Prdx1 Inhibits Tumorigenesis via Regulating PTEN/AKT Activity

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Citation

Published Version
doi:10.1038/emboj.2009.101

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Prdx1 inhibits tumorigenesis via regulating PTEN/AKT activity

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It is widely accepted that reactive oxygen species (ROS) promote tumorigenesis. However, the exact mechanisms are still unclear. As mice lacking the peroxidase peroxiredoxin1 (Prdx1) produce more cellular ROS and die prematurely of cancer, they offer an ideal model system to study ROS-induced tumorigenesis. Prdx1 ablation increased the susceptibility to Ras-induced breast cancer. We, therefore, investigated the role of Prdx1 in regulating oncogenic Ras effector pathways. We found Akt hyperactive in fibroblasts and mammary epithelial cells lacking Prdx1. Investigating the nature of such elevated Akt activation established a novel role for Prdx1 as a safeguard for the lipid phosphatase activity of PTEN, which is essential for its tumour suppressive function. We found binding of the peroxidase Prdx1 to PTEN essential for protecting PTEN from oxidation-induced inactivation. Along those lines, Prdx1 tumour suppression of Ras- or ErbB-2-induced transformation was mediated mainly via PTEN.

The EMBO Journal (2009) 28, 1505–1517. doi:10.1038/emboj.2009.101; Published online 16 April 2009

Subject Categories: molecular biology of disease

Keywords: oxidative stress; peroxiredoxin1; PTEN phosphatase; transformation; tumour initiation

Introduction

Peroxiredoxins (Prdxs) are a superfamily of small non-seleno peroxidases (22–27 kDa) currently known to possess six mammalian isoforms. Although their individual roles in cellular redox regulation and antioxidant protection are quite distinct, they all catalyse peroxide reduction to balance cellular H2O2 levels essential for signalling and metabolism (Rhee, 2006). Prdxs I–IV share the common CxxC motif and use thioredoxin (Trx) as an electron donor (Chae et al., 1994a). On reaction with peroxides, the Prdxs’ ‘peroxidatic’ cysteine (Cys51 in Prdx1) is oxidized into a sulfenic acid intermediate, which then forms a disulfide bond with the ‘resolving’ cysteine (Cys172 in Prdx1) from the other homodimer’s subunit. Catalytic reduction of peroxides results in different oxidation states of the catalytic cysteine. Lower oxidation states (sulfenic acid) are reduced back to thiol by the Trx system. Higher oxidation states such as sulfonic acid can be reduced back to thiol by sulfiredoxin, whereas the oxidation to sulfonic acid is irreversible and results in Prdx degradation (Neumann and Fang, 2007). Loss of Prdx1 shortens the life span of mice due to the development of hemolytic anemia and cancer. Analysis of cells and mice lacking Prdx1 suggested that it not only regulated H2O2 levels, but also that it possessed the properties of a tumour suppressor (Neumann et al., 2003), which was further supported by the finding that Prdx1 interacts with c-Myc thereby selectively inhibiting c-Myc transcriptional activity (Egler et al., 2005).

Peroxides are known to modify protein tyrosine phosphatases (PTPs) by oxidation. PTP catalytic property depends on a thiolate anion of a low pKₐ cysteine residue (pKₐ 4.7–5.4) located in the conserved motif of its active site (Zhang and Dixon, 1993; Peters et al., 1998). This highly nucleophilic group makes the initial attack on the phosphate group of the substrate, but renders the PTP extremely susceptible to oxidation resulting in its inactivation. The PTP and tumour suppressor PTEN exhibits phosphatase activity towards phosphoinositides and tyrosine phosphates and is known to be inactivated through H2O2-mediated oxidation or through growth factor signalling, including epithelial growth factor (EGF) or platelet-derived growth factor (PDGF) (Leslie et al., 2003; Kwon et al., 2004). Analysis of human recombinant PTEN revealed that two of the five cysteines in its N-terminal phosphatase domain (PTD) (Cys71 and Cys124) form a disulfide bond after oxidation, which resulted in the transient inhibition of its phosphatase activity and allowed oxidized PTEN to migrate faster on a non-reducing SDS–PAGE (Lee et al., 2002; Kwon et al., 2004). In stimulated macrophages, PTEN oxidation led to the temporary inhibition of its phosphatase activity and in the phosphorylation of Akt on Serine 473 (Leslie et al., 2003).

