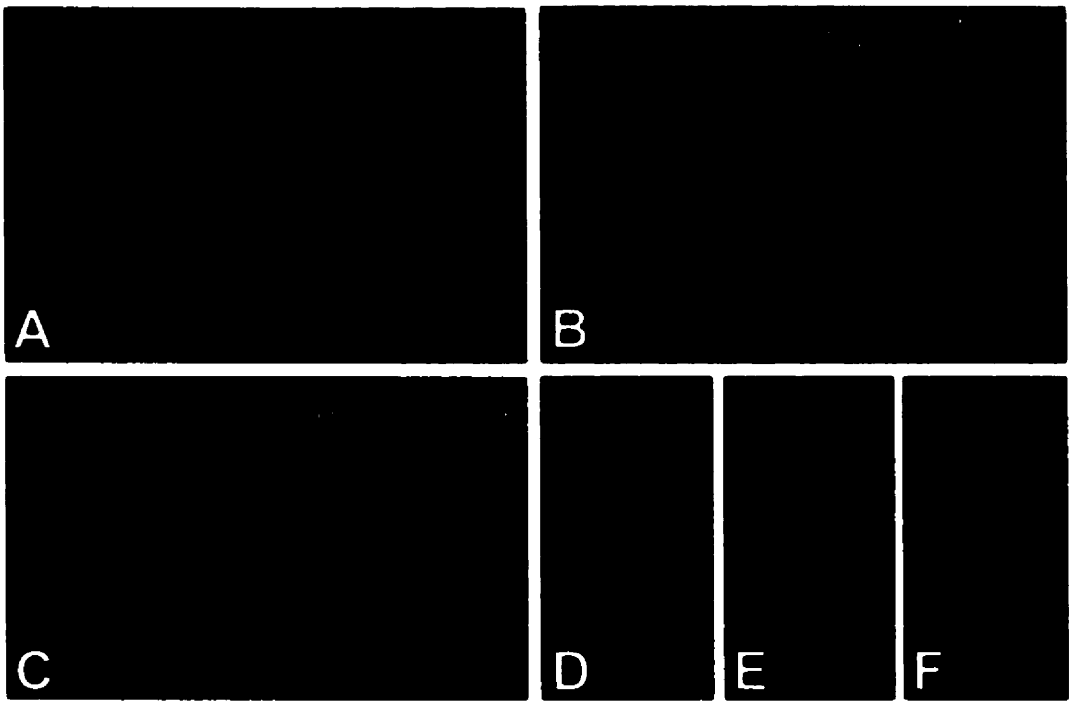

Figure 2

value for DTNB in the presence of 100 μM NADPH is 410 μM for TrxR-1^{mito} and, thus, is only slightly higher than the one determined for TrxR-1^{cyto} (380 μM; see also Kanzok et al., 2001). A more significant difference was observed with respect to the affinity of the two enzymes for *Drosophila* thioredoxin-1, a 12.4 kDa protein (Salz *et al., 1994;* Kanzok *et al.*, 2001). With thioredoxin-1 as substrate, a K_M value of 7 μM was obtained for TrxR-1^{cyto}, whereas the value obtained for TrxR-1^{mito} is almost 3-fold higher (K_M = 19 μM). Collectively, the data indicate that substrate turnover catalysed by TrxR-1^{cyto} and TrxR-1^{mito} is in a similar range (Table 1).

Mutations affecting different Trxr-1 functions

Previous results have shown that the *Trxr-1* locus maps to position 7D on the X chromosome, and we have recently identified two *Trxr-1* mutants, *Trxr-1^{481}* and *Trxr-1^{Δ1}* which represent a hypomorphic loss-of-function and a lack-of-function mutation, respectively (Missirlis *et al.*, 2001). Fig. 1A shows that P element mutation *Trxr-1^{481}* is caused by integration of *P{lacW}* into position 130,722 of the genomic clone AE03443 (Adams *et al.*, 2000). This position corresponds to exon 2 of the *Trxr-1* gene which contains the 5′untranslated region of the *Trxr-1^{mito}* transcript (Fig. 1A). The lack-of-function *Trxr-1^{Δ1}* allele represents a deletion of sequences of the open reading frame encoded by exons 3-5 common to both transcripts (Fig. 1A). *Trxr-1^{Δ1}* mutant larvae hatch; about 70% of these larvae survive as first instar but collectively die during the second instar larval stage without showing morphologically discernible phenotypes. In contrast, *Trxr-1^{481}* mutants survive into the third instar stage and 75% of the individuals develop into pupae with one day of delay.

Table 1: Biochemical characteristics of the two TrxR-1 variants of *Drosophila*

	TrxR-1^{cyto}	TrxR-1^{mito}
length of polypeptide (amino acids)	491	596
deduced molecular weight (kDa)	53.2	63.7
Isoelectric point (pH)	5.93	8.15
Mol. ext. coeff. (ε_{280}; mM^{-1} cm^{-1})	56.4	63.6
pH-optimum (pH)	7.1	7.1
K_M for NADPH (μM)*	1	1
K_M for DTNB (μM)*	380	410
K_M for thioredoxin-1 (μM)*	7	19

* All K_M values represent means of four independent determinations which differed by less than 10%

Only about 20% eclose into normal appearing adults the majority of which die within 2-3 days (Missirlis *et al.*, 2001).

In order to isolate mutants which affect the *Trxr-1* gene in a transcript-specific manner, we performed P element insertion mutagenesis using the $Trxr\text{-}1^{481}$ and the lack-of-function $Trxr\text{-}1^{\Delta 1}$ mutations as reference. We obtained a P element insertion located 34 base pairs downstream of the $Trxr\text{-}1^{481}$ insertion site (position 130,756 of the genomic clone AE03443; Adams *et al.*, 2000). This mutation fails to complement the $Trxr\text{-}1^{\Delta 1}$ mutation, indicating that we had isolated a novel *Trxr-1* allele, termed $Trxr\text{-}1^{477}$. Hemizygous $Trxr\text{-}1^{477}$ males and homozygous $Trxr\text{-}1^{477}$ females show a phenotype similar to $Trxr\text{-}1^{481}$ mutant individuals with two notable minor differences. $Trxr\text{-}1^{477}$ mutants spend on average one additional day as third instar larvae as compared to wildtype and, after metamorphosis, only about 2% of the pupae eclose. This observation suggests that $Trxr\text{-}1^{477}$ is a stronger mutant allele than $Trxr\text{-}1^{481}$. Interestingly, however, transheterozygous females of the genotype $Trxr\text{-}1^{481}/ Trxr\text{-}1^{477}$ show no mutant effects and develop into normal fertile adults. The fact that $Trxr\text{-}1^{477}$ complements the $Trxr\text{-}1^{481}$ allele implies that the two mutations affect different genetic functions both of which are uncovered by the non-complementing $Trxr\text{-}1^{\Delta 1}$ mutation that lacks the portion of the *Trxr-1* open reading frame common to both *Trxr-1* transcripts (Fig.1A).

The results suggest that $Trxr\text{-}1^{477}$ and $Trxr\text{-}1^{481}$ represent transcript-specific *Trxr-1* mutations. In order to find out whether this proposal is correct, we performed P element excision experiments designed to obtain a small and instructive deletion of exon 2. This exon contains the mitochondrial signal of $TrxR\text{-}1^{mito}$ (Fig. 1A). We obtained the $Trxr\text{-}1^{\Delta 2}$ mutation

(see Materials and Methods) which lacks a 534 bp DNA fragment corresponding to the N-terminal region of TrxR-1^{mito} (Fig. 1A). *Trxr-1$^{\Delta 2}$* mutants develop normally into late third instar larvae which, however, die without undergoing metamorphosis. *Trxr-1$^{\Delta 2}$* fails to complement *Trxr-1^{477}*, but is able to complement *Trxr-1^{481}*, as was observed with *Trxr-1^{477}*. These results establish that *Trxr-1^{481}* and *Trxr-1^{477}* are transcript-specific mutations and that *Trxr-1$^{\Delta 1}$* causes the lack of both TrxR-1 activities. In addition, the data imply that the mitochondrial and cytoplasmic variants provide independent and essential enzyme activities which are not interchangeable *in vivo*.

TrxR-1^{mito} cannot replace TrxR-1^{cyto} activity and vice versa

In order to demonstrate unambiguously that the two *Trxr-1* variants provide unique functions *in vivo*, we took advantage of the *UAS*/Gal4 system (Brand and Perrimon, 1993) to ubiquitously express each of the two isoenzymes from a cDNA-derived transgene in various mutant combinations (see Materials and Methods). The results summarized in Figure 3 show that expression of TrxR-1^{mito} rescues both the *Trxr-1^{477}* and the *Trxr-1$^{\Delta 2}$* mutants (Fig. 3A), but leads to only a partial rescue of the *Trxr-1$^{\Delta 1}$* lack-of-function mutation (Fig. 3B). In contrast, expression of TrxR-1^{cyto} has no rescuing activity with *Trxr-1^{477}* and the *Trxr-1$^{\Delta 2}$* mutants (Fig. 3A), but fully rescues the *Trxr-1^{481}* phenotype (Missirlis *et al.*, 2001) which is only partially rescued by the expression of TrxR-1^{mito} (Fig. 3B). Finally, combined TrxR-1^{cyto} and TrxR-1^{mito} expression resulted in a complete rescue of *Trxr-1$^{\Delta 1}$* mutants (Fig. 3B). These findings establish that *Trxr-1^{481}* and *Trxr-1^{477}* represent transcript-specific mutations and that each of the two *Trxr-1* variants provides a distinct function required for normal fly development. TrxR-1^{mito} activity in mitochondria and TrxR-1^{cyto} activity in the cytoplasm are

Figure 3: Rescue of the different *Trxr-1* alleles by transgenic overexpression of the correspondingly affected *Trxr-1* variants. Sibling analysis was performed with male progeny derived from females that were heterozygous for the different *Trxr-1* alleles. The ratio of mutant to wildtype males is depicted in the presence or absence of the different *UAS*-transgenes and the *actin-Gal4* driver in the individuals genome; there is an apparent leakiness of transgene transcription even in the absence of the driver. A) The two alleles $Trxr\text{-}1^{477}$ and $Trxr\text{-}1^{\Delta 2}$ affecting the $Trxr\text{-}1^{mito}$ transcript cannot be rescued by ubiquitous overexpression of TrxR-1^{cyto}, but require the activity provided by TrxR-1^{mito}. n is the number of wildtype progeny. Standard deviations are derived from the results of three independent experiments. Rescue was assessed for statistical significance by chi-squared analysis (Schor, 1968). For $Trxr\text{-}1^{477}$ and $Trxr\text{-}1^{\Delta 2}$, both p values were less than 0.0005. B) The mutant allele $Trxr\text{-}1^{481}$ affects the $Trxr\text{-}1^{cyto}$ transcript, whereas $Trxr\text{-}1^{\Delta 1}$ represents a lack-of-function allele for both activities. The first and third column of $Trxr\text{-}1^{481}$ are previously published results (Missirlis *et al.*, 2001), indicating that $Trxr\text{-}1^{481}$ represents an allele affecting TrxR-1^{cyto} activity. TrxR-1^{mito} cannot significantly rescue this allele ($p>0.1$). The true lack-of-function allele $Trxr\text{-}1^{\Delta 1}$, which deletes both *Trxr-1* variants, requires the activity of both enzymes for a full rescue. Note that expression of TrxR-1^{cyto} provides no rescue of $Trxr\text{-}1^{\Delta 1}$ individuals, expression of TrxR-1^{mito} provides only a partial rescue in $Trxr\text{-}1^{\Delta 1}$ individuals, but the concomitant overexpression of both variants leads to a full rescue of $Trxr\text{-}1^{\Delta 1}$, which lacks any TrxR-1 activity.

