Thioredoxin peroxidases can foster cytoprotection or cell death in response to different stressors: over- and under-expression of thioredoxin peroxidase in *Drosophila* cells

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Recently, we identified a set of five genes constituting the peroxiredoxin gene family in *Drosophila melanogaster* [Radyuk, Klichko, Spinola, Sohal and Orr (2001) Free Radical Biol. Med. 31, 1090–1100]. This set includes two abundant thioredoxin peroxidase (TPx) species, namely *Drosophila* peroxiredoxin DPx-4783, a cytosolic TPx and DPx-5037, a mitochondrial TPx. Overexpression of either one of them in *Drosophila* S2 cells conferred increased resistance to toxicity induced by hydrogen peroxide, paraquat or cadmium. To understand further the functional roles of these enzymes *in vivo*, we report in the present study the effects of decreased expression, using RNA interference, on the response of S2 cells to different stressors. When either of the TPxs was blocked, cells became relatively more susceptible to oxidative stress caused by

exposure to hydrogen peroxide or paraquat, but were unaffected when challenged with copper and heat stress. In contrast, TPx overexpressing cells were more susceptible to copper and heat stress when compared with control cells and exhibited DNA fragmentation. Furthermore, when cells were supplemented with *N*-acetyl-L-cysteine together with copper, there was a clear negative effect on cell survival, which was exacerbated by TPx overexpression. Manipulations in the levels of TPxs demonstrated that, under different stress conditions, these enzymes might have both beneficial and detrimental effects on *Drosophila* cell viability.

Key words: oxidative stress, peroxiredoxin, RNA interference.

INTRODUCTION

The antioxidative function of the thioredoxin peroxidases (TPxs), which eliminate hydrogen and organic peroxides, using thioredoxin as the electron donor, has been amply demonstrated. Since insects possess no detectable glutathione peroxidase activity, it is supposed that TPxs play a particularly central role in the enzymic removal of reactive oxygen species (ROS) in this group. Recent evidence that TPx can also possess peroxynitrite reductase activity further underscores the capacity of these enzymes to play a protective role against reactive species.

Previously, we reported the identification of three different TPxs in *Drosophila melanogaster*, which have distinct patterns of subcellular localization and expression [1]. Two of them (associated with cytosol and mitochondria) appear to be related to TPxs in mammals and yeast; they are relatively abundant, and are presumed to play a housekeeping role in mitigating the effects of pro-oxidants.

The purpose of the present study was to elucidate further the functional role of TPxs in *D. melanogaster* cells, using under- or overexpressing cell lines, exposed to treatments that have been demonstrated to elicit oxidative stress. Underexpressing cell lines were created by treatment with gene-specific double-stranded RNA (dsRNA), where expression of individual TPx genes was essentially blocked. Results indicate that these cell lines were susceptible to hydrogen peroxide as well as the superoxide generator paraquat, whereas stably transfected cell

lines overexpressing TPx were more resistant to oxidative stress. In contrast, with some treatments anticipated to lead to increased oxidative stress, such as elevated temperature or exposure to copper, cells overexpressing TPx demonstrated increased susceptibility, whereas underexpressors showed no increase in susceptibility relative to controls (and in fact were more resistant to heat treatment). In other words, these enzymes can have both beneficial and detrimental effects on cell viability, depending on the type of stressor.

MATERIALS AND METHODS

Cell culture

Schneider S2 cells were propagated in complete *Drosophila* expression system (DES) medium (Invitrogen, Carlsbad, CA, U.S.A.), containing 10 % foetal bovine serum and 50 µg/ml gentamicin (Sigma, St. Louis, MO, U.S.A.). For stable cell lines, the medium was supplemented with 300 µg/ml hygromycin (Sigma). Cell cultures were split 1:2 with fresh medium every 3–5 days, retaining one-third of the conditioned media.

dsRNA production and conditions for RNA interference (RNAi)

Ambion MAXIscript(tm)T7/T3 kit (Ambion, Austin, TX, U.S.A.) was used to generate dsRNAs specific for the *Drosophila* peroxiredoxin DPx-4783 and DPx-5037 genes. For the sake of

Abbreviations used: BSO, buthionine sulfoximine; DES, *Drosophila* expression system; dsRNA, double-stranded RNA; DPx, *Drosophila* peroxiredoxin; NAC, *N*-acetyl-L-cysteine; N₂ph, 2,4-dinitrophenyl; RNAi, RNA interference; ROS, reactive oxygen species; rp, ribosomal protein; RT, reverse transcriptase; TPx, thioredoxin peroxidase; cTPx, cytosolic TPx; mtTPx, mitochondrial TPx.