PTEN deficiency is a hallmark of many human tumours (Keniry and Parsons, 2008) and is accompanied by enhanced cell proliferation, decreased cell apoptosis and increased Akt activity (Stambolic et al., 1998). Akt is regulated by PI3K and PTEN, thus playing a central role particularly in Ras- and
ErbB-2-induced transformation. Akt activity has been proven crucial for the initiation of this transformation, both in vitro and in vivo (Sheng et al., 2001; Hutchinson et al., 2004; Lim and Counter, 2005) and phosphate inactive PTEN (PTEN Cys124Ser) is incapable of suppressing Ras-induced transformation either in vitro or in vivo (Tolkacheva and Chan, 2000; Koul et al., 2002).

Although it is known that PTEN activity is negatively regulated through oxidation, the existence of a protective mechanism inhibiting such inactivation has not been proposed. On the basis of our findings, we propose here that Prdx1 promotes PTEN tumour suppressive function by binding PTEN and protecting its lipid phosphate activity from H2O2-induced inactivation. This way, Prdx1 controls excessive cellular Akt activity and reduces the susceptibility to H-Ras and ErbB-2-induced transformation.

**Results**

**Prdx1 inhibits Ras-induced transformation through its peroxidase activity**

We tested first Prdx1 peroxidase activity under physiological conditions and measured endogenous H2O2 release in growing cells over time. Extracellular H2O2 buildup from Prdx1+/−/Prdx1WT MEFs was less compared with Prdx1−/−MEFs during the 240 min measured. In contrast, Prdx1+/−/Prdx1C51/172S MEFs resulted in an excess of extracellular H2O2 buildup (Supplementary Figure S1A). Rates calculated for extracellular H2O2 buildup (pmol/min) confirmed that Prdx1−/−/Prdx1WT MEFs released H2O2 at a 30% lower rate than Prdx1−/−MEFs, whereas Prdx1+/−/Prdx1C51/172S MEFs released H2O2-release 1.5-fold more than Prdx1+/+MEFs (Figure 1A, left panel). Western blotting analysis further confirmed that Prdx1 was functioning as a peroxidase, as Prdx1WT protein formed mostly DTT-reducible (data not shown) H2O2-scavenging dimeric structures (Figure 1A, right panel), whereas Prdx1Cys51/172S did not, as dimer formation depends on Cys51 and Cys172 disulfides (Chae et al., 1994b). The remaining Prdx1 monomers were not over-oxidized and therefore active (unlike Prdx1 monomers in H2O2-treated Prdx1−/−/Prdx1WT MEFs). Prdx1 peroxidase activity was required to suppress H-Ras-induced tumour suppression, as Prdx1−/−MEFs transduced with retrovirus expressing H-RasV12 formed two-fold more colonies in soft agar compared with Prdx1+/+H-RasV12MEFs (Figure 1B, left panel).

Expression of exogenous Prdx1WT reduced colony formation by 50% in Prdx1−/−/H-RasV12MEFs, and by 20% in Prdx1+/+H-RasV12MEFs, whereas exogenously expressed Prdx1C51/172S modestly increased colony formation in Prdx1−/−/H-RasV12MEFs and Prdx1+/+H-RasV12MEFs. Western blot analysis ensured that re-expression of Prdx1 proteins in Prdx1−/−MEFs did not exceed those Prdx1 levels found in Prdx1+/+MEFs (Figure 1B, right panel). Prdx1−/−/H-RasV12MEFs appeared also more spindle-like than Prdx1+/+H-RasV12MEFs, whereas expression of Prdx1WT in Prdx1−/−/H-RasV12MEFs reversed the spindle-like morphology. Expression of exogenous Prdx1C51/172S, however, promoted spindle-like cell morphology in both Prdx1−/−/H-RasV12MEFs and Prdx1+/+H-RasV12MEFs (Supplementary Figure S1B). Analysis of the Prdx1 peroxidase activity in H-RasV12-transformed MEFs showed that Prdx1−/−/Prdx1WT/H-RasV12MEFs released H2O2 at a 26% lesser rate than Prdx1−/−H-RasV12MEFs, which was comparable to the rate found Prdx1−/−/EVMEFs. In contrast, Prdx1−/−/C51/172S/H-RasV12MEFs had a 1.5-fold increased rate of H2O2 release compared with Prdx1−/−/Prdx1WT/H-RasV12MEFs (Figure 1C).