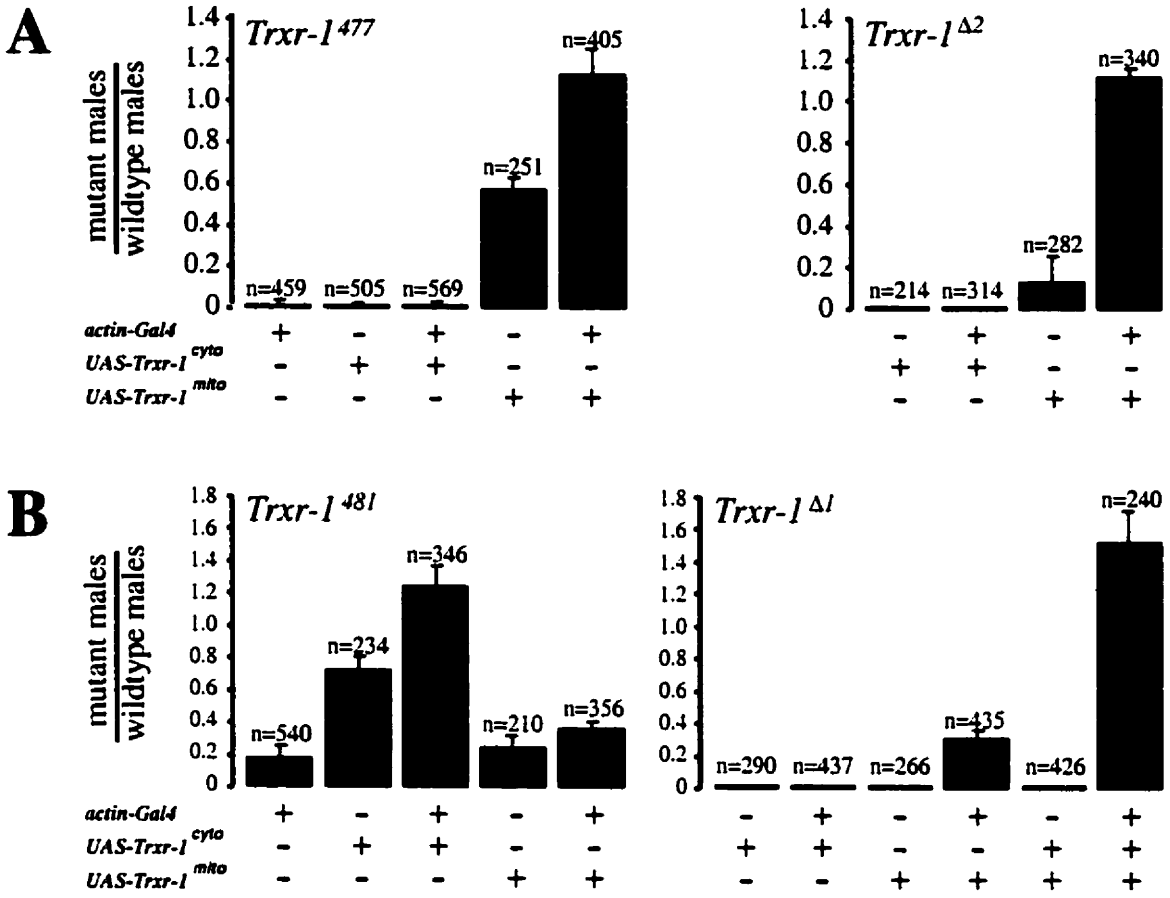
A
Trxr-1 477
Trxr-1 Δ2
mutant males / wildtype males
n=459
n=505
n=569
n=251
n=405
n=214
n=314
n=282
n=340
actin-Gal4
UAS-Trxr-1 cyto
UAS-Trxr-1 mito
B
Trxr-1 481
Trxr-1 Δ1
n=540
n=234
n=346
n=210
n=356
n=290
n=437
n=266
n=435
n=426
n=240

Figure 3

separate vital functions of the *Trxr-1* locus that arise by alternative splicing of a single primary transcript.

Lifespan is dependent on TrxR-1 activity

Impairing of antioxidant enzyme activities, such as superoxide dismutase (Phillips *et al.*, 1989), catalase (Griswold *et al.*, 1993) or glutathione-S-transferase (Toba and Aigaki, 2000), results in a shortened *Drosophila* adult lifespan. Furthermore, corresponding studies with the $Trxr\text{-}1^{481}$ mutant showed that impairement of TrxR-1^{cyto} activity diminishes the viability of the organism, ostensibly due to unbalanced redox homeostasis (Missirlis *et al.*, 2001). Since the $Trxr\text{-}1^{477}$ and $Trxr\text{-}1^{\Delta 2}$ mutations affect specifically mitochondrial TrxR-1 activity, which is functionally distinct from TrxR-1^{cyto} activity we next asked whether the reduced lifespan of these mutants can be restored by transgene-derived TrxR-1^{mito} and TrxR-1^{cyto} activities.

Figure 4 shows that ubiquitous TrxR-1^{mito} activity restores the lifespan of hemizygous $Trxr\text{-}1^{477}$ and $Trxr\text{-}1^{\Delta 2}$ mutant males from a few days up to the range of the wildtype lifespan. Furthermore, ubiquitous TrxR-1^{mito} expression not only partially rescues pre-adult lethality of the $Trxr\text{-}1^{\Delta 1}$ hemizygous males (see above and Fig. 3B), but also increases the lifespan of the rescued adults to about half the normal lifespan of wildtype flies (Fig. 4). TrxR-1^{mito} activity can therefore partially substitute for the total lack of *Trxr-1* gene expression to a degree that is beyond rescuing the mitochondrial aspect of *Trxr-1* gene activity only. In contrast, expression of TrxR-1^{cyto}, which rescues the shortened lifespan of $Trxr\text{-}1^{481}$ mutants (Missirlis *et al.*, 2001), has no discernable effect on hemizygous $Trxr\text{-}1^{\Delta 1}$ mutant males which

lack both *Trxr-1* activities (Fig. 3B). However, expression of TrxR-1^{cyto} in combination with TrxR-1^{mito} restores a normal adult lifespan to the *Trxr-1*$^{\Delta 1}$ mutants (Fig. 4).

The results presented here establish that the *Trxr-1* gene encodes two distinct non-selenocysteine-containing TrxRs with similar biochemical and kinetic properties. The lack of selenocysteine in *Drosophila* TrxRs is therefore not consistent with the paradigm established from studies of mammalian TrxRs showing that a selenocysteine residue in the activity center of the proteins is absolutely essential for their enzymatic function (Zhong and Holmgren, 2000). Genetic intervention with either or both of these activities of the *Trxr-1*gene, that arise through differential splicing of a single primary transcript, shows that each of the two isoenzymes provides an essential function and that these functions cannot be performed by the other enzyme *in vivo*. Furthermore, the putative activity of the other TrxR encoding gene in the fly genome, *Trxr-2* (Kanzok *et al.*, 2001), is not sufficient to compensate for the lack of either the cytoplasmic or mitochondrial *Trxr-1* activity. The results provide conclusive evidence for separate and compartmentalized ROS defence systems in the cytoplasm and mitochondria and that each system is required for cell viability, for successful eclosion, and for normal lifespan.

Figure 4: Lifespan determination of hemizygous *Trxr-1*^{477} (red boxes), *Trxr-1*$^{\Delta 2}$ (green rhomboids) and *Trxr-1*$^{\Delta 1}$ (black triangles and crosses) males, which express the alternative *Drosophila Trxr-1* cDNAs, individually or in combinations. Note that the lifespan of the eclosed hemizygous *Trxr-1*$^{\Delta 1}$ males overexpressing only the mitochondrial *Trxr-1* variant is severely reduced (black triangles) and that their shortened lifespan was rescued by simultaneous overexpression of both *Trxr-1* variants (black crosses). Transgene-derived ubiquitous *Trxr-1* expression was achieved by the Gal4/UAS system (Brand and Perrimon, 1993) using the *act5C-Gal4* driver on the third chromosome (Gonzales-Gaitan and Jäckle, 2000) in combination with *UAS-Trxr-1*^{mito}, *UAS-Trxr-1*^{mito} or both transgenes on the second chromosome.

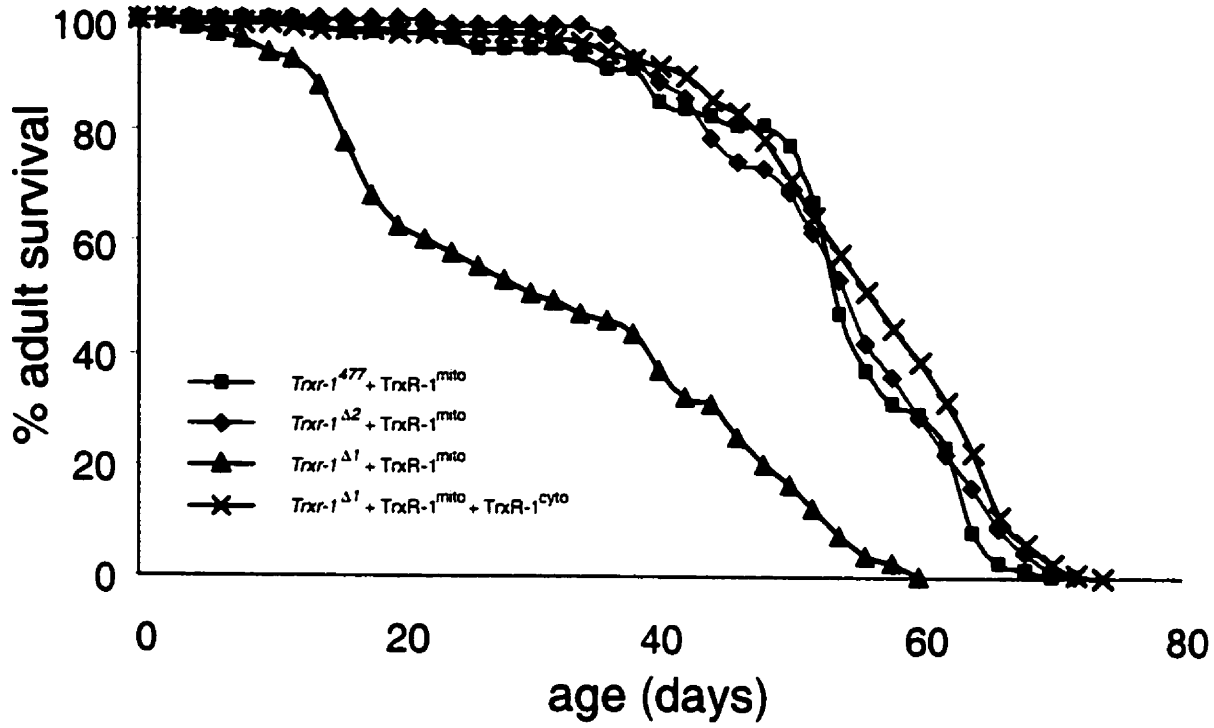
100
80
60
40
20
0
% adult survival
0
20
40
60
80
age (days)
Trxr-1^477 + TrxR-1^mito
Trxr-1^Δ2 + TrxR-1^mito
Trxr-1^Δ1 + TrxR-1^mito
Trxr-1^Δ1 + TrxR-1^mito + TrxR-1^cyto

Figure 4

Materials and Methods

Flystocks and generation of *Trxr-1* mutants

Flies were kept under standard conditions as described in Forjanic *et al.* (1997). In addition to the mutants and balancer chromosomes described in Lindsley and Zimm (1992), *P{lacW}* insertion lines *l(1)G0477* and *l(1)G0481* of the Göttingen X chromosome collection [Peters et al., submitted] and flies containing a transposase source on the second chromosome of the genotype *w; CyO/wg^{Sp}; TM6/Sb P{ ry^{+} Δ2-3}(*99B) (Robertson *et al.*, 1988) were used. *Trxr-1* mutant flies were balanced with *FM7i-pAct-GFP* (Reichhart and Ferrandon, 1998) which allowed identification of hemizygous male mutant larvae by the absence of GFP expression.

Reversion tests and generation of null mutants for *Trxr-1* involved remobilisation of the *P{lacW}* element of the line *l(1)G0477*. *l(1)G0477/FM7c* virgin females were crossed with *FM6/Y; TM2, ry P{ ry^{+} Δ2-3}*(99B)*/MKRS, Sb P{ ry^{+} Δ2-3}*(99B) (Robertson *et al.*, 1988). Female offspring with mosaic eyes were crossed with *FM6* balancer males; progeny which had lost the *P{lacW}* element were examined whether the excision event had restored wildtype function or generated a deletion corresponding to a *Trxr-1* null allele (see below and text). Two deletions, *Trxr-1^{Δ1}* and *Trxr-1^{Δ2}*, were used for the experiments described.