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Figure 1 Primers used for *in vitro* RNA generation and RT-PCR

(A) Primer names and sequences are shown with the T7 promoter sequence shaded. Primer positions for the two differentially spliced mRNAs [first variant (B); second variant (C)] of the cTPx gene are shown. Primer positions for the mRNA of the mtTPx gene (D) are shown. T7 sequence is indicated by dotted lines. Bars represent coding parts of the cDNAs, bold lines represent the non-coding 5' and 3' regions, and the regular line represents that part of the vector sequence containing T7 promoter sequence.

clarity, these genes will henceforth be referred to as cytosolic TPx (cTPx) and mitochondrial TPx (mtTPx) respectively, reflecting their cytosolic and mitochondrial localizations. DNA fragments for *in vitro* transcription were prepared by PCR using primers specific to each gene containing a 5'-end T7 RNA-polymerase-binding sequence as indicated in Figure 1. Two fragments, approx. 700 bp, were generated for the differentially spliced cTPx gene by using the T7-4-F1/T7-4-R and T7-4-F2/T7-4-R primer pairs. These fragments differ only in their 5' non-coding regions. A 300-bp DNA fragment for the mtTPx gene was generated by primers T7-5-F/T7-5-R and contains a 5' non-coding sequence and a part of the gene coding region. A 1000-bp fragment for the mtTPx gene was generated by primers T7-5-F/T7 encompassing the entire cDNA sequence. PCR products were purified by the Nucleotide Removal kit (Qiagen, Valencia, CA, U.S.A.) and transcribed *in vitro*, using the RiboProbe[®] In Vitro Transcription System (Promega, Madison, WI, U.S.A.). The RNA products were

precipitated by ethanol, dissolved in water and heated at 65 °C for 30 min followed by slow cooling to room temperature (25 °C) to allow annealing of the RNA. As a negative control, a 3 kb dsRNA was generated from the *Escherichia coli* pGEM[®] vector (Promega) using the T7 and T3 primers. The concentration of the dsRNAs was adjusted to 3 µg/µl and used in the treatment of S2 cells.

The introduction of dsRNA into *Drosophila* S2 cells was performed essentially as described by Clemens et al. [2]. Briefly, 1 ml of cells for each reaction was placed in a 35 mm plate at a final concentration of 1×10^6 cells/ml in DES serum-free medium (Invitrogen). dsRNAs (5–20 µg) were added to an approximate concentration of 40 nM and plates were swirled vigorously and subsequently incubated for 1 h at room temperature. After incubation, 2 ml of the complete DES medium was added and cultures were incubated for 3 days to allow protein turnover.

Reverse transcriptase (RT)-PCR and Western-blot analysis

Aliquots (100 μ l) of the cell suspension were centrifuged at 1000 g and protein lysates for immunoblot analysis were extracted by homogenizing the pellet in 20 μ l of lysis buffer containing protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) as described previously [3]. Protein extracts were then fractionated by SDS/PAGE, followed by transfer to PVDF membrane (Millipore, Bedford, MA, U.S.A.). Protein carbonyl content was determined as described by Yan et al. [4]. To visualize cTPx protein, antibodies raised against recombinant cTPx protein were used, as reported previously [1]. In a similar manner, mtTPx recombinant protein was purified from *E. coli* cells as described above and sent to Covance Research Products (Denver, PA, U.S.A.) for the preparation of antisera in rabbits. The immunoblots were visualized using the ECL[®] + Western-blotting detection system (Amersham) according to the manufacturer's instructions. Densitometric scanning of the gels or films was performed by the digital imaging analysis system with AlphaEase Stand Alone Software (Alpha Innotech Corp., San Leandro, CA, U.S.A.).

RNA for RT-PCR analysis was isolated using TRIzol[®] LS Reagent (Gibco BRL, Gaithersburg, MD, U.S.A.) and 200 ng was used for each assay. Reverse transcription was performed as reported previously [1]. Reverse primers were 4783-R for the cTPx gene and 5037-R for the mtTPx gene as indicated in Figure 1. RNA-DNA hybrids were denatured by heating for 5 min at 99 °C. The cDNA preparations were used in PCRs with primer pairs for each TPx gene and primers for the ribosomal protein (rp) 49 gene as the control. Forward primers were 4783-F-1 for the cTPx-1 variant, 4783-F-2 for the cTPx-2 variant and 5037-F for the mtTPx gene. Reverse primers used for PCR were the same as those used for reverse transcription. Primers for the amplification of rp49 were the same as used previously [5].

