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Regulation of the Activity of the Tumor Suppressor PTEN By Thioredoxin in *Drosophila Melanogaster*

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Abstract

Human Thioredoxin-1 (hTrx-1) is a small redox protein with a molecular weight of 12 kDa that contains two cysteine residues found in its catalytic site. HTrx-1 plays an important role in cell growth, apoptosis, and cancer patient prognosis. Recently, we have demonstrated that hTrx-1 binds to the C2 domain of the human tumor suppressor, PTEN, in a redox dependent manner. This binding leads to the inhibition of PTEN lipid phosphatase activity in mammalian tissue culture systems. In this study, we show that over-expression of hTrx-1 in *Drosophila melanogaster* promotes cell growth and proliferation during eye development as measured by eye size and ommatidia size. Furthermore, hTrx-1 rescues the small eye phenotype induced by the over-expression of PTEN. We demonstrate that this rescue of the PTEN-induced eye size phenotype requires cysteine-218 in the C2 domain of PTEN. We also show that hTrx-1 over-expression results in increased Akt phosphorylation in fly head extracts supporting our observations that the hTrx-1-induced eye size increase results from the inhibition of PTEN activity. Our study confirms the redox regulation of PTEN through disulfide bond formation with the hTrx-1 in *Drosophila* and suggests conserved mechanisms for thioredoxins and their interactions with the phosphatidylinositol-3-kinase signaling pathway in humans and fruit flies.

Keywords

PTEN; Thioredoxin; eye development; Akt; *Drosophila*

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Introduction

PTEN (phosphatase and tensin homolog deleted on chromosome ten) is a major tumor suppressor of human cancer that acts by hydrolyzing membrane phosphatidylinositol (PtdIns)-3-phosphates, preventing the activation of the survival signaling kinase Akt by PtdIns-3-kinase [1]. Recently, we have shown in human tissue culture systems, that human thioredoxin-1 (hTrx-1) binds in a redox dependent manner to PTEN and inhibits its PtdIns-3-phosphatase activity, resulting in increased Akt activity in cancer cells [2]. Molecular docking and site-specific mutation studies show that the binding of hTrx-1 to PTEN occurs through a disulfide bond between the active site Cys³² of hTrx-1 and Cys²¹⁸ of the C2 domain of PTEN. The binding of hTrx-1 to PTEN leads to a steric interference in the catalytic site of PTEN and to the inhibition of the C2 lipid membrane-binding domain [2].

In the fruit fly, *Drosophila melanogaster*, the PtdIns-3-kinase/Akt signaling pathway is conserved and plays an important role in the regulation of cell growth (an increase in cell mass) and, in some cases, the control of proliferation [3-5]. Mutations in the *Drosophila* PtdIns-3-kinase or Akt homologs reduce cell size, organ size and organism size. Ectopic expression of these kinases has the opposite effect [3, 4]. Huang and collaborators [6] showed that *PTEN* functions in the insulin signaling pathway and importantly, they reported that transgenic expression of either human or fly *PTEN* elicit similar phenotypic effects during *Drosophila* eye development. Specifically, they demonstrated that *PTEN* affects cell proliferation and apoptosis in a developmental context dependent manner. Several lines of genetic evidence indicate that dPTEN, like vertebrate PTEN, is a PtdIns-3-phosphatase whose activity is critical for the regulation of cell division and growth [6-8]. With the exception of *PTEN* from *Caenorhabditis elegans*, which encodes the protein DAF-18 with a molecular mass of 120 kDa, all known PTEN orthologs encode proteins of 40-50kDa. Additionally, it is also important to note that conservation of the C2 domain among PTEN proteins appears to be restricted to metazoans and is absent in *S. cerevisiae* and *S. pombe*. Finally, it has been reported that loss of dPTEN function results in enhanced growth and larger cells [6, 9, 10]. Conversely, over-expression of dPTEN has also been found to lead to a reduction in cell number and cell size [9]. Taken together, dPTEN antagonizes insulin signaling pathway to control growth and cell size during *Drosophila* development.

Adult T cell leukemia (ATL) was the first human cancer found to be caused by a retrovirus [11]. The ATL-derived factor (or ADF), an inducer of IL-2Ra chain produced by ATL cells has been shown to be the small redox protein human thioredoxin (hTrx-1). hTrx-1 has been implicated in many cellular functions such as DNA synthesis as electron donors [12], redox regulation of transcription factors such as NF- κ B [13] and AP1 [14] as well as the activity of several proteins including PKC [15], ASK1 [16] and PTEN [2]. Subsequently, hTrx-1 has been shown to be over-expressed in a large number of human tumors [17-22]. Elevated hTrx-1 is associated with increased tumor cell proliferation, inhibition of apoptosis, aggressive tumor growth and decreased patient survival [22]. Though, the molecular mechanisms for the promotion of tumorigenesis by hTrx-1 are not fully known, however, it is postulated that the interaction between hTrx-1 and PTEN may at least in part explain the biological effects of thioredoxins in mammals. Like other organisms, *Drosophila* possesses several thioredoxins that differ in their intracellular localization and their functions [23]. The ovary-specific DmTrx1, which is also called deadhead (*dhd*) protein, and the testis-specific TrxT are both localized in the nucleus and are arranged as a gene pair [23]. The cytosolic DmTrx, also called DmTrx-2, represents the metabolically prominent thioredoxin and is the preferred substrate of thioredoxin peroxidase [24]. Despite the difference in numbering, the physiological function of Trx-2 in *Drosophila* is thought to be analogous to that of Trx-1 in human [25].