Molecular studies

Genomic DNA was isolated from flies using the QIAamp Tissue Kit supplied from Qiagen (Hilden, Germany). *P{lacW}* excisions which caused a reversion of the mutant phenotype

into wildtype or generated the deletion mutants *Trxr-1*$^{\Delta 1}$ and *Trxr-1*$^{\Delta 2}$ (see above) were examined by PCR amplification followed by sequencing as described earlier (Missirlis *et al.*, 2001). The insertion site DNA fragment of a randomly picked wildtype reversion line was amplified with the primer pair trxr5' - trxr3'b. Sequence analysis revealed that a a precise excision of the *P{lacW}* had taken place. PCR amplification of various DNA fragments of the deletion mutants *Trxr-1*$^{\Delta 1}$ and *Trxr-1*$^{\Delta 2}$ involved the following primers:

P, 5′-CGACGGGACCACCTTATGTTATTTCATGATG-3′;

P-inverse, 5′-CATGATGAAATAACATAAGGTGGTCCCGTCG-3′;

trxr5', 5′-TTTACGTGGAGCACCTACCAACAAGC-3′;

trxr3'b, 5′-GATGGCGCAAATCATGTACTTCAGC-3′;

trxr3'c, 5′-TCTTCGGCGGTAGTGGGATGGATGC-3′;

trxr3'd, 5′-CAGCGACTTATCAATGGGTTGG-3′.

For position of the primers within AE03443 DNA (Adams et al., 2000) see Fig. 1A.

PCR using genomic DNA isolated from *Trxr-1*$^{\Delta 1}$*/FM6* and *Trxr-1*$^{\Delta 2}$*/FM6* females and primers P and trxr5' amplified a 1kb DNA fragment, whereas primers P and trxr3'b failed to amplify any DNA fragment. Thus, the 3' end of the *P{lacW}* element is present in both mutants whereas the 5' end is lacking. Furthermore, PCR with the primer pair P-inverse/trxr3'b and *Trxr-1*$^{\Delta 2}$ DNA amplified a 3.5 kb DNA fragment. Sequence analysis revealed a 534 bp deletion which specifically removes sequences corresponding to the TrxR-1^{mito} transcript with breakpoints located within *P{lacW}* DNA and at position 131,290 of the AE03443 clone (Adams *et al.*, 2000) (see Fig. 1A). PCR with *Trxr-1*$^{\Delta 1}$ DNA was performed with the primer pairs P-inverse/trxr3'b and P-inverse/trxr3'c of which only P-inverse/trxr3'd amplified a 4.5 kb DNA fragment. Thus, *Trxr-1*$^{\Delta 1}$ lacks the genomic region from position 130,756 to at

least position 134,738 of clone AE03443 (Adams *et al.*, 2000) which codes for sequences of the open reading frame common to both transcripts (see Fig. 1A).

Developmental Northern blot analysis was done with total RNA extracted from embryos, larvae, pupae and adult *Drosophila* (see Fig. 1B) using the RNeasy Maxi Kit (Qiagen, Hilden, Germany). RT-PCR analysis (Sambrook and Russel, 2001) was carried out with RNA of the corresponding stages. For RT reactions, 5 µg total RNA was first treated with the DNA-free kit according to the protocol of the manufacturer (Ambion, Huntingdon, UK). cDNA synthesis was performed using the SuperScript Choice system (Gibco BRL, Karlsruhe, Germany). First strand synthesis was carried out at 42°C for 60 min with an oligo$(dT)_{12\text{-}18}$ Primer (Gibco BRL, Karlsruhe, Germany). Double-stranded cDNA was purified by phenol/chloroform extraction, ethanol precipitated and resuspended in H_20. The developmental expression profile of the two *Trxr-1* splicing variants was also examined by PCR amplification on the cDNA using the following primers: TrxR-1 Ex1 5′-CTCCGCTTATTCGTTTCGTG-3′; TrxR-1 Ex2 5′-TCTCCTTCGGCTGGCATTAT-3′; TrxR-1 Ex3 5′-TCAGCTTCTTGGGAATGCAG-3′. For the position of the primers see Fig. 1A. PCR with the primer pair TrxR-1 Ex1 and TrxR-1 Ex3 amplifies a 370 bp DNA fragment corresponding to the *Trxr-1^{cyto}* transcript, wheras PCR with the primer combination TrxR-1 Ex2 and TrxR-1 Ex3 results in a 454 bp DNA fragment specific for the *Trxr-1^{mito}* transcript (see Fig. 1C).

Transgene construction and transformation

Construction of *UAS-Trxr-1^{cyto}* was described in Missirlis *et al.* (2001). For *UAS-Trxr-1^{mito}* construction, the EST clone LD06006 (Rubin *et al.*, 2000) was digested with XbaI and XhoI

and the DNA fragment was subcloned in pSL1180 (Pharmacia, Erlangen, Germany). The resulting pSLTrxr-1^{mito} DNA was digested BglII-XhoI and the DNA fragment containing *Trxr-1^{mito}* open reading frame was cloned into the pUAST vector. pUAS-Trxr-1^{mito} DNA was used for transformation of flies as described (Rubin and Spradling, 1982). For each experiment outlined in the text, results were confirmed by use of two independent transgenic lines.

Cell culture

TrxR-1^{cyto} and TrxR-1^{mito} were fused to EGFP in front of the CMV promoter, in the pEGFP-N2 vector (Clontech, Heidelberg, Germany). The respective DNA was PCR amplified with primers that inroduced a 5' HindIII site, a 3' XhoI site, and LD21729, LD06006 as the template DNA, respectively. The primers used were XhoIcytogfp5' GCCCTCGAGATGGCGCCCGTGCAAGGATCCTACGAC, XhoImitogfp5' GCCCTCGAGATGAACTTGTGCAATTCGAGATTCTCCG and HindIIIcmgfp3' TTCAAGCTTAAGCTGCAGCAGCTGGCCGGCGTGGG. The stop codon was mutated into a leucine. The corresponding plasmids were transfected into mouse NIH/3T3 cells, using the method of Chen and Okayama (1987). The cells were washed with phosphate-buffered saline (PBS), fixed in 4% formaldehyde and mounted in Vectashield containing DAPI purchased from Vector Laboratories, Inc. (Burlingame, California). The fusion proteins were detected after 24h using fluoresence microscopy (Fig. 2A-C). For localization of mitochondria, unfixed transfected cells were incubated for 1 h with 250 nM of Mitotracker® Orange CM-H_2TMRos (Molecular Probes, Leiden The Netherlands), washed with PBS and inspected by confocal microscopy (Fig. 2D-F)

Rescue experiments and statistics

Rescue experiments were performed with the *Trxr-1* mutants (see text) using the Gal4/UAS system (Brand and Perrimon, 1993) to drive ubiquitous expression of *UAS-Trxr-1*^{cyto} or *UAS-Trxr-1*^{mito} under the control of an *act5C-Gal4* driver as described recently (Missirlis *et al.*, 2001). Transgene-derived *Trxr-1* expression was monitored by *in situ* hybridization (Rivera-Pomar *et al.*, 1995). The frequency of pupal eclosion and lifespan measurements of hemizygous mutant males bearing the Gal4/UAS combination of transgenes were monitored and compared with hemizygous mutant males that contain only the *act5C-Gal4* transgene and siblings that carried the balancer chromosome *FM6* in place of the mutant X chromosome, respectively. Results presented in Table3 were compared pair-wise using chi-squared analysis as described in Schor (1968). The chi-squared determination for each comparison was calculated by the formula $[(a*d-b*c)^2 * (a+b+c+d)]/[(a+b)(c+d)(a+c)(b+d)]$. For other controls see Figure 3 and Missirlis *et al.* (2001).

Lifespan measurements

Up to 10 eclosed males (0-24hrs old) of the genotype described in the text were kept in small food vials and transferred into new vials every second day. Survival of flies was monitored in 48 hr intervals. For each experiment described in Figure 4 at least 150 males were monitored and experiments were repeated with different batches of food and at different seasons during the year.

Recombinant proteins

TrxR-1^{cyto} and TrxR-1^{mito} were recombinantly produced and purified as described before (Kanzok *et al.*, 2001). *Drosophila* thioredoxin-1 was kindly placed at our disposal by Holger

Bauer and Heiner Schirmer, Heidelberg University. The open reading frame (ORF) of TrxR-1^{mito} (Fig. 1A) was PCR amplified from LD06006 (Rubin *et al.*, 2000), using primers XhoI5'alt GCCCTCGAGATGAACTTGTGCAATTCG and HindIII3'alt TTCAAGCTTTAGCTGCAGCAGCTGGC introducing a 5' XhoI and a 3' HindIII restriction sites, respectively. The ORF was subsequently subcloned in-frame into the pRSETA vector (Invitrogen, Karlsruhe, Germany) and sequenced. The resulting fusion protein contains an amino-terminal hexahistidyl tag, which allowed purification (see below). All chemicals used were of the highest available purity and were obtained from Roth or Sigma. Ni-NTA matrices for purification of His-tagged protein were purchased from Qiagen (Hilden, Germany).

Expression and purification

The *E. coli* strain BL-21 was used for expression of the *D. melanogaster Trxr-1^{mito}* gene. Competent cells were transformed with the respective pRSETATrxr-1^{mito} plasmid. Starter cultures from single colonies were grown overnight and 12 ml were used for inoculation of 600 ml LB-medium containing carbenicillin (100 µg/ml). Cells were grown at 37°C to an OD_{600} of 0.5; subsequently, the expression was induced by adding 1 mM IPTG. Cells were grown over night, harvested, and directly used for protein-purification or were frozen at -20°C. For purification, the cells were disintegrated by sonication in the presence of protease-inhibitors. After centrifugation, the supernatant was loaded onto a Ni-NTA column equilibrated with 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. After washing the column with increasing imidazol concentrations, the respective protein was eluted with 75 mM imidazol; collected fractions were tested for enzymatic activity and for purity by 10% SDS gel electrophoresis. Active fractions were pooled and concentrated via ultrafiltration.

Enzyme assays

TrxR activity was determined spectrophotometrically in 100 mM potassium phosphate, 2 mM EDTA, pH 7.4, at 25°C in 2 different assay systems: (a) at 412 nm in the presence of 200 μM NADPH and 3 mM 5,5`-dithiobis(2-nitrobenzoate) (DTNB) measuring the production of 2-nitro-5-thiobenzoate ($\varepsilon_{412\ nm}$ = 13.6 mM^{-1} cm^{-1}) or (b) at 340 nm in the presence of 100 μM NADPH and various concentrations of TrxR-1 following the oxidation of NADPH ($\varepsilon_{340\ nm}$ = 6.22 mM^{-1} cm^{-1}) (Gromer *et al.*, 1998a; Kanzok *et al.*, 2001). For determination of K_M-values substrate concentrations were systematically varied. All K_M-values represent means of 4 independent determinations.

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Manuscript 3

A thioredoxin-dependent peroxidase expands the repertoire of oxidative stress protection in *Drosophila*

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Abstract

Molecular oxygen is key to aerobic life, but is also converted into cytotoxic byproducts referred to as reactive oxygen species (ROS). The fruit fly *Drosophila* possesses most of the repertiore of ROS detoxifying enzymes that have been reported for other vertebrate and lower eukaryotic organisms excluding glutathione reductase and possibly also glutathione peroxidase activities. Here, we report the sequence identification of two non-selenium containing glutathione peroxidase-like genes in the genome of *Drosophila* and present an initial biochemical characterization of one of these enzymes. The results reveal that the protein is actually a thioredoxin peroxidase (TPx) rather than a glutathione-dependent peroxidase. We also show that transgene-dependent overexpression of the TPx gene increases resistance to experimentally induced oxidative stress, but does not compensate for the loss of catalase (Cat), an enzyme which, like TPx, functions to eliminate hydrogen peroxide. Furthermore, transgene-derived overexpression of TPx in mutant flies lacking Cu/Zn superoxide dismutase (Sod1), an antioxidant enzyme which protects cells from superoxide radical toxicity, is detrimental in contrast to transgene-derived overexpression of Cat which partially rescues the *sod1* mutant, indicating that TPx1 and Cat function in genetically distinct pathways or cellular compartments.