Experimentally induced stress and cell viability

Untransfected Schneider S2 cells as well as stable transfected cell lines overexpressing each of the TPx genes [1] or transfected with vector only and cell lines treated with dsRNAs were grown at 22–24 °C in DES complete media for 1–2 days until a density of approx. 10⁶ cells/ml was reached. Cell cultures were exposed to 10 mM paraquat, 20 mM hydrogen peroxide, 0.5 mM CdCl₂, 2 mM CuSO₄, or incubated at 32 and 42 °C for the different time intervals. In addition, the cells were incubated with 0.5 mg/ml *N*-acetyl-L-cysteine (NAC; Sigma) or 1 mM buthionine sulfoximine (BSO; Sigma) for 2–24 h before exposure to heat, heavy metal or hydrogen peroxide. After treatment, cells were stained with Trypan Blue according to the manufacturer's instructions (Invitrogen) and counted in a haemocytometer. Viability was determined as the ratio of the percentage of live cells to the total number of cells.

Apoptosis

DNA fragmentation as a characteristic of apoptosis was evaluated by agarose-gel electrophoresis [6]. Briefly, total DNA was isolated from S2 cells by using the Puregene kit (PCG Scientific Corp., Frederick, MD, U.S.A.) according to the manufacturer's instructions, except that DNA precipitation by propan-2-ol was performed by incubation on ice for 30 min. DNA concentration was determined spectrophotometrically at 260 nm and equal amounts were loaded on to a 1.2% agarose gel. After electrophoresis, gels were examined under UV.

Figure 2 RT-PCR analysis of the dsRNA-mediated impact on TPx mRNA transcription in *Drosophila* S2 cells

Transcriptional levels of variant 1 of the cTPx gene (cTPx-1) are shown in (A) and of the mtTPx gene (mtTPx) in (B). PCR signals for cTPx were obtained using the primers 4783-F-1/4783-R, and the signals for mtTPx were obtained using the primers 5037-F/5037-R. As an internal control, PCR fragments were generated using primers for the rp49 gene. RNA samples for PCR were extracted from cell cultures treated with 700 bp dsRNA generated from the cTPx-1 variant (lane 1), 700 bp dsRNA from the cTPx-2 variant (lane 2), 300 bp dsRNA from mtTPx (lane 3), 1000 bp dsRNA from mtTPx (lane 4), non-specific dsRNA (lane 5) and from untreated cells (lane 6).

Statistical analysis

All experiments were performed three times and the results are means $\pm$ S.D., determined using INSTAT 3.00 or GraphPad Prism 3.0a software.

RESULTS

Gene silencing in *Drosophila* cells

The efficiency and kinetics of gene silencing, mediated by RNAi, were determined in S2 cells at 2, 3, 7 and 45 days after treatment, using both immunoblot analysis and RT-PCR. As reported previously [1], the cTPx gene (DPx-4783) has two alternatively spliced transcripts that differ from each other in their 5' non-coding segments. RT-PCR was used to examine the efficiency of gene silencing of both variants by two dsRNAs, 700 bp, that had approx. 500 bp of coding domain in common as well as upstream sequence specific to each of the two variants. Relative abundance was first assessed with primer pairs that were within the domains used to generate the dsRNAs. On the 7th day after treatment, a marked perdurance of the dsRNAs was observed (results not shown), indicating that their propagation was quite efficient.

To differentiate between endogenous mRNAs and the added dsRNAs, primer pairs (4783-F-1/4783-R and 4783-F-2/4783-R) were devised to target mRNA sequence outside the region used in the generation of dsRNA. A severe reduction in variant 1 mRNA levels in the cell line, treated with cTPx-1 dsRNA (Figure 2A, lane 1), was found approx. 3 days after treatment. cTPx-1 transcript levels were not altered in control non-treated cells (Figure 2A, lane 6), in cells treated with non-specific dsRNA (lane 5) or with dsRNA targeting the mtTPx gene (lanes 3 and 4). Partial suppression of mRNA levels was observed in cells treated with the dsRNA, which specifically targeted the cTPx-2 variant (lane 2).