**Loss of Prdx1 promotes PTEN oxidation and Akt activation**

We investigated now whether Prdx1 regulates H-Ras effector pathways known to promote transformation, including PI3K/Akt, RalGEP or MAPK pathways (Li et al., 2004; Lim and Counter, 2005). Treatment of MEFS with H2O2 (50 μM) for increasing periods of time enhanced Akt phosphorylation on Ser473 (pAktSer473) and Thr308 (pAktThr308) at a higher level in Prdx1+/−MEFs compared with Prdx1+/+MEFs (Figure 2A). This finding correlated with a steady increase of PTEN oxidation over time in Prdx1−/−MEFs, whereas in Prdx1+/+/MEFs PTEN oxidation plateaued after 6–8 min (Figure 2B and C). Phosphorylation of Akt on serine 473 (pAktSer473) and PTEN oxidation increased also in Prdx1−/−MEFs compared with Prdx1+/+MEFs when exposed to increasing amounts of H2O2 (Supplementary Figure S2A and B). Similar data were obtained by inducing H2O2 endogenously through treating cells with PDGFR, as it led to a larger increase in (1) pAktSer473 and pAktThr308 (Figure 2D) and (2) PTEN oxidation in Prdx1−/−MEFs when compared with Prdx1+/+MEFs (Figure 2E). Although PTEN oxidation steadily increased in Prdx1−/−MEFs, it decreased in Prdx1+/+MEFs over time (Figure 2E and F). Levels of pAktSer473 and PTEN oxidation was also increased in Prdx1−/−MEFs compared with Prdx1+/+MEFs when exposed to increasing amounts of H2O2 or PDGFR (Supplementary Figure S2A–D). Lastly, we confirmed that lack of Prdx1 enhanced basal and H2O2-induced phosphorylation of Akt substrates in Prdx1−/−MEFs more than in Prdx1+/+MEFs (Figure 2G).

**Prdx1 interacts with PTEN**

PTEN forms a complex with endogenous and epitope-tagged HA–Prdx1 (Figure 3A). This complex was regulated by oxidative stress, as co-expressed epitope-tagged Myc–PTEN and HA–Prdx1 dissociated under increasing concentrations of oxidative stress (Figure 3B, upper panel). In contrast, increasing dosages of H2O2 did not disrupt binding of Prdx1Cys51/72Ser with PTEN (Figure 3C). Immunoblotting confirmed equal expression of proteins (Supplementary Figure S3A and B). Peroxidatic Cys51 may regulate the H2O2-induced Prdx1:PTEN complex disruption as Prdx1C51S and Prdx1C51S172S proteins bind more to wild-type PTEN than Prdx1C172S (Figure 3D). We further confirmed a physical association between Prdx1 and PTEN by using GST-pull down of GST–Prdx1 and His–PTEN (Figure 3E). Mutational analysis suggested that Prdx1 interacts within the C2 domain of PTEN (amino acids 186–274) (Figure 3F; Supplementary Figure S3C and D) and PTEN with the N terminus of Prdx1 (amino acids 1–40) and the C terminus of Prdx1 (amino acids 157–199) (Figure 3F, lower schematic and Supplementary Figure S3G). Computational analysis allowed us to narrow those interaction surfaces (Figure 3G). The images of Prdx1 and PTEN were generated from coordinates from the protein database 1d5r (Lee et al., 1999) and 2z9s (Matsumura et al., 2008), respectively. As shown for PTEN, the region of 186–251 consists of two double-stranded anti-parallel sheets con-
connected through two flexible loops. Similarly, the 1–40 region of Prdx1 consists of a single double-stranded anti-parallel sheet and another, surface exposed single sheet strand. In Figure 3G, the Prdx-1 protein is shown only as the monomer and the helical region that lies at the dimer interface, residues 183–199, are also positioned in a surface exposed position.
that is obviously well poised to mediate a protein–protein interaction. Deleting the identified interaction sites in PTEN and Prdx1 altered enzyme activity of both enzymes (data not shown), which did not allow us to study the role of a physical interaction in protecting PTEN lipid phosphatase activity.