Introduction

In an aerobic environment, oxidative stress of organisms and individual cells derives from reactive oxygen species (ROS) generated as byproducts of oxygen metabolism (Halliwell and Gutteridge, 1999). Primary ROS include the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\bullet}$). The numerous secondary and tertiary products, such as alkyl hydroperoxides and lipid peroxides, are generated by the interaction of primary ROS with cellular targets (Girotti, 1998). High concentration of ROS has damaging effects on membrane lipids, nucleic acids and proteins (Stadtman and Levine, 2000; Termini, 2000). To cope up with these detrimental effects of ROS, organisms have evolved antioxidant defense systems (Yu, 1994). These include the enzymes superoxide dismutase (Sod) and catalase (Cat) as well as the glutathione (GSH) and thioredoxin (Trx) systems (Halliwell and Gutteridge, 1999).

Sod and Cat constitute an evolutionary conserved ROS defense system; Sod converts $O_2^{\bullet -}$ to H2O2 and Cat prevents $OH^{\bullet}$ formation by breaking down H2O2 into oxygen and water (Chance *et al.*, 1979). The GSH system comprises of glutathione reductase (GR), Glutaredoxins (Grxs), GSH-S-transferases (GSTs) and glutathione peroxidases (GPxs). GR reduces oxidized glutathione (GSSG) to GSH, which is in turn used as an electron donor by Grxs, GSTs and GPxs (Fahey and Sundquist, 1991). Trxs, on the other hand, constitute a family of small thiol proteins (approximately 12 kDa) which reduce oxidized cysteine groups on proteins. Trxs undergo NADPH-dependent reduction by thioredoxin reductases (TrxRs)

and in turn supply reducing equivalents to thioredoxin peroxidases (TPxs) (Miranda-Vizuete *et al.*, 2000).

TPx catalyzes the reduction of H_2O_2 or organic hydroperoxides to water and alcohols, respectively, using Trx as an electron donor (Chae *et al.*, 1994). GPx performs essentially the same reaction, except for using GSH as an electron donor (Mills, 1957). A large number of proteins have been biochemically and molecularly characterized as GPxs and TPxs (Chae *et al.*, 1994; Chen *et al.*, 2000; Ketterer and Meyer, 1989; Pedrajas *et al.*, 2000; Rahlfs and Becker, 2001; Saito *et al.*, 1999; Ursini *et al.*, 1995). This group of proteins is characterized by considerable amino acid sequences heterogeneity. Here, we focus on a GPx family which includes the mammalian selenocysteine-containing GPxs and homologs that contain cysteine instead of selenocysteine in the reactive center of the GPx enzymes (Ursini *et al.*, 1995).

Mammals express at least 5 homologous proteins of the GPx family (Arthur, 2000), among which is GPx1 that reduces only soluble hydroperoxides and was the first GPx identified (Mills, 1957). Investigations of the biological function of this enzyme, including overexpression studies and knock-out mice, demonstrate a protective role for GPx1 against the $O_2^{\bullet -}$ generating reagent paraquat (Bus *et al.*, 1974) and H_2O_2 toxicity, but not against hyperoxia and thermal stress (Cheng *et al.*, 1998; de Haan *et al.*, 1998; Ho *et al.*, 1997; Mirochnitchenko *et al.*, 1995). Phospholipid hydroperoxide GPx4 is unique in acting on hydroperoxides integrated in membranes (Thomas *et al.*, 1990). GPx4 was shown to have an additional function in the testis. It not only exists as a soluble peroxidase in spermatids, but also acts as an enzymatically inactive, oxidatively cross-linked structural protein of the

mature spermatozoan (Ursini *et al.*, 1999). The two GPx proteins described above contain selenocysteine at their active sites (Ursini *et al.*, 1995). In contrast, the vast majority of invertebrate and plant GPx-like proteins contains a cysteine residue in the corresponding position of the catalytic domain (Ursini *et al.*, 1995). This is also true for the three *Saccharomyces cerevisae* GPx homologs (Inoue *et al.*, 1999) which were recently reported to exhibit activity against phospolipid hydroperoxides (Avery and Avery, 2001).

Suprisingly, a number of proteins which, according to molecular criteria, should belong to the GPx family, lack GPx activity. For instance, *Synechocystis* PCC 6803 homologs do not accept GSH as an electron donor, but are NADPH-dependent peroxidases (Gaber *et al.*, 2001). Furthermore, human plasma GPx3 and *Plasmodium falciparum* TPx accept GSH *in vitro*, but are more active with Trx as the electron donor and were thus re-classified as TPxs (Bjornstedt *et al.*, 1994; Sztajer *et al.*, 2001). These results showed that proteins which, based on molecular criteria, belong to the GPx family may either be GSH-dependent, may represent structural proteins such as GPx4 (see above) or may depend on electron donors different from GSH and thus exert TPx activity.

Early biochemical work on several insect species led to the widely-held belief that insects lack glutathione peroxidase activity (Ahmad *et al.*, 1988; Allen *et al.*, 1983; Smith and Shrift, 1979). These initial observations were later reassessed and some GPx activity was reported using cumene hydroperoxide, instead of H_2O_2 as a substrate (Ahmad *et al.*, 1989). However, the activities measured were still considerably low. These observations led to the expectation that reduction of intracellular H_2O_2 would be the sole domain of Cat (Orr and Sohal, 1994;

Parkes *et al.*, 1993). Recently, however, the existence of an extracellular GPx active towards H_2O_2 was reported from the bug *Rhodnius prolixus* (Paes and Oliveira, 1999), re-opening the possibility that at least some insects may contain a true GPx. Survey of the recently sequenced *Drosophila* genome (Adams *et al.*, 2000) revealed two genes with homology to the GPx gene family but which appeared to encode a cysteine residue instead of selenocysteine in their active center. We wondered whether they encoded true GPxs as was shown for their yeast homologs (Avery and Avery, 2001) or whether they encoded TPxs as was the case with the *Plasmodium* protein (Sztajer *et al.*, 2001).

Drosophila melanogaster has been a popular genetic model for oxidative stress research (Le Bourg, 2001; Parkes *et al.*, 1999) focusing primarily on the Sod/Cat system (Griswold *et al.*, 1993; Orr and Sohal, 1994; Parkes *et al.*, 1998; Phillips *et al.*, 1989; Sun and Tower, 1999) and GST analysis (Parkes *et al.*, 1993; Singh *et al.*, 2000; Singh *et al.*, 2001; Toba and Aigaki, 2000). In contrast, the GSH and Trx systems have not been examined in great detail up to now. Drosophila was subsequently reported to lack a true GR homolog (Kanzok *et al.*, 2001), implying that it must depend upon another reducing system like Trx. Subsequent identification of the two essential components of such a system, Trx (Salz *et al.*, 1994) and TrxR (Kanzok *et al.*, 2001), and the finding that lack-of-function mutations for either Trx or TrxR are lethal (Missirlis *et al.*, 2001; Salz *et al.*, 1994), strengthened the idea that reductant recycling in Drosophila may be largely if not entirely carried out by the Trx/TrxR system. Consequently, we broadened our search to include criteria that would capture TPxs as well as GPxs.

Here we present the cloning and a functional characterization of one of two GPx-like genes identified in the *Drosophila* genome. Biochemical studies indicate that the product of this gene exhibits peroxidase activity towards H202 and to alkyl and aromatic hydroperoxides, and that Trx, rather than GSH acts as the reducing agent. We present evidence that overexpression of this gene, termed *dmtpx1*, encodes a thioredoxin peroxidase, DmTPx1, that protects against toxicity confered by hyperoxia and paraquat. However, *dmtpx1* overexpression fails to rescue a Cat-null mutant and, unexpectedly, is detrimental to mutants which lack Cu/Zn Sod1 activity. These results suggest that DmTPx1 is a functional part of the *Drosophila* Trx antioxidant defense system with the capacity to reduce organic peroxides, and that it plays a role distinct from that of Cat in detoxifying H_2O_2 *in vivo*.

Results and Discussion

Two GPx-like genes are present in the *Drosophila* genome

Analysis of the *Drosophila* genome sequence revealed two genes with high degree of sequence homology to the GPx gene family corresponding to the annotated transcripts CG12013 and CG15116 (Adams *et al.*, 2000). These two transcripts are predicted to encode proteins with 51% amino acid sequence identity. When compared to the human GPxs, they show the highest degree of identity (44% and 38%, respectively) with GPx4. An alignement of the two protein sequences with homologs from human, yeast and *Plasmodium falciparum* is shown in Fig. 1. All conserved domains and amino acids implicated in the catalytic function are present in the *Drosophila* proteins. Notably, however, both gene products contain a cysteine residue in position of the catalytic selenocysteine found in the human homologs (Ursini *et al.*, 1995).

To initiate the biochemical and functional analysis of the first GPx-like gene product in an invertebrate organism, we examined one of the two genes, CG12013 and its encoded GPx-like protein. Because cysteine-containing homologs of this GPx family in yeast (Fig. 1) had already been shown to function as phospholipid hydroperoxide GPxs (Avery and Avery, 2001) whereas the cysteine-containing homolog of *Plasmodium falciparum* turned out to be a TPx (Sztajer *et al.*, 2001), we first asked whether CG12013 codes a GPx, a TPx or whether the protein product exhibits both activities.

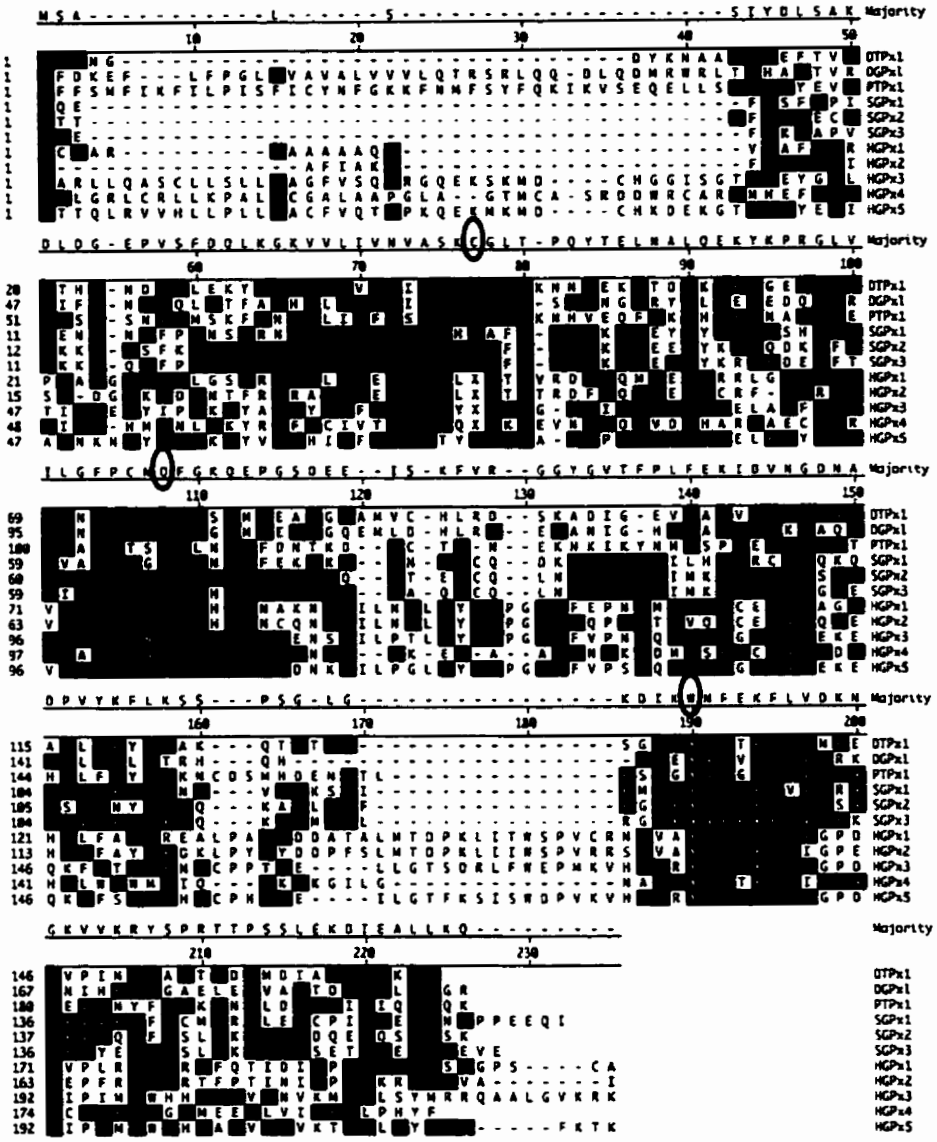

Figure 1: Comparison of the deduced amino acid sequences of CG12013 (shown as DTPx1) and CG15116 (shown as DGPxl, for *Drosophila* glutathione peroxidase-like protein) with those of GPxs from humans and yeast , and the *Plasmodium falciparum* GPx-like protein recently qualified as a TPx . The alignement was performed by the ClustalW computer programme . Conserved residues are shown as white letters in a black background. The top line shows the amino acid residue represented in most sequences. The amino acids of the catalytic triad are circled. X is selenocysteine.