For the mtTPx gene, the primers 5037-F/5037-R were used to identify specifically the mRNA rather than the dsRNA in the cell line treated with the 300 bp dsRNA. The use of a 300 bp dsRNA did not result in discernible gene silencing, as measured by transcript levels. However, when a 1000 bp dsRNA was used, a significant decrease in RNA levels relative to controls was observed approx. 7 days after treatment (Figure 2B, lane 4).

Figure 3 Immunoblot analysis of proteins isolated from S2 cell lines after exposure to dsRNAs

Cells were treated with dsRNAs and crude lysates were prepared on day 3 and subjected to immunoblot analysis. Cells were treated with 300 bp dsRNA corresponding to mtTPx (lane 1), 1000 bp dsRNA corresponding to mtTPx (lane 2), 700 bp dsRNA from the cTPx-1 variant (lane 3), 700 bp dsRNA from the cTPx-2 variant (lane 4), non-specific dsRNA (lane 5), or untreated (lane 6). Signals were obtained with cTPx, mtTPx and CuZn-superoxide dismutase antibodies.

To assess the effects of RNAi on protein production, immunoblot analysis was performed using antibodies raised against cTPx and mtTPx proteins. After treatment with either cTPx variant, a significant decrease in the level of cTPx protein was observed by day 2, and by day 3 it was virtually undetectable (Figure 3B, lanes 3 and 4). At the same time, a significant decrease in the level of mtTPx protein was observed in the cell line treated with 1000 bp dsRNA of the mtTPx gene (Figure 3A, lane 2) and to a lesser extent in the cell line treated with 300 bp dsRNA of the mtTPx gene (Figure 3A, lane 1). Antibodies raised against CuZn-superoxide dismutase protein were used to control protein-loading amounts (Figure 3C). The block in cTPx protein accumulation continued for 45 days in the cell line treated with the cTPx-1-specific dsRNA variant and reached pretreatment levels only after 60 days (results not shown). Cell lines underexpressing TPx were first assessed in the absence of any environmental challenge with respect to steady-state levels of oxidative damage and susceptibility to apoptosis. To estimate the impact of TPx underexpression on steady-state levels of oxidative damage, crude cell lysates were analysed for protein carbonyl content, using anti-2,4-dinitrophenyl (N_2 ph) monoclonal antibodies (Sigma), and the results are presented in Figure 4(A). The carbonyl content was substantially lower in the control line when compared with cell lines where the production of TPx was blocked. In addition, preliminary analysis indicated that the cell lines underexpressing TPx showed a greater susceptibility to apoptosis, as shown by the ladderized DNA fragmentation observed in Figure 4(B).

Effects of over- and under-expression of TPx genes on S2 cell viability under different stress conditions

To assess the impact of the over- and under-expression of cTPx and mtTPx genes on cell physiology, cells were challenged with different stressors supposed to increase oxidative stress. In these experiments, only those 'null' lines were used where phenotype was established and confirmed; specifically, the cell line cTPx-RNAi, generated by treatment with 700 bp dsRNA derived from cTPx-1, and the cell line mtTPx-RNAi, generated by treatment with 1000 bp dsRNA derived from mtTPx. As negative controls, cell lines treated with non-specific dsRNA and cell lines transfected only with the pMTV5 vector were used.

Effects of hydrogen peroxide and paraquat

It was demonstrated previously that the cTPx and mtTPx gene products possess thioredoxin-dependent peroxidase activity

Figure 4 Analysis of protein and DNA extracts from RNAi cell lines

(A) Immunoblot analysis of protein carbonyl content in different cell cultures. Protein extracts were subjected to dinitrophenyl hydrazine treatment and separated by PAGE followed by immunoblot analysis using anti- N_2 ph antibodies. (B) Gel electrophoresis of DNA isolated from different cell cultures. Total DNA preparations (5 μ g) were loaded for each sample. Samples isolated from cells treated with dsRNA derived from the cTPx-1 gene variant are denoted as cTPx, whereas samples from cells treated with dsRNA derived from the mtTPx gene are denoted as mtTPx. Control, S2 cells treated with non-specific dsRNA; M, the Hi-Lo DNA molecular-mass marker. Arrows indicate DNA fragments that correspond to 180 bp increments.

in vitro and protect S2 cells against oxidative damage caused by hydrogen peroxide exposure [1]. In the present study, we observed that cell lines with decreased TPx expression were more susceptible to hydrogen peroxide exposure, as measured by cell viability (see Figure 5A). Similar results were obtained when cells were challenged with paraquat (Figure 5B). Cell lines overexpressing either cTPx or mtTPx proteins were more resistant to paraquat relative to vector-only cell lines (Figure 5C). Taken together, these observations strongly suggest that cTPx and mtTPx play an antioxidative cytoprotective role in *Drosophila* cells.