**Prdx1 interaction with PTEN protects and promotes PTEN lipid phosphates activity under oxidative stress**

PTEN lipid phosphatase activity was fully protected by Prdx1 in cells under mild oxidative stress (25 μM H₂O₂) (Figure 4A), where Prdx1 was found to bind PTEN (Figure 3B). However, under higher oxidative stress treatment (500 μM H₂O₂), which resulted in decreased binding of PTEN and Prdx1 (Figure 3B), impaired PTEN’s lipid phosphatase activity. Western blotting confirmed equal amounts of PTEN protein in the lipid phosphatase assay (Figure 4A, lower panel). To complement these results, we exposed recombinant purified His-tagged PTEN to H₂O₂ in the absence or presence of increasing amounts of purified recombinant Prdx1 and assayed PTEN for PI(3,4,5)P₃ utilization. As expected (Figure 4B), in the presence of H₂O₂, we found that low molar amounts of Prdx1 protein weakly protected PTEN lipid phosphatase activity, whereas equimolar Prdx1 fully restored and further increases in Prdx1 protein amount did not enhance PTEN lipid phosphatase activity any more. Precipitation of PTEN in this assay with N-and C-terminal Prdx1 peptide replica (P1 and P2, respectively; peptide se-

**Figure 2** (A) Prdx1⁻/⁻ MEFs and Prdx1 ⁺/⁺ MEFs were stimulated with H₂O₂ as indicated. Protein lysates were collected under argonized conditions by scraping cells into argon-purged lysis buffer (see Materials and methods) and analysed under non-reducing conditions on SDS–PAGE. Akt phosphorylation was detected on Ser473 and Thr308. Akt protein as loading control. (B) Prdx1⁻/⁻ MEFs and Prdx1 ⁺/⁺ MEFs protein lysates were treated as described under (A) and analysed for oxidized PTEN proteins. Actin as loading control. (C) Levels of oxidized PTEN proteins were evaluated by quantifying oxidized and reduced PTEN using a Fuji imaging system (LAS 3000) and related software (ImageGuage). Quantifications of staining intensities were obtained by analysing protein bands from the same ECL obtained film exposure. The y-axis represents staining intensities in arbitrary units of oxidized PTEN. Curves represent data from three independent experiments. (D) Prdx1⁻/⁻ MEFs and Prdx1 ⁺/⁺ MEFs were stimulated with PDGF as indicated. Protein lysates were collected and analysed as described under (A). (E) Prdx1⁻/⁻ MEFs and Prdx1 ⁺/⁺ MEFs protein lysates were treated as described under (D) and analysed for oxidized PTEN proteins. Actin as loading control. (F) Data analysis was done as described under (C). All experiments shown are representative of at least three independent studies including two sets of MEF clones from Prdx1 littersmates. (G) Serum starved MEFs were stimulated with H₂O₂ as indicated. Protein lysates were collected and analysed as described under (A). Akt substrates were detected by western blotting using a phospho-Akt substrate antibody (SRXRXXS/T).
Figure 3 (A) Epitope-tagged PTEN was expressed in 293T cells. Cell lysate was prepared under anaerobic conditions and immunoprecipitates were prepared as described in Materials and methods. A measure of 1000 μg of protein was immunoprecipitated (anti-PTEN antibody; Santa Cruz). Proteins were analysed by SDS–PAGE. PTEN and Prdx1 proteins were detected by staining membranes with anti-PTEN (138G6, Cell Signaling) and anti-Prdx1 antibodies, respectively. (B) Epitope-tagged PTEN and Prdx1 wild type were co-expressed in 293T cells. Before lysis, cells were treated with increasing dosages of H2O2 as indicated for 15 min. Cell lysates were prepared under anaerobic conditions, and precipitated over night using HA-conjugated agarose beads. IPs were washed four times with argon-purged lysis buffer and analysed by western blotting. Proteins were detected with anti-PTEN, anti-Prdx1-SO2/-SO3 and anti-Prdx1 antibodies. Epitope-tagged Prdx1 migrates slower electrophoretically than endogenous Prdx1, labelled as HA–Prdx1. Epitope-tagged PTEN is labelled as Myc–PTEN. Anti-Prdx1-SO2/-SO3 can cross-react with Prdx2-4 and stains more intense in the IP proteins due to over night incubation and longer exposure to atmospheric oxygen. In contrast, lysates were immediately frozen (Supplementary Figure S3A and B). (C) Myc–PTEN and HA–Prdx1 C51/172S were analysed as described under (B). Anti-Prdx1-SO2/-SO3 does not bind Prdx1C51/172S. (D) Recombinant His–PTEN (100 nM) was applied to equimolar GST or GST–Prdx1 and incubated over night. Glutathione sepharose (GST) beads were added for GST-pull down and analysed by western blotting. Input represents 1/50 of the total. (E) HA–Prdx1 wild type and cysteine to serine mutants of catalytically active Prdx1 cysteines were co-expressed with Myc–PTEN wild type. HA–IPs were processed as under (B). Left side shows co-immunoprecipitations, right side shows expression of PTEN and Prdx1 cysteine to serine mutants. (F) Schematic domain structure of potential interaction sites of Prdx1 with PTEN (upper schematic) and PTEN with Prdx (lower schematic). (G) Identification of potential interaction sites using computer modelling (SwissPdb Viewer version 4; http://www.expasy.org/spdbv) (Guex and Peitsch, 1997). *Prdx1 N terminus.
oxidation to NADP⁺ were linear and the Prdx1 inhibition was proportional to molar amount of PTEN and reached saturation (completed inhibition) at PTEN/Prdx1 = 1:1 (mol/mol) ratio. Moreover, an increase in PTEN ratio lowered the velocity of Prdx1 peroxidase activity (Figure 4E).