Biochemical and kinetic properties of the CG12013 gene product

To produce the CG12013 protein for analysis, a full-length cDNA of the CG12013 gene was inserted into the expression vector pRSETA (see Materials and Methods). Freshly transformed *E. coli* BL21 cells were used for production of the recombinant protein with a yield of 25-30 mg protein/l cell culture. The N-terminal hexahistidyl-tag contributed by the pRSETA vector allowed purification of the his-tagged CG12013 protein over Ni-NTA agarose columns. As assessed by silver stained SDS-gels, the resulting protein was >99% pure and its size agrees with the calculated molecular mass of 23.2 kDa (data not shown). The native CG12013 protein comprises of 169 amino acids and has a deduced molecular weight of 18.7 kDa (Table 1).

We first asked whether the recombinant protein exhibits GSH-dependent peroxidase activity using H2O2, cumene hydroperoxide or tert-butyl hydroperoxide (tBOOH) as substrates. With these substrates, no activity was observed in a GSH-coupled assay. This implies that CG12013 is not a GPx, provided that the recombinant protein is active *per se*. We next assayed the recombinant protein for Trx-dependent activity. In the presence of NADPH, *P. falciparum* TrxR, which serves as a thioredoxin regenerating system, and *D. melanogaster* Trx-1 or Trx-2, CG12013 efficiently catalyses the reduction of H_2O_2, cumene hydroperoxide and tBOOH (Table 1). At 25°C and in the presence of 10 μM DmTrx-2 and 200 μM peroxide substrate the specific activities were 5.3, 11.1, and 2.9 U/mg, respectively. The pH-dependence of the activity carried out in the presence of 10 μM DmTrx-2 and 200 μM H_2O_2 indicated an optimum at pH 7.2. To test the influence of salt concentration on enzyme

Table 1. Biochemical properties of DmTPx1

length of polypeptide (amino acids)	169	
deduced molecular weight (kDa)	18.7	
Isoelectric point (pH)	7.91	
Mol. ext. coeff. (ε280; mM^{-1} cm^{-1})	16.0	
pH-optimum (pH)	7.2	
Substrate		
K_M for DmTrx-1	10 μM	
K_M for DmTrx-2	3 μM	
	K_M	**k_{cat}***
H_2O_2	180 μM	300 min^{-1}
t-Butylhydroperoxide	3.2 mM	440 min^{-1}
Cumene hydroperoxide	150 μM	290 min^{-1}

*assuming substrate saturation with DmTrx-2 and peroxide

activity, assays run in 50, 100, and 200 mM KCl, respectively, resulted in markedly decreased enzyme activity (data not shown).

In the presence of 200 μM H_2O_2, K_M values of 11 μM and 3 μM were determined for DmTrx-1 and DmTrx-2, respectively (Table 1). Trx concentrations >25 μM produced substrate inhibition. In the presence of 10 μM DmTrx-2, K_M values could also be obtained for the peroxide substrates, 180 μM for H_2O_2, 150 μM for cumene hydroperoxide and 3.2 mM for tBOOH. Substrate inhibition was reached at appr. 1 mM, 300 μM and 10 mM, respectively. Based on these data and assuming a substrate saturation with DmTrx-2, the V_{max} values were calculated to be 13.1 U/mg for H_2O_2, 12.7 U/mg for cumene hydroperoxide, and 18.9 U/mg for tBOOH. These values correspond to k_{cat} values of 300 min^{-1}, 290 min^{-1} and 440 min^{-1} respectively.

The results demonstrate that the recombinant CG12013 gene product, which has GPx-like sequence homologies, is a *bona fide* Trx-dependent peroxidase. In this respect, the protein is functionally related to the recently identified TPx from *Plasmodium falciparum* (Sztajer *et al.*, 2001) and, despite the molecular similarity, differs from the phospholipid hydroperoxide GPxs from *Saccharomyces cerevisae* (Avery and Avery, 2001). Based on the biochemical characteristics described above, we refer to the CG12013 gene product as DmTPx1.

Structure, chromosomal location and expression of *dmtpx1*

Several expressed sequence tagged (EST) clones from the Berkeley Drosophila Genome Project (BDGP) corresponded to the DmTPx1 gene (Rubin *et al.*, 2000). We sequenced one

of these clones, GH13101, as well as three cDNA isolates from an embryonic cDNA library (see Materials and Methods). The four independent clones contained an identical open reading frame sequence and intron-exon structure as depicted in Fig. 2a. *dmtpx1* contains two introns and the translation initiation start is within exon 2. *In situ* hybridization of DmTPx1 DNA Dig-labeled probes on polytene chromosomes revealed that the gene is localized on the left arm of the third chromosome at position 63C (Fig. 2b), which is in accordance with the location of the gene proposed by the genome project (Adams *et al.*, 2000).

To visualize the spatial and temporal patterns of *dmtpx1* expression in the *Drosophila* embryo, we performed *in situ* hybridization of antisense RNA probes to whole mount preparations of embryos at different stages of development (Rivera-Pomar *et al.*, 1995). The results show that *dmtpx1* transcripts are provided maternally to the embryo (Fig. 2c). During embryogenesis, evidence of *dmtpx1* expression is initially detected in the germline progenitor cells (Fig 2d), later in the amnioserosa and macrophages (Fig 2e) and finally in the fat bodies and oenocytes (Fig. 2f), where transcripts reside until larval stages. Interestingly, expression of *dmtpx1* in the fat body and oenocytes overlaps with the expression of the *cat* gene (Missirlis *et al.*, 2001).

To investigate the subcellular location of DmTPx1 we constructed plasmid vectors to express DmTPx1 as a fusion protein with Green Fluorescent Protein (GFP), which is used as a marker for gene expression (Chalfie *et al.*, 1994). DmTPx1 was inserted at the N-terminus of EGFP, a red-shifted variant of GFP designed for brighter fluorescence (see materials and methods). The plasmid DNA was transiently transfected into Schneider cells and the cellular

Figure 2: Genomic structure, chromosomal location and expression of *dmtpx1*. a) a physical map of the *dmtpx1* transcript and its location within AE003477 DNA (Adams *et al.*, 2000). Boxes represent exons, black regions correspond to the open reading frame of TPx1. b) *In situ* hybridization of dig-DNA *dmtpx1* probes to polytene chromosomes. Arrow shows that *dmtpx1* resides at position 63C on the left arm of the third chromosome, which is in aggreement with the localization proposed by the Genome project (Adams *et al.*, 2000). c-f) *In situ* hybridization of antisense RNA probes to whole mount preparations of embryos (Rivera-Pomar *et al.*, 1995). c) DmTPx1 transcripts are provided maternally. d) Zygotic expression occurs first in germ line progenitors (arrow), e) during germ-band extension in the macrophages and amnioserosa and f) during the later stages of embryogenesis in the oenocytes and fat bodies (asterisks and arrow, respectively). Stages of *Drosophila* embryogenesis are described in (Campos-Ortega and Hartenstein, 1985). g) Schneider II cells were transfected with TPx1-EGFP-expression plasmids and viewed by fluorescence microscopy. DNA was monitored by DAPI staining, shown in blue. Note that TPx1-EGFP resides outside the nucleus and is concentrated in subcellular compartments. h) Schneider II cells were transfected with TPx1-EGFP-expression plasmids and viewed by confocal microscopy. Mitochondria are stained red with Mito Tracker Orange CM-H2TMRos. Note that TPx1-EGFP fluorescence does not co-localize with the mitochondrial staining. i) *In vivo* expression of TPx1-EGFP in the adult midgut using the UAS/Gal4 system (Brand and Perrimon, 1993) and the 1407-Gal4 driver. j) *In vivo* expression of TPx1-EGFP in the adult fat bodies using the FB-Gal4 driver. Black circles represent lipid vacuoles, TPx1-EGFP is concentrated also in this tissue in a distinct inracellular compartment resembling the endoplasmatic reticulum.

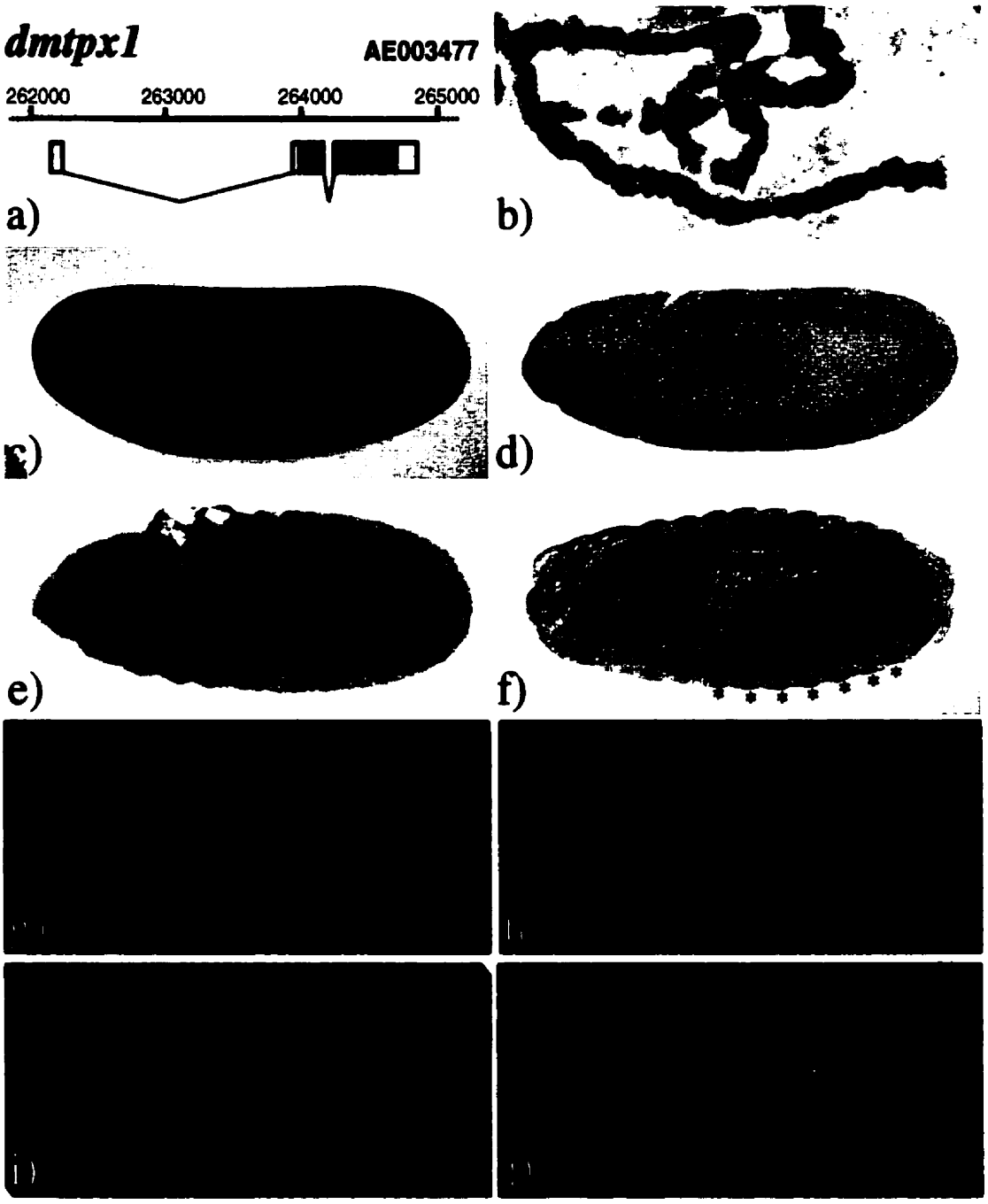

Figure 2.

distribution of the fluorescent fusion protein was detected by fluorescence microscopy. Cells transfected with EGFP alone showed ubiquitous distribution of fluorescence in the cytoplasm and in the nucleus (data not shown). In contrast, TPx1-EGFP is excluded from the nucleus (Fig. 2g) and resides in the cytoplasm, with clear enrichment in some perinuclear structures. Parallel staining with Mito Tracker orange CM-H2TMRos, a mitochondrial marker (see materials and methods), indicated that TPx1-EGFP is excluded from the mitochondria (Fig. 2h). In addition to cell culture experiments, we expressed the DmTPx1-EGFP fusion protein *in vivo* by means of the Gal4/UAS system (Brand and Perrimon, 1993). For this purpose we generated transgenic strains containing the DmTPx1 open reading frame fused to EGFP under the control of UAS regulatory elements. We induced expression of the transgene in the adult midgut (Fig 2i) using the 1407-Gal4 driver, and in the fat bodies (Fig 2j) with the FB- Gal4 driver. In both tissues, the protein is found in the cytoplasm of cells with a clear enhancement in subcellular structures that resemble the endoplasmatic reticulum (ER).