Effects of temperature

Cells, which were under- and overexpressing TPxs, were exposed to heat stress (32 °C) and heat shock (42 °C). In cell lines lacking either cTPx or mtTPx, there was no detectable difference in cell viability relative to controls under heat shock (results not shown) and there was some increase in cell viability for the dsRNA-treated cells at 32 °C that was abrogated after 48 h of incubation (Figure 6A). Cells overproducing either cTPx or mtTPx were more susceptible to heat treatment at both 32 and 42 °C. At 42 °C, a difference in viability was first observed as early as 30 min after the initiation of heat shock (Figure 6B), whereas the effects of heat stress were observed after 24 h (Figure 6C).

Effects of heavy metals

The concentrations of the metals (0.5 mM $CdCl_2$ and 2 mM $CuSO_4$) were selected based on their ability to cause 40–60% cell death in control cells after a 24 h incubation period. Cells subjected to dsRNA treatment did not differ from control cells in

Figure 5 S2 cell viability after treatment with hydrogen peroxide and paraquat

S2 cells depleted of cTPx (cTPx-RNAi) or mtTPx (mtTPx-RNAi) were exposed to 20 mM hydrogen peroxide (A) or 10 mM paraquat (B). Cell lines overexpressing cTPx (cTPx[+]) or mtTPx (mtTPx[+]) were exposed to 10 mM paraquat (C). As a control for cTPx-RNAi and

their resistance to either cadmium or copper (results not shown). Cell lines overexpressing either of the TPx variants were more resistant to cadmium exposure when compared with control cells (Figure 7A). In contrast, treatment with 2 mM CuSO₄ led to increased mortality in cell lines overexpressing either cTPx or mtTPx (Figure 7B).

Effects of NAC and BSO

Different agents that modify cellular glutathione concentrations [7,8] and thereby increase or decrease oxidative stress were tested for their ability to sensitize or protect S2 cells exposed to different environmental insults. Cells were exposed either to 0.5 mg/ml NAC, a glutathione precursor, or to 1 mM BSO, a glutathione-depleting agent. NAC and BSO, taken separately, had no effect on cell survival characteristics at the chosen concentrations for a period of 24 h. After 36 h of incubation with BSO, cell viability decreased to approx. 70% in control cells, whereas a 48 h incubation period was required to achieve a comparable effect in the cTPx cell line (results not shown). The cell line overexpressing mtTPx remained 90–95% viable after 48 h incubation with BSO, indicating significant sparing effects of TPx overexpression at decreased glutathione levels.

No statistically significant differences were observed between samples subjected to heat stress (Figure 8A) or heat shock (results not shown) either alone or in combination with NAC or BSO.

Cytotoxicity after 24 h exposure to cadmium, which resulted in more than 50% cell mortality in control cells, was substantially reversed in cells exposed to NAC, which was comparable with that observed for cells overexpressing TPx (Figure 8B). When co-treated with cadmium and BSO, the TPx overexpressing cell lines were more resistant to cadmium when compared with control cells. The effects of copper treatment contrasted markedly with those of cadmium (Figure 8C). In control cells, a combination of copper and NAC led to a decrease in cell viability when compared with cells treated with copper alone and this effect was significantly enhanced in cells overexpressing cTPx and to a greater extent in cells overexpressing mtTPx. On the other hand, BSO treatment conferred greater resistance to cells overexpressing TPx relative to controls. To complement this analysis, cells underexpressing either cTPx or mtTPx were exposed to similar treatments. These RNAi cell lines did appear to exhibit a slightly greater resistance to Cu + NAC or Cu + BSO in combination, but the differences were not statistically significant (results not shown).