In this study, we show that the increased dPTEN activity resulting in decreased eye size can be rescued by ectopic expression of hTrx-1 in the fly eye. The inhibition of PTEN activity appears to be dependent on the binding of hTrx-1 to PTEN. This interaction occurs at a specific conserved cysteine, (Cys²¹⁸) in the C2 domain of PTEN and results in an inhibition of the phosphatase activity, which in turn increases the levels of phosphorylated dAkt. Moreover, we used a molecular homology model for dPTEN, and docking studies with hTrx-1 to show that the interaction between PTEN and thioredoxin occurs in fruit flies to regulate PTEN activity thereby modulating dAkt phosphorylation levels.

Experimental Procedures

Drosophila stocks and genetics

Stocks of *Drosophila melanogaster* are maintained on standard cornmeal medium and all crosses are carried out at 25°C using standard genetic methods unless otherwise noted. Fly stocks were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). UAS-dPTEN transgenic flies were kindly provided by Dr. Tian Xu (Howard Hughes Medical Institute New Haven, CT) and by Dr. Clive Wilson (Research School of Biosciences, Canterbury, UK). P-element transformation was conducted according to standard procedures. To drive the expression of the PTEN and/or the hTrx-1 proteins in the eyes, the GAL4-UAS enhancer trap system was used [26]. In this system, the transcription factor *GAL4* is controlled by an enhancer element specific for the eyes. This drives the expression of the genes placed downstream of *GAL4* Upstream Activating Sequences (UAS). Throughout these studies, we used the enhancer traps GMR-GAL4 and EYE-GAL4 to drive the expression in the eyes posterior to the morphogenetic furrow. GMR-GAL4 and EYE-GAL4 lines were described by Freeman [27] and by Quiring and collaborators [28]. These enhancer traps have been successfully used by others to study components of the PtdIns-3-Kinase/Akt pathway in the eye and the wings [29, 30].

Molecular Biology

The cDNAs for hTrx-1 - human wild type thioredoxin-1 cDNA and hTrxB (Cys³² and Cys³⁵ double mutant) were obtained from Dr. Powis (MD Anderson, Houston, TX), recovered by BamHI and XbaI from PCR 3.1 vector, and inserted into the pUASP or pUAST vector that was previously digested by BglII and XbaI. hPTEN cDNA was obtained from Alan Hall [31], and three different wild type human PTEN constructs were made: a) Wild type hPTEN (wt10) cDNA was recovered by standard PCR, primer 5'-TTTAAAGCGGCCGCATGACAGCCATCATCAAAGAGATCG-3', and 5'-TCTGCAGGAATTCTCAGACTTTT-3', digested by NotI and EcoRI, and cloned in pUASP. b) myc-tagged wild type hPTEN digested by EaeI and inserted into a NotI site of pUASP. c) *wth10* for wild type hPTEN with drosophila PTEN-2, 5' and 3' untranslated region. A two-step PCR approach was used involving the synthesis of mega-primers. First step, with drosophila PTEN-2 as template to get 5' and 3' UTR's of dPTEN, two PCR products were made using primers 5'-CCAGCAACCAAGTAAATCAAC-3', 5'-ACAAGTCTTTAAACCTTAAAAATATGACAGCCATCATCAAAGAGAT-3', and 5'-TACACAAATTACAAAAGTCTGATCAATGCTTATTACGGACTT-3', 5'-GTCACACCACAGAAGTAAGGT-3'. The two PCR products were then used as the forward and reverse primer (mega primers) to amplify the wild type hPTEN cDNA. Digested by NotI and XbaI and then cloned into pUAST. The chimera construct (as referred to DHH) of drosophila PTEN and human PTEN consists in drosophila PTEN N terminal (1-182) and human PTEN C2 domain (175-403). Drosophila PTEN template was recovered by standard PCR of drosophila genomic DNA bearing dPTEN2 (cp114, [6]. Primers 5'-CCAGCAACCAAGTAAATCAAC-3' and 5'-TTTGGAATAACTGTACGTATCGACGCTGAGATGG-3', digested by NotI and

SnaBI. Human PTEN template was constructed with *with10* (mentioned above), primers 5'-CCCAGTCAGAGGCGCTACGTATATTATTATAGCTAC-3' and 5'-GTCACACCACAGAAGTAAGGT-3', digested by SnaBI and XbaI. Then these two fragments were ligated into PUASt vector digested by NotI and XbaI. The mutant chimera construct (DH218) where Cys²¹⁸ is mutated into a Serine was made following the standard protocol of QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The two complimentary oligonucleotides containing the desired mutation were 5'-CCTCAGTTTGTGGTCAGCCAGCTAAAGGTGAAG-3' and 5'-CTTCACCTTTAGCTGGCTGACCACAACTGAGG-3'. All constructs were sequenced before injection to confirm that no error was introduced during PCR amplification or cloning.