In summary, *dmtpx1* shows a highly regulated tissue and temporal expression pattern during embryogenesis. The data imply a tight transcriptional control of the gene. The gene product, DmTPx1, is a non-nuclear, non-mitochondrial enzyme, which functions in the cytoplasm and possibly accumulates in the ER.

DmTPx1 protects from paraquat toxicity and hyperoxia

We had previously demonstrated that the Trx and Sod1/Cat systems cooperate in overall antioxidant defense metabolism in *Drosophila* (Missirlis *et al.*, 2001). Because the Trx-dependent peroxidase activity demonstrated here for DmTPx1 qualifies this enzyme as a

component of the *Drosophila* Trx system, we hypothesized that *dmtpx1* functions as an integral component of the Trx redox system *in vivo*. To test this hypothesis, we asked whether *dmtpx1* contributes to protection against oxidative toxicity imposed by exposure of Drosophila to paraquat and hyperoxia.

We generated DmTPx1 overexpressing transformants (Rubin and Spradling, 1982) that carry multiple copies of a*dmtpx1* genomic transgene. The *dmtpx1* transgene contains a 7kb genomic fragment (containing *dmtpx1* flanked by 5.5 kb and 1 kb of upstream sequences and downstream sequences, respectively) inserted upstream of a lacZ reporter gene in the pCaspeRAUG-ßGal vector (see Materials and Methods). Plasmid DNA was injected into *white* recipient embryos and w^+ transformants, termed *P(tpx1⁻)*, were recovered. Transgenes were chromosomally localized by the established balancing procedure (Greenspan, 1997). *In situ* hybridization using the LacZ probe on whole mount preparations of transgenic embryos showed that the LacZ gene is expressed in the same spatial and temporal patterns as the endogenous *dmtpx1* gene (data not shown).

Two independent *dmtpx1* transformants were brought to homozygocity by *inter se* crosses, thereby retaining the genetic background of the parental *white* mutant strain which was then used as a control strain. The *mini-white* phenotypic marker carried by the pCaspeR vector enabled the identification of transgene heterozygotes and homozygotes by eye color intensity. *P(tpx1⁺)* insertions resided on the X- and on the second chromosome, respectively.

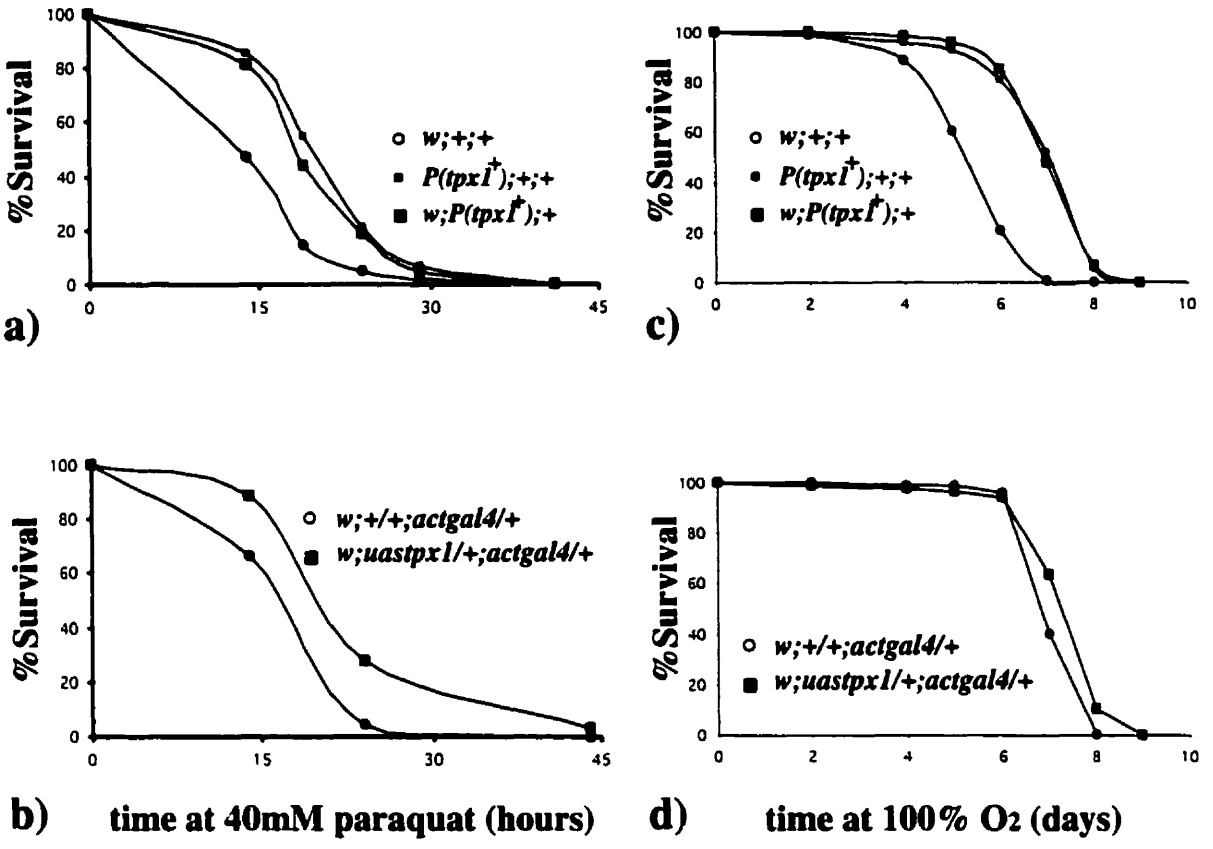

Figure 3: Transgene-derived overexpression of DmTPx1 confers resistance to paraquat toxicity and hyperoxia. Survival of two-days old adult male *P(TPx1⁺)* transgene-bearing flies, was compared to that of mutant *white* controls of the same age and genetic background after exposure (a) to 40mM Paraquat and (c) to 100% O_2 levels. Note enhanced survival of the DmTPx1-overexpressors under both experimentally induced conditions of oxidative stress. Similar results were obtained using the UAS/Gal4 induction system (b+d). Ubiquitous DmTPx1 expression was achieved by a *uastpx1* transgene in combination with the *actin-Gal4* driver. Experiments involved a minimum of 200 individuals (see Materials and Methods).

To determine the impact of augmenting DmTPx on resistance to paraquat toxicity, hemizygous *P(tpx1$^+$)*, homozygous *P(tpx1$^+$)* and *white* mutant adult males were exposed to 40mM paraquat (see Materials and Methods). *P(tpx1$^+$)* flies survived the paraquat treatment significantly better than the corresponding *white* mutant control animals (Fig. 3A). Enhanced resistance to paraquat was reproducibly exhibited by both independent transgenic strains. Similar results were also obtained at lower paraquat concentrations (data not shown).

To determine the impact of augmenting DMTPx on resistance to hyperoxia, flies of the same genotypes described above were exposed to 100% oxygen (see Materials and Methods). Transgenic *P(tpx1$^+$)* flies survived much longer than *white* mutant controls under hyperoxia (Fig. 3C).

We also took advantage of the UAS/Gal4 induction system (Brand and Perrimon, 1993) to ectopically overexpress DmTPx1 during development and in the adult. For this experiment, we cloned the *dmtpx1* cDNA into pUAST, *uastpx1* transgenic strains were generated by P-element transformation (Rubin and Spradling, 1982), and crossed to an actin-Gal4 driver line (Gonzales-Gaitan and Jäckle, 2000) to ubiquitously express DmTPx1 throughout the lifecycle. To assess the impact of ectopic overexpression of DmTPx1 on resistance to oxidative stress, *uastpx1/+; actgal4/+* and *+/+; actgal4/+* flies were exposed to paraquat and hyperoxia as described above. In Fig. 3B, results of the paraquat treatment are depicted. As shown in Fig. 3B, ectopic overexpression of TPx1 provided robust protection against paraquat toxicity but minimal if any protection against hyperoxia (Fig. 3D). Collectively, the results in Fig. 3 demonstrate that overexpression of DmTPx1 in either of two different modes

provides significant protection against experimentally applied oxidative stress. We conclude that DmTPx1 can function *in vivo* as an important component of the *Drosophila* antioxidant defense system.

Overexpression of DmTPx1 cannot substitute for the loss of Cat activity *in vivo*

DmTPx1 and Cat are both implicated in the removal of hydrogen peroxide (see above and Griswold *et al.*, 1993). They also appear to be expressed in similar patterns, at least in the *Drosophila* embryo (Fig 2 and Missirlis *et al.*, 2001). We therefore asked whether overexpression of *dmtpx1* could rescue the *cat*-null mutants as was previously shown in *Schizosaccharomyces pombe* (Yamada *et al.*, 1999). We generated flies carrying the *P(tpx1$^+$)* transgene on the second chromosome and the cat^{n1} allele on the third chromosome. Because cat^{n1} is a semi-lethal mutation, i.e. only 10% of the homozygous flies eclose successfully (Griswold *et al.*, 1993), we reasoned that if DmTPx1 can compensate for Cat, transgenic augmentation of *dmtpx1* expression would increase the eclosion efficiency of cat^{n1}. To do this, we crossed siblings of the genotype *w;P(tpx1$^+$)/+;*ca^{n1}*/TM3* and scored the progeny for the genotypes *w;+/+;*cat^{n1}/ca^{n1}, *w;P(tpx1$^+$)/+;*cat^{n1}/cat^{n1} and *w;P(tpx1$^+$)/P(tpx1$^+$);*cat^{n1}/cat^{n1}, on the basis of a white, orange and red eye color, respectively. No rescue would yield an expected Mendelian 1:2:1 segregation of the three genotypes, while metabolic rescue would increase the proportion of cat^{n1} homozygous adults carrying one or two *P(tpx1$^+$)* trangenes. The results (Table 2) show a 1:2:1 ratio of the three genotypes, indicating that the *P(tpx1$^+$)* transgene confers no capacity for enhanced survival on cat^{n1}. The same result is also obtained using control strains carrying a wildtype 3rd chromosome.