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Figure 4  (A) Epitope-tagged Myc-PTEN and HA–Prdx1 were co-expressed in 293T cells, which were treated with H₂O₂ for 15 min, as indicated. Cell lysates were prepared under anaerobic conditions, and precipitated overnight as described above by using anti-PTEN antibody.  PTEN-immunoprecipitates were analysed for PI(3,4,5)P3-utilization in a 96-well plate assay as described in Materials and methods (triplicate samples). All results are presented as mean ± s.e. (or difference) for at least two independent experiments. The PTEN protein amount was confirmed by western blotting. (B) 500 µM of H₂O₂ was added to either 20 pmoles of recombinant His-purified PTEN in the presence of different amounts of recombinant purified Prdx1 as indicated and described in Materials and methods. Experiment shown is representative of three independent studies. (C) PTEN activity was measured as described in (B) in the presence of peptides interfering with Prdx1 binding to PTEN. P1 (peptide 1) is replica of first N-terminal 21 amino acids (AS) of Prdx1; P1 Sc, scrambled sequence of P1; P2 (peptide 2), replica of last C-terminal 26 AS of Prdx1; P2 Sc, scrambled sequence of P2. The results are presented as mean ± s.e. (or difference) for at least two independent experiments. (D) Prdx1 peroxidase activity was measured by a standard thioredoxin (Trx)/thioredoxin reductase (TrxR)/NADPH-coupled spectrophotometric assay (see Materials and methods for details). A measure of 20 pmoles of purified Prdx1 (Sigma) was incubated with 500 µM H₂O₂ and increasing molar amounts of purified His–PTEN as indicated. Prdx1 peroxidase activity (without PTEN) was about 68 nmol/mg protein/min under those conditions (extinction coefficient 6.62 mM⁻¹ cm⁻¹ for NADPH at 340 nm). Experiment shown is representative of three to four independent studies. (E) Relative reaction velocity of Prdx1 peroxidase activity from (D) was determined by plotting linear regression coefficient K (y = a – Kx, where y is the absorbency at 340 nm and x is time) versus various PTEN/Prdx1 (mol/mol) ratios. The results are presented as mean ± s.e. (or difference) for at least two independent experiments.