Western blot of total protein extracts

Flies were raised at 25 °C, 20µg total fly head lysate protein extracts were size fractionated via sodium dodecylsulfate (SDS)/Poly-acrylamide Gel Electrophoresis (PAGE). Briefly samples are boiled for 5min., loaded on a 10% acrylamide/bisacrylamide gel and proteins are separated by electrophoresis at 135V for 45min. Proteins are then electrophoretically transferred to polyvinylidene fluoride membranes, pre-incubated in blocking buffer (137mM NaCl, 2.7mM KCl, 897µM CaCl₂, 491µM MgCl₂, 3.4mM Na₂HPO₄, 593mM KH₂PO₄ and 5% bovine serum albumin (BSA)), and incubated with antibody. Phospho-Ser⁵⁰⁵-dAkt is an antibody from Cell Signaling (Beverly, MA), which detects endogenous levels of Akt only when phosphorylated at serine 505. This antibody does not recognize drosophila Akt when phosphorylated at other sites, nor does it recognize related kinases such as PKC or p70 S6 Kinase according to the manufacturer. Total Akt antibody (Cell Signaling, Beverly, MA) detects endogenous levels of total Akt1, Akt2 and Akt3 proteins. Human PTEN antibody (BD Biosciences, Franklin Lakes, NJ) recognizes human and mouse PTEN. A peptide representing amino acids 387-400 at the C-terminus of human PTEN was used as the immunogen. Human PTEN is a polypeptide of 403 amino acids and is observed to migrate at ~55 kDa in SDS/PAGE.

Scanning electron microscopy procedure and eye size measurement

Fly samples were dehydrated through a graded series of ethanol (70%, 80%, 95%-30 min each; 100% - 3 × 30 min). Samples were then dried using hexamethyldisilazane and mounted on stubs and sputters coated with 20nm gold. Samples were examined under a Philips XL30 scanning electron microscope at 10 k.v. Fly eyes and ommatidia sizes were measured by pixels of selected area using Adobe Photoshop CS 8.0. Statistics were performed using student *t* test using Sigma Plot version 10.0 (Systat Software, Inc).

RT-PCR for hTrx-1

Total RNA is extracted from 20-40 adult fly heads using a FastRNA Pro Green Kit (Qbiogene, Inc., Irvine, CA.) according to the manufacturer's instructions. The RNA is further purified with an RNAeasy kit (Qiagen, Valencia, CA). The SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) was used to synthesize first-strand cDNA from purified total RNA. Primers 5'-ctgcttttcaggaagccttg-3' and 5'-tggtggcatgcatttgactt-3' targeted a 202bp product of human thioredoxin-1 gene. RP49 (ribosomal protein 49: Dm2151827_g1) was used as internal control during the PCR procedure. The RP49 primers were the following: 5'-atgaccatcgccagcatata-3' and 5'-tacctcgttcttcttagacgc-3'.

Homology model for DPTEN and docking studies

For modeling and docking studies of dPTEN, hPTEN and hTrx-1, we used Insight II 2000.3L modeling software running on Linux. (See footnote 1) The PDB files for PTEN (PDB Id: 1D5R) [32] and hTrx-1 (PDB Id: 1ERU) [33] were used. In order to build the homology model for *Drosophila* PTEN, the protein sequence was subjected to BLAST program to search for homologous template proteins [34]. The alignment model of *Drosophila* PTEN protein with the human PTEN protein sequence was generated using multiple sequence alignment from CLUSTAL-W program [35]. This alignment of protein sequences was used to build model of *Drosophila* PTEN-2 from the co-ordinates of hPTEN using Biopolymer module of Sybyl 7.0 program (see footnote 2). The homology model was generated by replacing the aminoacids of hPTEN protein by the corresponding residues of the *Drosophila* PTEN-2 protein sequence. The resulting model was then minimized using the Discover 3.0 program within Insight II software. For performing docking studies dPTEN-2, hPTEN or the chimeric construct was kept stationary and hTrx-1 was rotated and translated manually. The complex of PTEN and hTrx-1 structure was then immersed in a box of TIP3P water molecules [36]. The structure of the complex was then minimized and the most stable low energy structure with the best interactions was then used to make the covalent disulfide interaction. Two stage molecular dynamics simulations were performed at 300 K. This involved restrained MD simulations with 40 ps equilibration and 100 ps simulations with trajectory snapshots collected at every 0.1 ps. Trajectories were analyzed on the basis of potential energy and frame with lowest potential energy was further minimized with 5000 steps of Discover 3.0 program. This energy-minimized structure was analyzed for protein-protein interactions.