Table2. Non-Mendelian *P(tpx1$^+$)* segregation in Sod1 mutants

	$\frac{+}{+}$	$\frac{P(tpx1^+)}{+}$	$\frac{P(tpx1^+)}{P(tpx1^+)}$	total number of flies scored
+	1	2.1	0.9	n=769
cat^{n1}	1	2.0	0.7	n=485
$sod1^{n108}$	1	0.8	0.02	n=322
$sod1^{x39}$	1	0.7	0.08	n=141

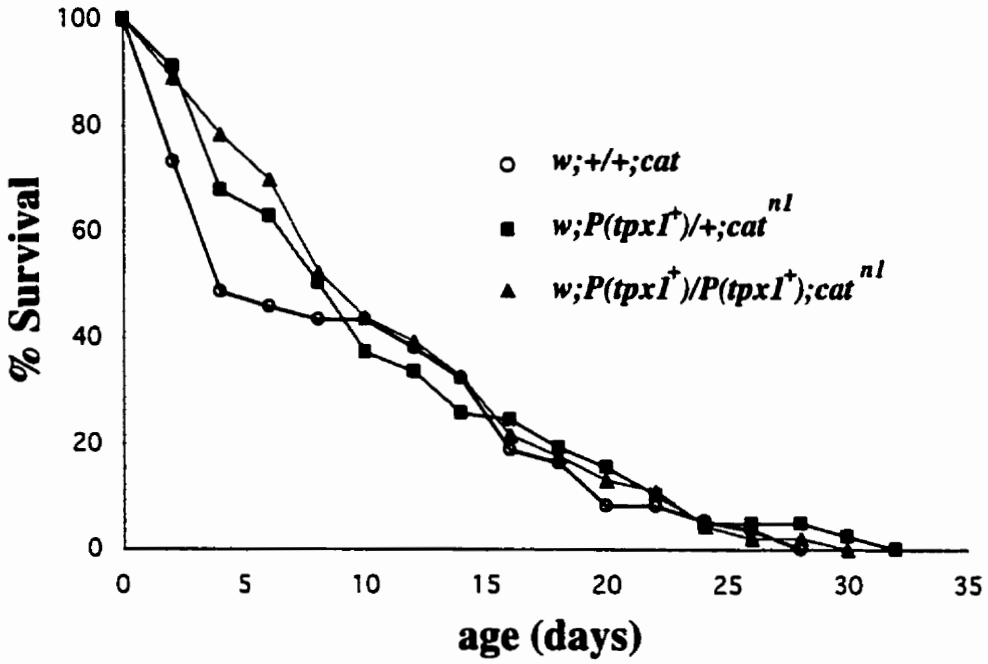

Figure 4: The reduced lifespan of cat^{n1} mutant flies cannot be restored by the presence of one or two extra *P(TPx1$^+$)* transgenes.

The capacity of DmTPx1 to compensate for Cat was also investigated by comparing the lifespans of the above three cat^{n1} genotypes. Consistant with the eclosion results, *P(tpx1⁺)* transgenes have no signifiant impact on the severely truncated lifespan of the cat^{n1} null mutant (Fig 4). We therefore conclude that, as assessed by restoration of lifespan or resistance to oxidative stress, DmTPx1 cannot functionally substitute for Cat.

Overexpression of DmTPx1 is toxic in *sod1* mutants

We continued to ask if *dmtpx1* functions in the redox system of *Drosophila* by investigating if it interacts genetically with the *sod1* mutants, which have been shown to be sensitive to as yet uncharacterized enhancer and supressor mutations (JP and AH; unpublished results). As described above for *cat*, we generated progeny from *inter se* crosses of *w;P(tpx1⁺)/+;sod1/TM3* flies and compared their relative viabilities. The results shown in Table 2 demonstrate unambiguously that the presence of extra copies of *dmtpx1* has a negative impact on *sod1* mutant flies. Two extra copies of P(*tpx1⁺*) dramatically reduces the frequency of eclosion of *sod1* mutants (Table 2; the ratio of their siblings with a wildtype second chromosome is 0.02 - 0.08, instead of 1) whereas one extra copy has a less pronounced effect (0.7-0.8 instead of 2). This result and the dosage-dependence effect were both confirmed using two independent null alleles of *sod1*, namely $sod1^{n108}$ and $sod1^{x39}$.

We also took advantage of a recombinant *DaGal4, sod1*^{x39} chromosome to drive ubiquitous expression of DmTPx1 and Cat in the sod^{x39} mutant. The results summarized in Table 3 indicate that overexpression of *uastpx1* does not have any positive impact on $sod1^{x39}$ viability. The reduction from 7.9% to 6.0% is not statistically significant ($p>0.05$). In

Table3. Effect of overexpressed hSod1, DmTPx1 and Cat on *sod1* mutant

activated transgene	copies	$\frac{sod1^{x39}}{TM3}$	$\frac{sod1^{x39}}{sod1^{x39}}$	$\frac{sod1^{x39}}{sod1^{x39}}$	$\frac{sod1^{x39}}{sod1^{x39}}$
		experimentally determined	expected according to Mendelian 2:1 ratio	experimentally determined	% of expected
+		4228	2114	168	**7.9**
uashsod1	2	1653	827	460	**55.7**
uastpx1	2	1741	871	52	**6.0**
uascat	2	1342	671	263	**39.2**

contrast, overexpression of *uascat*, enhances $sod1^{x39}$ viability ($\chi 2=99.2$; $P<0.0005$; Table3). Collectively, these results confirm that overexpression of DmTPx1 is harmful to flies lacking Sod1. In addition, the results provide evidence that overexpression of Cat can partially rescue the *sod1* mutant. Notably, they point out that DmTPx1 does not function in the same *in vivo* context as Cat. Increased Cat was also shown to partially rescue a loss-of-function mutation of thioredoxin reductase (*dmtrxr-1*) in *Drosophila* (Missirlis *et al.*, 2001). Overexpression of DmTPx1 in the $dmtrxr\text{-}1^{481}$ mutant proved to be deleterious (data not shown), arguing for a different mode of action than Cat in a different redox paradigm. Although both proteins can catalyze the removal of H2O2, it appears that their differential *in vivo* functions extend beyond their purely biochemical and enzymatic properties to reflect differences in their subcellular localization and to their dependence upon (TPx) or independence from (Cat) a continuous supply of reducing equivalents.

Materials and Methods

Isolation of genomic and cDNA clones of *dmtpx1*

PCR (Sambrook and Russel, 2001) was performed on genomic DNA isolated from wildtype (Oregon-R) *Drosophila melanogaster*. A pair of degenerate primers was designed against sequences corresponding to the conserved regions of the GPx proteins FPCNQFG and WNFEKFL respectively (Fig. 1). The primers used were GPx375-5' GCNTTYCCNTGYAAYCARTTYGG and GPx564-3' TCVATVARRAAYTTNGTRAARTTCCACTT. A band of approximatelly 200 bp was amplified and subcloned in PGEMT vector (Promega, Mannheim, Germany). Sequence analysis showed that it encoded for a homolog of the corresponding GPx family. Subsequently this clone was used as a probe for screening a *Drosophila* genomic library in the Lambda FIX II vector (Stratagene, Amsterdam, The Netherlands). We isolated and analyzed by Southern blotting 3 independent phages. A 7kb EcoRI genomic fragment containing the entire *dmtpx1* locus (5.5kb upstream - 1kb downstream) was isolated from one of the phages and subcloned in pBstKS resulting in pBstKStpx1gen.

In addition, an embryonic lambda ZAP cDNA library of Drosophila (Stratagene, Amsterdam, The Netherlands) was screened using the *tpx1* probe. We isolated and sequenced three clones, all of which contained the full-length cDNA of *tpx1* in pBst KS (pBstKStpx1cDNA).

Gene expression and purification of the recombinant proteins

The ORF of DmTPx1 was PCR amplified, using primers that inroduced an XhoI site at both the 5' and 3' ends. The primers used were TPx1GFP5' and TPx1SET3' CCGCTCGAGTCTAGACATCTACAGCAGC. The 500 bp fragment was XhoI digested and inserted in frame at the XhoI site of the vector pRSETA (Invitrogen, Karlsruhe, Germany) resulting in an aminoterminal hexahistidyl tagged fusion protein. The *E. coli* strain BL21 was used for expression of the *D. melanogaster* peroxidase gene. Competent cells were transformed with the respective PRSETAtpx1 plasmid. Five ml LB-medium was inoculated with a single colony and used as a starter culture for 250 or 500 ml cultures. Cells were grown at 37°C in LB medium containing ampicillin (100 μg/ml) to an OD_{600} of 0.5; subsequently, expression was induced by adding 1 mM IPTG. Cells were grown for additional four hours, harvested, and directly used for protein-purification or frozen at -20°C. For purification, the cells were disintegrated by sonication in the presence of protease-inhibitors. After centrifugation, the supernatant was loaded onto a Ni-NTA column equilibrated with 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. After washing the column with increasing imidazol concentrations, the protein was eluted with 50-75 mM imidazol; collected fractions were tested for enzymatic activity and for purity by 12% SDS gel electrophoresis. Active fractions were pooled, concentrated via ultrafiltration, and equilibrated with 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, prior to use. The concentrated peroxidase was stable at 4°C over weeks. Thioredoxin-1 of *Drosophila* and thioredoxin reductase of *Plasmodium falciparum* were recombinantly produced and purified as described previously (Kanzok *et al.*, 2001; Kanzok *et al.*, 2000). Cloning, expression and characterization of thioredoxin-2 from *Drosophila* will be described elsewhere (Bauer *et al.*, submitted). Protein concentrations were

determined spectrophotometrically on the basis of the calculated extinction coefficient of the respective protein.

Thioredoxin peroxidase assay

TPx-assays were carried out at 25°C in an assay mixture of 1 ml consisting of 50 mM Hepes, pH 7.2, with 100 µM NADPH, 100 mU *Plasmodium falciparum* thioredoxin reductase (as determined in the presence of 100 µM NADPH and 3 mM DTNB as substrate), 10 µM DmTrx1 or 2, and 200 µM of the second substrate (hydrogen peroxide (H_2O_2), t-butyl hydroperoxide (tBOOH), and cumene hydroperoxide, respectively). For further kinetic characterization DmTrx1 or 2 and concentrations of the second substrate were systematically varied. The assay was started with TPx and NADPH consumption ($\varepsilon_{340\,nm}$ = 6.22 mM^{-1} cm^{-1}) was monitored spectrophotometrically at 340 nm. In this coupled assay system constantly high concentrations of reduced thioredoxin were maintained by the NADPH/TrxR-system. The peroxidase activity effected by the TrxR/Trx system was monitored in each assay before the addition of DmTPx-1 and subtracted from the activity of the complete system.

Cell culture

The ORF of *dmtpx1* was PCR amplified, using primers that inroduced an XhoI site at both the 5' and 3' ends, and pBstKStpx1cDNA as the template DNA. The primers used were TPx1GFP5' CCGCTCGAGATGTCTGCTAACGG and TPx1GFP3' CCGCTCGAGGCACATCAACAGCAGC. The stop codon was mutated into a Leucine. The 500bp fragment was XhoI digested and inserted in frame at the XhoI site of the vector PEGFP N1 (Clontech, Heidelberg, Germany) resulting in a fusion protein of DmTPx1 with

EGFP. Note that this plasmid confirs kanamycin resistance to bacteria. *Drosophila* embryonic Schneider II cells were transfected with the PTPx1-EGFP plasmid. Transfections were done using Effectene Transfection Reagent kit from Quiagen according to their procedures. Cells were viewed using fluorescent or confocal microscopy. For Dapi staining, cells were incubated into 5μM DAPI in PBT for 30' min. As a mitochondrial marker we used Mito Tracker Orange CM-H2TMRos (from Molecular Probes), and followed the instructions of the provider.