Overexpression of TPx levels, carbonyl content and DNA fragmentation analysis

Protein carbonyls were measured to assess the levels of oxidative damage caused by different stressors (Figures 9A, 9C and 9F). In control cells, treatment with paraquat, cadmium and hydrogen peroxide elicits more carbonyl accumulation relative to non-treated cells as well as cells subjected to elevated temperature and treatment with copper (Figure 9A). The effect was reduced for paraquat and hydrogen peroxide treatments in cell lines overexpressing TPx (Figures 9C and 9F). No correlation was

mtTPx-RNAi cells, a mock-treated cell line was used. As a control for cTPx[+] and mtTPx[+] cell lines, a pMTV5 vector-transfected cell line was used. Percentage cell survival was determined at various time intervals. Bar charts show cell survival of the experimental cell lines relative to controls. Inset: percentage of live cells for control and experimental cell lines. Statistically significant differences (**P* < 0.05) between treated and non-treated cells are indicated. Note that the vector-only control (C) is more susceptible to paraquat when compared with the mock-treated control (B), presumably due to the presence of the additional genetic component.

Figure 6 S2 cell viability after heat stress and heat shock conditions

S2 cells depleted of cTPx (cTPx-RNAi) or mtTPx (mtTPx-RNAi) were exposed to a 32 °C heat stress (A). Overexpressing cell lines cTPx[+] and mtTPx[+] were exposed to a 42 °C heat shock (B) or a 32 °C heat stress (C). Percentage cell survival was determined at the indicated time intervals. Bar charts show cell survival of the experimental cell lines relative to controls.

observed between the levels of protein carbonyls and the levels of overexpressed TPxs as measured by immunoblot analysis with anti-V5 antibodies (Figures 9E and 9H). For example, expression levels were similar in samples treated with hydrogen peroxide, paraquat or heat, but the levels of protein carbonylation were higher in samples treated with paraquat and hydrogen peroxide when compared with heat (Figures 9C, 9E, 9F and 9H). These results have been confirmed using antibodies specific for cTPx and mtTPx (results not shown), showing that, in all instances, TPx expression in the overexpressing cell lines was elevated relative to the endogenous levels of the control cells. Finally, the significant variations in TPx expression observed in response to different stressors in the present study may be partly ascribed to transcriptional control, but additional levels of post-transcriptional or post-translational regulation cannot be ruled out.

Analysis of DNA fragmentation has been used to evaluate apoptotic changes in cell lines exposed to different stress conditions (Figure 10). No DNA degradation was observed in samples isolated from non-treated cells and from cells subjected to short periods of exposure to hydrogen peroxide, heat shock and treatment with cadmium. Barely detectable DNA laddering was observed in samples after paraquat treatment. A typical internucleosomal fragmentation in 180–200 bp multiples was observed after longer exposures to hydrogen peroxide, heat stress and treatment with copper. In samples treated with copper and heat stress, DNA fragmentation appeared more pronounced in cell lines overexpressing TPx when compared with the control.

DISCUSSION

The use of RNAi in *Drosophila* cell culture has proved to be a very effective and rapid means to characterize possible functional roles of the TPx genes by permitting significant and specific down-regulation. A significant reduction in mRNA levels was observed approx. 72 h after dsRNA administration, which is similar to that reported previously [9]. A surprising finding was that the RNAi effect was relatively long lasting, up to 45 days in one case, which suggests that the dsRNA is quite stable and can cause effective gene silencing at very low levels. These findings are consistent with the notion of a post-transcriptional mechanism where a nuclease incorporates the dsRNAs as guide to target specifically the mRNAs, as proposed by Hammond et al. [10].

The efficiency of RNAi was dependent on the length and sequence of the dsRNAs. Consistent with other reports for *Drosophila* [11], we observed that suppression was generally effective when the length of the dsRNA was in the 500–1000 bp range (the present study; S. N. Radyuk, R. S. Sohal and W. C. Orr, unpublished work). Overall, it seems that the ease, duration and selectivity by which target genes may be 'knocked-out' in cell culture render this approach an effective tool for functional analysis.

In a recent study, we showed that overexpression of either the cTPx or mtTPx in *Drosophila* cells conferred greater resistance to hydrogen peroxide treatment [1]. In the present study, we demonstrate that blocking the TPx expression had the opposite effect, making cells relatively more susceptible to hydrogen peroxide treatment. This finding was further extended by exposing cells over- and underexpressing TPx to paraquat, a source of superoxide radical generation. Again, cells overexpressing either of the TPx variants were more resistant when compared with

Inset: percentage of live cells for control and experimental cell lines. Statistically significant differences (* $P < 0.05$) between treated and non-treated cells are indicated.