Results

Human thioredoxin-1 increases cell growth in the fly eye

Human thioredoxin-1 (hTrx-1) is able to stimulate cell proliferation [37] and interacts with many signaling proteins, including the tumor suppressor, PTEN [2]. To examine the interaction of hTrx-1 and PTEN signaling pathway *in situ*, we generated transgenic flies bearing an inducible human Trx-1 transgene. We observed a big eye phenotype when hTrx-1 was ectopically expressed in the eye (Figure 1A). Importantly, this eye phenotype was only detected in the catalytically active form of hTrx, and not the inactive form, hTrxB (where both Cys³² and Cys³⁵ were mutated), which was expressed at similar levels (Figure 1C). This observation is in agreement with the function and role of the hTrx-1 double mutant in mammalian cell culture systems (Figure 1A). In order to quantify these observations, we analyzed the size of the eye as well as the ommatidia size of the control, hTrx-1 and hTrxB over-expressing animals (Figure 1B). Statistical analyses of the fly eye sizes and the ommatidia sizes showed significant increased eye sizes compared to the sizes of the wild type eye and of the hTrxB eye (Figure 1B). In conclusion, our results suggest that human Trx-1 is able to maintain its functions in *Drosophila*. These results also indicate that the eye phenotype induced in flies is analogous to the increased cell growth previously described in several mammalian systems [37-39].

Over-expression of human thioredoxin-1 inhibits PTEN activity

To investigate the potential relationship between the tumor suppressor PTEN and hTrx-1 in *Drosophila*, we over-expressed *Drosophila* PTEN (dPTEN) in the fly eye. As reported previously by Huang and collaborators [6] as well as Goberdhan and collaborators [9], eye

¹Insight II 2000.3L Molecular Modeling Software, Accelrys Inc., 9685 Scranton Road, San Diego, CA, 92121.

²Sybyl 7.0 molecular modeling program is available from Tripos Inc. St. Louis, MS

specific over-expression of dPTEN causes a small eye phenotype (Figure 2A, panels B and F as compared to panels A and E). Human Trx-1 (UAS-hTrx-1) was then co-expressed with dPTEN using the same GMR-GAL4 driver and the results are shown in Figure 2A, panels D and H. Intriguingly, the small eye phenotype caused by the over-expression of dPTEN was rescued by the expression of hTrx-1 (panel D and H). To ensure that the increased eye size is not due to the additional UAS sequences, another transgene UAS-GFP was also co-expressed with dPTEN (panels C and G). This control showed no change in the dPTEN induced eye size phenotype. Statistical analyses of the fly eye sizes and the ommatidia sizes of the corresponding animals confirmed that both the eye size and ommatidia size were increased significantly in flies over-expressing dPTEN and hTrx-1 as compared to flies over-expressing only dPTEN and the flies that over-expressing dPTEN and UAS-GFP (Figure 2B). To further test our hypothesis that hTrx-1 may interact and regulate the function of the tumor suppressor dPTEN, we performed western blots probed for phospho-Ser⁵⁰⁵-dAkt using fly heads extracts. As shown in Figure 2C, the over-expression of dPTEN in fly eyes suppressed Akt phosphorylation, but co-expression of both dPTEN and hTrx-1 restored Akt phosphorylation (Figure 2C). Again, no supplemental changes were observed in Akt phosphorylation in the control flies with the UAS-GFP control transgene. These results suggest strongly that human thioredoxin-1 acts as an antagonist of dPTEN activity, and that the rescue of the phenotype observed in Figure 2B is, at least in part, through the PTEN-PtdIns-3-Kinase-Akt signaling pathway.

Chimeric constructs of PTEN have identical activities as compared to wild type *Drosophila* PTEN

Human PTEN has been reported to be functionally equivalent to *Drosophila* PTEN based on the observations that ectopic expression of human PTEN or over expression of *Drosophila* PTEN result in an indistinguishable small eye phenotype [6]. However, we were unable to replicate the described human PTEN over-expression phenotype (Figure 3A, panels G-I). Even though the hPTEN protein was expressed at significant levels in the fly eye protein extracts (Figure 3C), it was not functional as shown by the blotting with phospho-dAkt antibodies (Figure 3C). Thus, to test our hypothesis that hTrx-1 interacts with the C2 domain of human PTEN as demonstrated in mammalian systems [2], we generated two chimeric constructs of hPTEN and *Drosophila* PTEN (called thereafter DHH and DH218 – See cartoon Figure 3D), which consisted of *Drosophila* PTEN N-terminal domain (amino acids 1 to 182 of dPTEN) linked to the human PTEN C-terminal (amino acids 175-403 of hPTEN). DH218 refers to the Cys²¹⁸ previously identified as the critical residue for making a disulfide bridge with the Cys³² of hTrx-1 in mammalian tissue culture cell systems [2]. Driven by GMR-GAL4 or by EYE-GAL4, DHH over-expressing animals exhibited an indistinguishable small eye phenotype relative to the dPTEN-induced phenotype (Figure 3A, panels E, F). Similar observations were made for the mutant DH218, in which Cys²¹⁸ in the C-terminal was mutated into a serine (Figure 4A, panel D). It should be noted that DHH and DH218 protein expressions could be detected using the anti-human PTEN antibody against hPTEN C-terminus (amino acids 387-400) by western blots of fly eye extracts (Figure 3B). Taken together, although both proteins hPTEN and chimeric PTEN were expressed at similar levels, only chimeric PTEN exhibited a small eye phenotype reinforcing our statement that hPTEN does not induce a smaller eye phenotype.