Drosophila stocks and transgenics

All flies were kept under standard conditions as described (Forjanic *et al.*, 1997). Genetic crosses were performed at 25°C. Mutants used were cat^{n1} (Griswold *et al.*, 1993), sod^{n108} (Phillips *et al.*, 1989), sod^{k39} (Parkes *et al.*, 1998), $dmtrxr\text{-}1^{481}$ (Missirlis *et al.*, 2001). Balancer chromosomes are described in Lindsley & Zimm (1992). The *DaGal4, sod*^{k39} recombinant was a gift from Tony Parkes. *Actin-Gal4* is described in (Gonzales-Gaitan and Jäckle, 2000), *1407-Gal4* in (Ferveur *et al.*, 1997) and *FB-Gal4* in (ref). For *P(tpx1⁻)* transgene construction, pBstKStpx1 was digested with EcoRI and the 7kb genomic fragment containing 5.5 kb upstream genomic sequences, the entire ORF and 1 kb downstream sequences of *tpx1* was inserted in the EcoRI site of PCaspeRAUGß-Gal 5' (Thummel and Pirrotta, 1992) in front of the *ß-Gal* gene. For *uastpx1*, pBstKStpx1 was digested EcoRI/XhoI and the full length cDNA of *tpx1* was inserted in PUAST using the same restriction sites. For *uastpx1-EGFP*, PTPx1-EGFP (see cell culture section above) was digested with BglII/NotI and inserted directionally into the PUAST vector using the same sites. PCaspeRß-Galtpx1, Puastpx1 and Puastpx1-EGFP were injected in fly embryos

(Rubin and Spradling, 1982), and transgenic lines were selected and named respectively, *P(tpx1⁻)*, *uastpx1* and *uastpx1-EGFP*.

Assay for paraquat toxicity

Adult males (24- to 48- hours old) were exposed for 48 h at 25 °C in vials (20 flies per vial) containing one disk of Whatman 3M filter paper (diameter 32mm) saturated with 400 µl of an aqueous solution of 40 mM paraquat (methyl viologen) in 1% sucrose. Survival of flies was scored per vial after indicated time intervals of exposure to paraquat. We show results of a representative experiment with 300 flies. Controls exposed at 1% sucrose showed no lethality during the period of the assay.

Hyperoxia assay

Newly eclosed adult males (24- to 48-hours old), 200 individuals per genotype, were collected and transferred to standard shell vials containing commeal (10 individuals per vial) and covered with nylon mesh. They were subsequently exposed to 100% O2 at 21-23°C and 101.3kPa in a leucite chamber (O2 concentration was monitored using a Servomex model 570 portable oxygen analyzer). At daily intervals vials were removed and flies scored for survival. Chamber recharge time was less than 10 min.

Lifespan measurements

Siblings (0-24hrs old) of the genotypes $w;+/+;cat^{nl}/ca^{nl}$, $w;P(tpx1^{+})/+;cat^{nl}/cat^{nl}$ and $w;P(tpx1^{+})/P(tpx1^{-});cat^{nl}/cat^{nl}$ were kept in small food vials at 25°C and transferred into new

vials every second day. Survival of flies was monitored at 48 hr intervals. A minimum of 40 flies per genotype was used.

Genetics and statistics

All crosses described in text were performed at 25°C and on standard yeast-containing, cornmeal food (Forjanic *et al.*, 1997). Results presented in Table3 were compared pair-wise using chi-squared analysis as described in Schor (1968). The chi-squared determination for each comparison was calculated by the formula $[(a*d-b*c)^2 * (a+b+c+d)]/[(a+b)(c+d)(a+c)(b+d)]$.

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CONCLUSIONS

1) The antioxidant defense system of *Drosophila melanogaster* includes Sod1, Sod2, Cat and the newly-identified TrxR-1 and DmTPx1 that are conserved in mammals. A notable exception is the absence of GR from *Drosophila*. TrxR-1, a GR-like protein, uses the Trx system for reduction of GSH (Kanzok et al., 2001).

2) The genes *sod1*, *cat*, *Trxr-1* and *dmtpx1* are expressed in overlapping tissue- and temporal-specific patterns during *Drosophila* embryogenesis. Sites of expression include tissues that may require robust protection from ROS toxicity such as prospective germ cells, the midgut, fat bodies and oenocytes.

3) Two *Drosophila* antioxidant genes, *Trxr-1* and *dmtpx1*, were molecularly and biochemically characterized. The *Trxr-1* transcript is alternatively spliced and thus encodes cytoplasmic and mitochondrial TrxR isoforms with distinct N-terminal amino acid sequences but which exhibit similar biochemical properties. *dmtpx1* codes for a protein which by molecular criteria is similar to mammalian GPxs, but functions biochemically as a TPx.

4) The two alternatively spliced variants of *Trxr-1* were shown to have distinct functions *in vivo*. Transcript-specific *Trxr-1* mutations confer similar phenotypes, e.g. pupal lethality and

reduced adult lifespan. The absence of both variants leads to early larval death. Transgene-derived overexpression of the *Trxr-1* variants in the different mutants reveals that they cannot substitute for one another.

5) Transgene-derived overexpression of DmTPx1 augments protection against oxidative stress.

6) A complex pattern of genetic interactions between the different components of the antioxidant defenses was elucidated. Overexpression of Cat partially compensates for a reduction of TrxR-1 activity and for the absence of Sod1 activity. In contrast, overexpression of DmTPx1 is detrimental in *Trxr-1* loss-of-function mutants and *sod1* lack-of-function mutants. Moreover, overexpression of DmTPx1 is without effect in the Cat lack-of-function mutant. Hence, despite the participation of both Cat and DmTPx1 in the removal of intracellular H_2O_2, as occurs in mammalian cells, Cat and DmTPx1 appear to function in different biological pathways in *Drosophila*. Finally, genetic analysis of *sod1*, *cat* and *Trxr-1* mutants suggests that the burden of ROS metabolism in *Drosophila* is shared by these enzymes.

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APPENDIX

A question arising from the work presented in my thesis was whether overexpression of DmTPx1 will result in a corresponding increase of the fly's average lifespan. In order to answer this question, I used the stocks described in my paper "A thioredoxin-dependent peroxidase from *Drosophila melanogaster* protects from oxidative stress". A critical factor that should be considered when doing lifespan comparisons is the genetic background of experimental and control animals. In the first set of experiments, I controlled for the genetic background effect by two independent ways:

a) The homozygous *P(tpx1$^+$)* animals used for the analysis, were brought to homozygocity by intercrosses. Thus, experimental flies were not crossed to a different genetic background than that of the control animals, which were the same parental *white* mutant strain which was used for generating the *P(tpx1$^+$)* transformants.

b) I used independent *P(tpx1$^+$)* insertions on different chromosomes, to verify the results.

The experiment was reproduced twice, at different times of the year and with different batches of food. The results show that overexpression of TPx1, via a genomic transgene, leads to a significant extension of lifespan (Fig. 1).

In a second set of experiments aimed to further control for the genetic background, I maintained the *P(tpx1$^+$)* stocks as heterozygous flies for 2-5 generations, allowing for

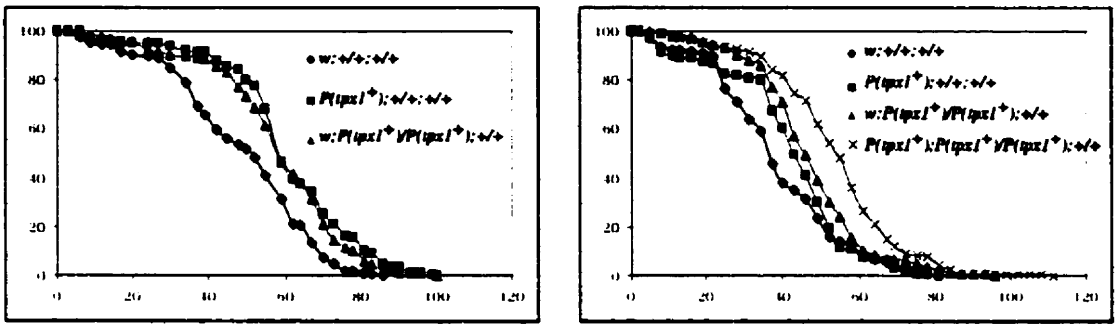
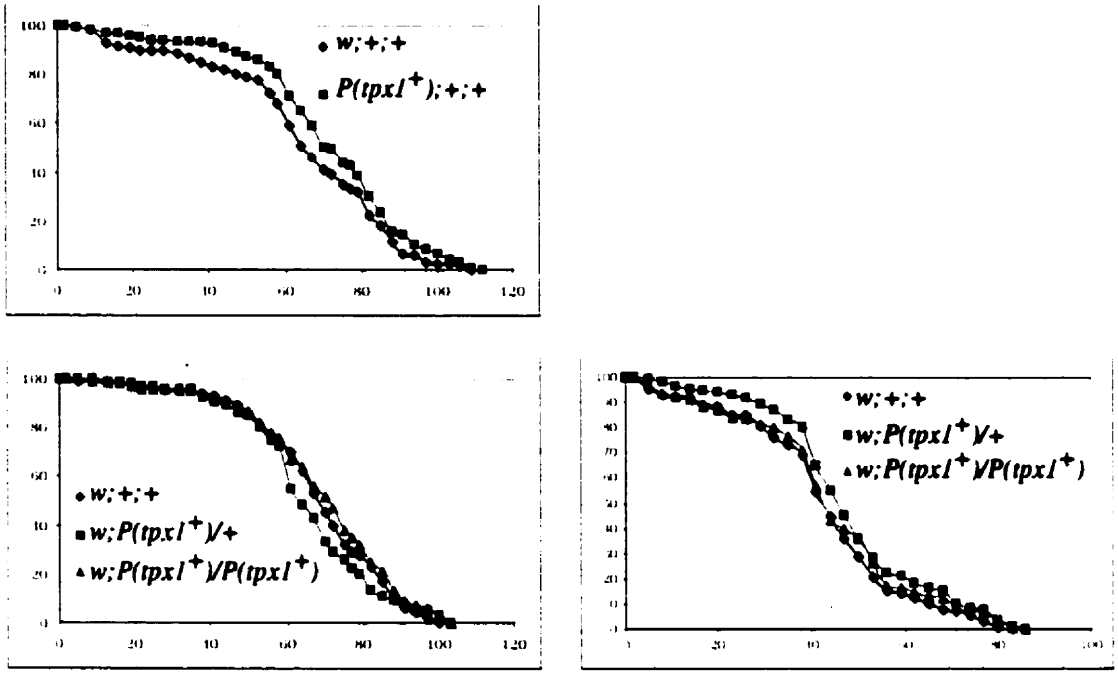


Figure 1

A)



B)

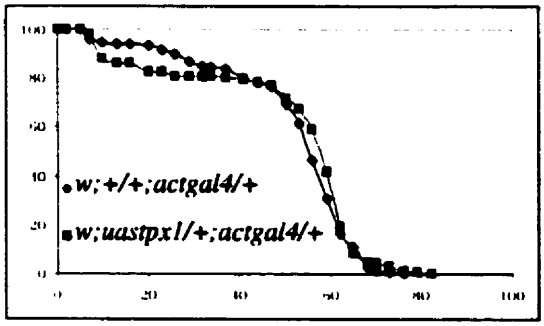


Figure 2

recombination to take place in all parts of the genome. Next, I performed sibling analysis, where control, heterozygote and homozygote animals derived from the same genetic pool. The results showed no significant difference between experimental and control animals (Fig. 2A). This result implies that the positive effect seen in the first set of experiments (Fig. 1) is due to a genetic background difference of the compared animals, but not directly associated with the presence of the *P(tpx1$^+$)* transgene.

Could this favorable genetic background (in terms of increased lifespan) arise two times independently by chance? Although this would provide an explanation for the results I obtained, it seems quite an unlikely possibility. I therefore propose the following hypothesis: **The presence of a DmTPx1-transgene in a *Drosophila* population caused genetic selection of alleles that result in increased lifespan.** This hypothesis is testable: It predicts, that if homozygous *P(tpx1$^+$)* stocks and *white* mutant controls are re-established from the heterozygous parents and reproduce separatelly for a few generations they will show again a difference in their lifespans, similar to the one I observed before.

Finally, in a single experiment, I monitored if ubiquitous overexpression of TPx1 would increase the lifespan of flies, using the *actinGal4/uastpx1* system (Fig 2B). No difference between flies containing and flies lacking the transgene was observed. However, in this experiment both the driver and the *uastpx1*-transgene were present as single copies. In addition, the Sod1-overexpression-dependent increase of *Drosophila's* lifespan was shown to depend also on the choice of the Gal4 driver (Parkes et al., 1999). Last, if the hypothesis mentioned above holds true, one would not expect an extension of lifespan by inducing

overexpression of TPx1 in a given individual. In summary, more experiments are needed before we can draw final conclusions with respect to whether or not overexpression of DmTPx1 affects the lifespan of flies.