Figure 7 S2 cell viability after cadmium (A) and copper (B) treatments

Stable overexpressing cell lines cTPx[+] and mtTPx[+] were exposed to 0.5 mM CdCl₂ (A) or 2 mM CuSO₄ (B) and percentage cell survival was determined at the indicated time intervals. Bar charts show cell survival of the transfected cell lines relative to controls. Inset: percentage of live cells for control and transfected cell lines. Statistically significant differences (**P* < 0.05) between treated and non-treated cells are indicated.

control cells, whereas cells underexpressing TPx were more sensitive. Furthermore, hydrogen peroxide- and paraquat-induced protein oxidative damage, indicated by carbonyl content, was lower in TPx overexpressing cell lines relative to the control (Figure 9). This finding demonstrates the role of TPxs in directly shielding cells against elevated ROS. In the absence of any environmental challenge, modulation of TPx activity, either up or down, had no impact on cell growth rate and cell survival. It is surprising that the absence of TPx appears to have little negative impact under normal conditions, but this result may be ascribed to overlapping functions of many intracellular antioxidants [12,13]. These results are also in agreement with results obtained from manipulations of TPx expression in yeast cells [14]. Although our TPx 'knock-out' cell lines appear unaffected under normal conditions in terms of cell growth rate and viability, there are increased levels of protein carbonyls as well as signs of DNA laddering, suggesting that these cells are under some degree of

Figure 8 Viability of stably transfected TPx overexpressing cell lines after treatment with stressors in combination with glutathione-modulating agents

(A) Heat stress (Hstr) in combination with NAC or BSO. Overexpressing cell lines cTPx[+] and mtTPx[+] were exposed to 0.5 mg/ml NAC or 1 mM BSO for 2 h at room temperature followed by a 24 h incubation at 32 °C. (B) Cadmium treatment in combination with NAC or BSO. Overexpressing cell lines cTPx[+] and mtTPx[+] were exposed to 0.5 mg/ml NAC or 1 mM BSO with or without 0.5 mM CdCl₂ for 24 h. (C) Copper treatment in combination with NAC or BSO. Overexpressing cell lines cTPx[+] and mtTPx[+] were exposed to 0.5 mg/ml NAC or 1 mM BSO with or without 2 mM CuSO₄ for 12 h. Bar charts show percentage of live cells.

stress. Overall, these results point to an important role for these TPx species, namely protecting against direct oxidative damage leading to cell necrosis and/or preventing progression towards apoptosis.

Stressors, such as elevated temperature and copper, have been reported to act, at least in part, via oxidative stress-related mechanisms. In the present study, however, their impact on cells over- and underexpressing TPx was quite distinct from treatments involving a more direct application of ROS, such as hydrogen peroxide and paraquat. Indeed, cell lines overexpressing either cTPx or mtTPx were more susceptible to these stressors

Figure 9 Protein carbonyl content of TPx overexpressing cell lines subjected to various stressors

The protein extracts were derived from the overexpressing cell line cTPx[+] (C–E) or mtTPx[+] (F–H) or from control cells transfected with the pMTV-5 vector (A, B). Protein carbonyl content, obtained using anti-N₂ph antibodies, is shown in (A), (C) and (F). Coomassie Blue protein staining is shown in (B), (D) and (G), whereas signals obtained with anti-V5 antibodies are shown in (E) and (H). Cells were cultured to a density of 10⁶ cells/ml and exposed to the following stressors: 42 °C (t°) for 1 h, 2 mM CuSO₄ (Cu) for 6 h, 10 mM paraquat (PQ) for 24 h, 0.5 mM CdCl₂ (Cd) for 24 h, 20 mM hydrogen peroxide (H₂O₂) for 1 h, or untreated (–). The bar chart shows relative protein carbonyl content. Statistically significant differences (**P* < 0.05) between control and overexpressing cell lines are indicated. OD, absorbance.

relative to vector-only control cell lines, whereas cells with decreased TPx levels were either more resistant or similar in resistance to controls. In contrast, an earlier study by Lee and Park [15] determined that a mutant yeast strain lacking TPx was more thermosensitive when compared with its isogenic wild-type control. Moreover, they demonstrated that protein damage, measured as carbonyl content, was higher in the ‘knock-out’ cell line relative to wild-type, and carbonyl content increased when cells were challenged with heat. They concluded that the antioxidative function of TPx facilitates the cell’s defence against heat shock. We also observed a higher protein carbonyl content in the TPx ‘knock-out’ cell lines under normal conditions, but observed no change in carbonyl levels after exposure to either heat stress or heat shock.