Cys²¹⁸ of the C2 domain of hPTEN is required for the negative regulation by human thioredoxin-1

In our previous study, we have showed that thioredoxin-1 binds to C2 domain of PTEN directly and inhibit PTEN's activity in tissue culture cells [2]. The binding was redox controlled and occurred between Cys³² of Trx-1 and Cys²¹⁸ of the C2 domain of hPTEN [2]. To evaluate the effects of thioredoxin on human PTEN *in situ*, we over-expressed the

chimeric constructs of PTEN, DHH and DHH218. Expression of both of these constructs exhibited a small eye phenotype (Figure 4A, panels B and D respectively). When hTrx-1 was co-expressed with DHH, the reduced eye size induced by DHH was partially rescued by hTrx-1 (Figure 4A, panel C). However, when we examined the hPTEN C2 domain with the Cys²¹⁸ > Ser mutation, we found that hTrx-1 is no longer capable of rescuing the phenotype. (Figure 4A, panel E). Statistical analyses of the fly eye sizes and the ommatidia sizes showed hTrx-1 can significantly increase both the eye size and the ommatidia size of chimeric PTEN transgenic flies, but not of the Cys²¹⁸ over-expressing mutant flies.

To test whether this inhibition occurred through the alteration of PtdIns-3-Kinase/Akt signaling pathway, we examined dAkt phosphorylation by Western blot analysis of the matching fly head extracts. As shown in Figure 4C, chimeric PTEN construct (DHH) C-terminal can be recognized by anti-human PTEN antibody, and phospho-dAkt can be detected by anti-phospho-Ser⁵⁰⁵-dAkt antibody. The expression of DHH or DH218 induced a decrease in Akt phosphorylation suggesting that these chimeric forms of PTEN are active in the fly eye and may, at least in part, be responsible for the smaller eye phenotype observed in Figure 4A. The co-over-expression of hTrx-1 with DHH restored Akt phosphorylation but could not increase phospho-Akt when DH218 was over-expressed. Taken together, these results indicate Cys²¹⁸ in the C2 domain of hPTEN is critically required for the interaction with human thioredoxin-1 *in situ*.

Structural evidence for the human thioredoxin-1 and human PTEN functional interaction

Molecular docking studies using the published crystallographic structures of PTEN (1DR5) [31], hTrx-1 (1ERT) [32] and DTrx-2 (1XWA) [25] were employed for the study of the interaction of PTEN and hTrx-1. A homology model for the *Drosophila* PTEN was build based on the crystal structure of the human PTEN and the alignment of the sequences (Figure 5A and B). The model for dPTEN was superimposed onto the structure for hPTEN and revealed a very strong overlap and similarities. We have previously published a molecular model for the interaction between hTrx-1 and human PTEN [2] (Figure 5C, left panel). The C2 domain of hPTEN folds into a β sandwich consisting of two antiparallel β sheets with two short α helices between the strands. The analysis of the hTrx-1/PTEN (Cys³²-Cys²¹²) complex clearly indicated that this interaction fulfills a number of complementary interactions favoring an energetically stable complex. For example, Trp³¹ in the active site of hTrx-1 packs against Phe²⁵² of hPTEN stabilizing the complex while Met³⁷ and Asp⁶⁰ of hTrx-1 are in the close proximity of Lys²¹⁵ and Lys²⁶¹ of hPTEN (d = 4.0 Å) (Figure 5C). The middle panel in Figure 5C represents the model for the dPTEN/hTrx-1 complex. Several amino acids appear to stabilize this complex and involve the following amino acids between dPTEN and hTrx-1: Glu⁵¹ with Glu⁹⁵; Lys²⁶⁶ with Cys³²; Glu²⁶⁸ with Thr³⁰; Asp²⁷⁵ with Cys³⁶ and Glu⁸¹ with Asn⁹³. Finally, the far right panel in Figure 5C represents the modeled interaction between the chimeric PTEN with hTrx-1. Figure 5D represents the model of the interaction between *Drosophila* PTEN and DTrx-2 showing that several hydrogen bonds can be formed between the following amino acids from dPTEN and dTrx-2: Lys⁴⁹ with Asn¹⁰⁵; Asn¹⁰² with Ser⁷⁴; Phe⁵³ with Glu⁸⁸; Asp⁹⁹ with Glu⁸⁹; Glu⁵¹ with Asn¹⁰⁵.

Discussion

The small redox protein Thioredoxin (Trx-1) was recently shown to regulate the activity of the tumor suppressor, PTEN in mammalian tissue culture cells [2]. This study demonstrates that hTrx-1 binding to PTEN and inhibition of the PTEN lipid phosphatase activity is conserved during eye morphogenesis in the phylogenetically distant species *Drosophila melanogaster*. Translation of our *in vitro* studies into a small organism model such as *Drosophila melanogaster* appeared essential to confirm pathway crosstalks, protein-protein

interactions as well as to acquire a better understanding of PTEN regulation. The fact given that both *PTEN* and *Thioredoxin* genes are highly conserved in the fruit fly, this allowed us to use experimental genetics as a powerful tool to discover a previously overlooked difference between human and *Drosophila* PTEN function as well as potential differences in the thioredoxins in the fruit fly (See below). Nevertheless, in agreement with our previous published observations [2], mutational studies showed that conversion of human Cys²¹⁸ into a serine residue in the C2 domain of PTEN had no observable effect on enzymatic activity, but did abrogate the ability of hTrx-1 to negatively regulate PTEN activity.

Docking studies using the X-ray crystal structures of PTEN (1DR5) and hTrx-1 (1ERT) as well as our own homology model for *Drosophila* dPTEN-2 confirm that the binding of hTrx-1 to PTEN occurs through a disulfide bond between the Cys³² of Trx-1 and human Cys²¹⁸ of the C2 domain of PTEN *in situ*. Importantly, this was further confirmed by site-specific mutagenesis studies of the Cys²¹⁸ in the C2 domain of hPTEN, which abolished the binding of hTrx-1 to the chimeric protein PTEN. Although the docking studies suggest that Cys³²-Cys²²² form a disulfide bridge covalently connecting hTrx-1 to the C2 domain of dPTEN, we were not able to directly show this interaction due to the lack of phenotype of the flies over-expressing mutant dPTEN-Cys²²² (Data not shown). In fact the single mutation of Cys²²² in dPTEN totally abolished the eye phenotype normally observed when dPTEN is over-expressed. Ongoing studies are underway in our laboratory to fully examine the role of this specific conserved cysteine in the C2 domain, already noted by Lee and collaborators as a hot spot for mutation [32]. However, the construct of the chimeric protein DH218 demonstrated that the interaction with hTrx-1 is abolished in *Drosophila*, suggesting an important role for this specific cysteine as already suggested by our group [2]. This bond appears to create a significant steric hindrance blocking substrate access to the catalytic site of the phosphatase domain of PTEN and thus may provide an explanation for the inhibition of PTEN's lipid phosphatase activity (See Figure 5). Interestingly, similar observations were made previously between hPTEN and hTrx-1 in mammalian tissue culture systems [2]. Importantly, the inhibition of dPTEN activity could be correlated with an increase in dAkt phosphorylation in fly tissue culture cells and *in situ* during *Drosophila melanogaster* eye morphogenesis. Taken together, these results establish this important interaction between PTEN and hTrx-1 is conserved between the phylogenetically diverse animals.

Recently, at least three alternatively spliced form of dPTEN have been identified, differing in the C-terminus of the protein [40]. However, only the isoforms dPTEN-3 was shown to significantly down-regulate *Drosophila* Akt in fly tissue culture studies [41]. Interestingly, we showed that dPTEN-3 and to a lesser extent dPTEN-2 could induce the eye size reduction which correlated with a reduction in Akt phosphorylation in S2 cells as well as whole protein extracts from the corresponding transgenic fly heads. This difference in dPTEN-2 and its effects on Akt may partially be due to the model system used i.e. *in vitro* kinase assay and S2 cell transfection [41] *versus* over-expression of the transgene *in situ* during *Drosophila* eye morphogenesis (in this study).

In spite of the many and diverse functions, the 3D structures of thioredoxins are highly conserved throughout the species. The Trx fold consists of five central β -strands surrounded by four α -helices. The typical active-site motif C32-G-P-C35 (nomenclature of human Trx1 and dTrx-2) resides between the β -2 strand and the α -2 helix. hTrx-1 levels are known to be elevated in many human primary cancers [36-38] and are associated with increased proliferation and inhibited apoptosis [36]. In patients with colon cancers and non-small cell lung cancers increased Trx-1 expression is associated with decreased patient survival [22]. Thus, inhibition of hPTEN by elevated hTrx-1 might account for some of the oncogenic effects of hTrx-1. Additionally, the fact that this interaction occurs also in a small organism system has allowed us to use powerful genetic tools to address the mechanisms through

which a small redox protein regulates an important the tumor suppressor, PTEN. Importantly, we have reproduced the effects of dPTEN in the fly eye i.e. over-expression of dPTEN leads to a reduction in eye size, as already described by Huang and collaborators [6] and shown that this small eye phenotype is dramatically rescued by ectopically expressing hTrx-1. These data strongly support our hypothesis that the interaction between these two proteins is a strongly conserved mechanism of growth control.