Heat stress is known to enhance cytotoxicity, although the precise mechanisms involved in cell death are currently not well defined. In some instances, oxidative stress has been considered to play a role in hyperthermia-induced cell death [16,17]. However, it was found that increased ROS did not necessarily correlate with increased cytotoxicity [18], an observation supported by our finding that modulation of oxidative status by NAC, a glutathione precursor, or by BSO, an inhibitor of GSH synthesis, had no impact on the rate of cell death under heat-stress conditions. A role for NO as an intermediary messenger in the cytoprotective effect

has also been postulated and could be relevant, as peroxiredoxins have been shown to possess peroxynitrite reductase activity [19]. One possible explanation for detrimental effects of TPx overexpression on cell survival after thermal challenge may be an alteration in the balance between the antioxidant defences required to minimize oxidative stress and sustain an optimal ROS- and RNS-mediated redox state for signalling mechanisms. The observed increase in DNA degradation and laddering in heat-treated cell lines overexpressing TPxs is consistent with a role for signalling mechanisms in the mediation of heat-induced cytotoxicity. Alternatively, it is possible that TPx is labile to hyperthermia and, when present at high levels, could form aggregates that cause cytotoxicity [20]. These possibilities are currently being investigated.

The exposure of cell lines, underexpressing TPx, to cadmium and copper was found to have no impact on cell viability. On the other hand, TPx overexpressing cells were more resistant to cadmium treatment but more susceptible to copper treatment. It is known that the elevation in the concentrations of transition metals often results in pathological conditions that are consistent with oxidative damage. Cadmium induces oxidative stress as a secondary response, causing the depletion of glutathione [21] and protein-bound thiol groups, resulting in increased free radical generation [22] and lipid peroxidation [23]. Cadmium is also

Figure 10 DNA fragmentation analysis of TPx overexpressing cell lines subjected to various stressors

DNA preparations were derived from the overexpressing cell lines cTPx[+] and mtTPx[+] or from control cells transfected with the pMTV-5 vector. Other than the gel with untreated cells, where the first lane is a HiLo DNA marker (M), the first lane is the control, the second is the cTPx overexpressor and the third is the mtTPx overexpressor. The conditions used are as indicated.

known to inhibit all of the enzymes of the thioredoxin system via interaction with vicinal thiol groups [24]. The lack of any significant effect of cadmium on viability in the underexpressing cell lines as well as in yeast TPx knockouts suggests that any detrimental effect on cell survival caused by cadmium treatment is not due to inactivation of TPx. According to our findings, resistance to cadmium was increased in TPx overexpressing cell lines to the same extent as in control cells treated with NAC, which delivers the intracellular free cysteine required for glutathione synthesis. When TPx overexpressing cell lines were treated with BSO, an inhibitor of glutathione synthesis, they were found to be more resistant than control cells, confirming the overlapping functional roles of glutathione and TPxs in the cell. We suggest that the main protective role of TPxs against cadmium-induced cytotoxicity is due to its substitution by glutathione.

By contrast, cells overexpressing TPx were more vulnerable to copper when compared with control cells. Studies have shown that copper may undergo redox cycling, resulting in the production of hydroxyl radicals via Fenton chemistry [25]. There is evidence that some antioxidants, including those containing thiol groups, such as homocysteine, could initiate cytotoxic effects by the induction of oxidative stress in the presence of Cu^{2+} [26,27]. In the proposed scheme, Cu(II) is reduced to Cu(I) and subsequently re-oxidized in a Fenton-like reaction with hydrogen peroxide and O_2 , leading to the formation of $\text{O}_2^{\cdot-}$, $\cdot\text{OH}$ or other ROS [28]. In our experiments, NAC had a clear detrimental effect on cell survival when combined with copper, an effect that was further enhanced by TPx overexpression (Figure 8). This effect was particularly pronounced when TPx is overexpressed in the mitochondrion, which is perhaps not unexpected given the presumed role that this compartment plays in oxidative stress. We propose that Cu(II) may be reduced by thiol groups of cysteine in the active site of TPx, thus competing with the reduction of hydrogen peroxide. Our results (Figure 10) suggest that the formation of ROS during this process may then trigger apoptosis.

Overall, manipulations in levels of TPx demonstrated that under different conditions of stress these enzymes might have both

beneficial and detrimental effects on the viability of *Drosophila* cells.

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