However, during our investigations for the interaction between hPTEN and hTrx-1 in flies, we have discovered two major differences related to PTEN as well as to the thioredoxins. First, in contrast to what has been published by Huang and collaborators [6], the hPTEN gene in flies does not have similar function as compared to its fly homologue, dPTEN. We were unable to reproduce the data showing an eye phenotype induced by hPTEN expression using the transgenic strains originally described by Huang et al. [6] nor with hPTEN strains did we construct. In fact, the over-expression of hPTEN, which was verified using western blotting analyses, did not cause an eye phenotype (See Figure 3C). Also, consistent with our failure to reproduce the phenotype, we found no changes in Akt phosphorylation in tissues expressing hPTEN (Figure 3C). This unexpected finding tends to suggest that human PTEN may require additional proteins or signaling pathways that do not exist in *Drosophila* in order to regulate the activity of the protein. To overcome this failure to obtain the PTEN over-expression phenotype in our system using the hPTEN, we generated chimeras of PTEN swapping the human and the drosophila C2 domains. The chimeric protein formed by a *drosophila* PTEN catalytic domain and a human C2-PTEN domain successfully produced the reduced eye phenotype when over-expressed in the eye. Consequently, the phenotype was rescued by hTrx-1. Although the amino acid portion of dPTEN is highly similar to that of hPTEN with an identity of 58% within the catalytic core motif responsible for the $\text{PtdIns}(3,4,5)\text{P}_3$ phosphatase activity, we found that this region of the human PTEN was not functional in the fly even when fused to the fly C2 domain (Data not shown). Interestingly also was the discovery of the single mutation in dPTEN of cysteine 222 as noted above. Such differences are of interest and under investigation as to whether this specific cysteine may be involved in a structural bridge with another cysteine of the catalytic site of the protein. Finally, the second difference observed during the course of this work is related to dTrx-2. While this thioredoxin is thought to be the analog of hTrx-1 [25], our attempts to genetically manipulate endogenous dTrx-2 levels in the fly eye failed to produce any change in eye size (Brabant et al., manuscript in preparation and personal communication). In addition, using dTrx-2 deletions (two lines EP(2)2118 and GS 2118 have a P-element insertion in the 5'-UTR [42]), the removal of dTrx-2 did not change the eye size. Finally, dTrx-2 null flies have no obvious phenotype indicating that one or more of the other *Drosophila* thioredoxin can provide the orthologous function of hTrx-1 necessary for life.

Despite these unexpected findings related to hPTEN, dPTEN-Cys²²² and dTrx-2, we were able to demonstrate that the activity of PTEN (either dPTEN or DHH) can be modulated by the manipulation of hTrx-1 expression in *Drosophila*. These important results confirm the conservation of such interaction and regulation of the tumor suppressor and suggest the potential importance, yet to be discovered, of the proteins in the thioredoxin family that are expressed in *Drosophila*. Studies unraveling the role of these thioredoxin analogs may be of particular interest in the better understanding cancer cell proliferation mechanisms.

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The abbreviations used are

PTEN	phosphatase and tensin homolog deleted on chromosome 10
PtdIns	phosphatidylinositol
Trx	thioredoxin
ATL	adult T cell leukemia

Figure 1. hTrx-1 over-expression in fly eye increases both eye size and ommatidia size

Figure 1A depicts the phenotype for the animal over-expressing human thioredoxin-1 (hTrx-1) in their eye observed by scanning electron microscopy (SEM; 150 $\times$). Panel A is for GMR-GAL4/+; B, GMR-GAL4/hTrx-1 and C; GMR-GAL4/hTrxB. High magnification SEM (500 $\times$) eye pictures (Panels D-F) also show that there is an increase of ommatidia size. Figure 1B represents the quantification of the eye size (Upper Graph) and ommatidia size (Lower Graph) of wild type control, hTrx-1 over-expressing flies and hTrxB over-expressing flies. The bars represent the means of 5 eyes or 10 ommatidias, the error bars correspond to the Standard Deviation (SD), with **, $P < 0.01$. Figure 1C represents the RT-PCR analysis of hTrx-1 mRNA levels in GMR-GAL4 control, hTrx-1 transgene only control, hTrx-1, and hTrxB. Rp49 is the internal loading control used for the RT-PCR and was carried out as described in the *Experimental Procedures* section. Note the presence of a band only in samples containing hTrx-1 or hTrxB with their appropriate drivers and which correspond to the right size of the transgene (202 bp for Trx and for 460 bp rp49, as pointed by the arrows).

Figure 2. Over-expression of human thioredoxin-1 inhibits PTEN activity

Figure 2A represents SEM pictures at 100 $\times$ of panel A, GMR-GAL4/+; B, GMR-dPTEN/+; C, GMR-dPTEN/UAS-GFP; and D, GMR-dPTEN/UAS-hTrx-1. Panels E-H are the SEM of the above-mentioned flies at higher magnification (500 $\times$). The small eye phenotype caused by the over-expression of *Drosophila* PTEN can be partially rescued by co-expressing human thioredoxin-1, and the rescue is not due to the additional UAS sequences since UAS-GFP did not alter the eye size. The quantified comparisons of eye sizes (top graph) and ommatidia sizes (bottom graph) for the flies over-expressing dPTEN, co-expressing dPTEN with hTrx-1, and over-expressing a control GFP as compared to control flies are shown in figure 2B. The bars represent the means of 5 eyes or 10 ommatidias, the error bars correspond to the Standard Deviation (SD), with **, $P < 0.01$. Figure 2C. *Drosophila* Akt phosphorylation in fly heads with over-expression of hTrx-1, dPTEN, and co-expression of dPTEN with hTrx-1. 40 μ g of total protein extracts were loaded on 10% Bis-Tris gel and analyzed by western blot using anti-drosophila Akt, anti-phospho-Ser⁵⁰⁵ drosophila Akt and anti-drosophila actin antibodies.

Figure 3. Chimeric constructs of PTEN have identical activities as compared to wild type *Drosophila* PTEN

Figure 3A represents the scanning electron micrograph (SEM) eye images of GMR-GAL4/+ (panel A), drosophila PTEN-2 driven by GMR-GAL4, which shows a flat, small eye phenotype (B. GMR-GAL4/dPTEN-2). When driven by EYE-GAL4, the eye shows an even smaller, rough eye phenotype (C. EYE-GAL4/dPTEN-2). Panels D-F are the SEM eye images of wild type (D. GMR-GAL4/+), of over-expression of chimeric PTEN-DHH driven either by the GMR diver (E. GMR-GAL4/DHH) or by the Eyeless drive (F. EYE-GAL4/DHH). Panels G-I are the SEM eye images of wild type control (G. GMR-GAL4/+) compared to flies over-expressing human wild type PTEN (H. GMR-GAL4/hPTEN, I. EYE-GAL4/hPTEN). Figure 3B represents the detection of PTEN by western blot. The samples are from total fly heads extracts of GMR-GAL4/+ control flies, chimeric DHH PTEN over-expressing flies, chimeric DHH PTEN with Cys218 mutation over-expressing flies (as referred to DH218) and wild type human PTEN over-expressing flies. Figure 3C represents a typical western blot for total protein extracts from the fly heads isolated from the GMR-GAL4 control flies and Myc tagged wild type hPTEN driven by GMR-GAL4 transgenic flies. Anti-human PTEN antibodies was used to detect hPTEN expression in the fly head extracts, anti-myc Tag (clone 9B11) was also used to detect the expression of the hPTEN-myc tagged protein. P-Ser505-dAkt was not decreased in GMR-GAL4/Myc-hPTEN as compared to GMR-GAL4 control animals. Figure 3D represents the cartoon for the structure of DHH chimeric PTEN with *Drosophila* PTEN N-terminal (amino acids 1-182 of dPTEN) and human PTEN C-terminal (amino acids 175-403 of hPTEN), and DH218 – a mutation form of DHH with human PTEN Cys218 of C2 domain mutated to Serine.

Figure 4. Cys²¹⁸ of PTEN C2 domain is required for the negative regulation by thioredoxin
 Figure 4A represents the SEM eye images for A. GMR-GAL4/+; B. GMR-GAL4/DHH; C. GMR-GAL4>DHH/hTrx-1; D. GMR-GAL4/DH218 and E. GMR-GAL4>DH218/hTrx-1. Note that hTrx-1 rescues chimeric PTEN small eye phenotype. SEM eye images (panels D-E) show that DH218 is a functional mutant of chimeric PTEN (small eye phenotype as compared to control), but the Cys²¹⁸ mutation in the human C2 domain of PTEN did not allow hTrx-1 to rescue the small eye phenotype caused by over-expression of chimeric PTEN. High magnification SEM (500×) eye pictures (Panels F-J) show that there is an increase of ommatidia size when hTrx-1 is co-expressed with chimeric PTEN. Figure 4B represents the quantifications and their statistic analyses of the eye size (Upper Graph) and ommatidia size (Lower Graph) of the respective pictures of A-E and F-K from the above panels in Figure 4A. The bars represent the means of 5 eyes, the error bars correspond to the Standard Deviation (SD), with *, P<0.05 and **, P<0.01. Figure 4C is the representative western blots of *Drosophila* Akt phosphorylation, total *Drosophila* Akt and chimeric PTEN in the fly head extracts. PTEN antibody recognizes human PTEN C-terminal (amino acids 387-400) of chimeric PTEN. While co-expression with hTrx-1, chimeric PTEN-DHH failed to inhibit *Drosophila* Akt phosphorylation. However, co-expression of hTrx-1 with DH218 mutant had no obvious effect on phospho-Akt, indicating that hTrx-1 does not interact with DH218.

Figure 5. Molecular interactions between hTrx-1 and PTEN proteins

Panel A represents the superposition of hPTEN crystal structure (red) with the homology model built for dPTEN (yellow). Note that both proteins appear to be very similar in their structure. Panel B is a sequence alignment by ClustalW of the dPTEN variants with hPTEN. The conserved amino acids are highlighted in red. Panel C is on the far left the model of hPTEN interaction with hTrx-1, in the center is the model for dPTEN with hTrx-1 and on the far right is the chimeric protein modeled for PTEN (DHH) with hTrx-1. hTrx-1 is in yellow, PTEN protein is in red and DHH is in green. Panel D represents the model of the interaction between DTrx-2 with dPTEN.