Prdx1 suppresses H-Ras and ErbB-2-induced transformation mainly via promoting PTEN activity

Next, we examined in PTEN⁻/⁻ MEFS whether Prdx1 has an important function in PTEN-induced tumour suppression. Although Prdx1 knock down in PTEN⁻/⁻ MEFS decreased
pAktSer473 levels, H2O2 treatment of MEFs increased it equally in PTEN−/− MEFs and PTEN+/+ MEFs (Figure 5A). Prdx1 is neither over-oxidized nor does it form less peroxidase active dimers in PTEN−/− MEFs compared with PTEN−/− MEFs reconstituted with GFP–PTEN, suggesting that in PTEN-null cells, Prdx1 activity is not compromised after H2O2 treatment (Figure 5B). PI3K/Akt signalling is also essential for oncogenic ErbB-2-induced transformation (Maroulakou et al., 2007), and Akt1 deficiency sufficiently suppresses tumour development in PTEN−/− mice (Chen et al., 2006). As shown for H-Ras (Figure 1B), Prdx1WT also prevented ErbB-2/neuT-induced transformation depending on its peroxidase activity (Supplementary Figure S5A). By using small hairpin (sh) RNA targeting Prdx1 mRNA (shPrdx1) in PTEN−/− MEFs, 

**Figure 5** (A) PTEN−/− MEFs were serum starved for 48 h and treated with H2O2 as indicated for 10 min. Protein lysates were analysed for pAktSer473 as described before. (B) PTEN−/− MEFs were infected with retrovirus expressing GFP–PTEN. After 5-day selection in puromycin (2 μg/ml), MEFs were serum starved, exposed for 10 min to H2O2 and analysed for Prdx1 oxidation and formation of dimeric structures by non-reducing SDS–PAGE. *Prdx1 hetero-dimer formation with other Prdxs. (C) PTEN−/− MEFs were infected with retrovirus expressing shPrdx1, GFP–PTEN, H-RasV12 or various vectors carrying resistance gene only (pBabe, pMKO1). MEFs were plated in soft agar. Colonies were counted after 21 days. *P<0.001 (Student’s t-test). (D) As under C, except MEFs express ErbB-2/neuT instead of H-rasV12. **P<0.039 (Student’s t-test). (E) Immunoblotting of MEFs from (C and D).
transformed by either H-RasV12 or ErbB-2/neuT, we showed that Prdx1 suppressed transformation mainly by regulating PTEN (Figure 5C–E). Although shPrdx1 expressed in PTEN+/−/H-RasV12MEFs or PTEN−+/−/ErbB−/2/neuT MEFs did not increase colony formation compared with PTEN+/−/H-RasV12MEFs or PTEN−+/−/ErbB−/2/neuT MEFs showed a 1.5-fold increase in colony formation compared with PTEN+/−/H-RasV12MEFs and PTEN−+/−/–shPrdx1, GFP−/−/PTEN−/−/H-RasV12MEFs and PTEN−+/−/–shPrdx1, GFP−/−/PTEN−/−/ErbB−/2/neuT MEFs and 1.35-fold increase in colony formation compared with PTEN+/−/−/pMKO1, GFP−/−/PTEN−/−/ErbB−/−/2/neuT MEFs. Furthermore, a slight increase of Prdx1 oxidation was noted in PTEN+/−/−/pMKO1, H−/−/RasV12MEFs compared with PTEN−+/−/−/−/pMKO1, H−/−/−/RasV12MEFs. By using minimum dosages of two well-known PI3K inhibitors, Wortmannin (WM) and Ly294002 (Ly) that inhibited H2O2-induced Akt activity in Prdx1+/+/−/−/MEFs less efficiently than in Prdx1+/+/+MEFs (Supplementary Figure S5B), we found that partial inhibition of PI3K/Akt activity translates into less efficient inhibition of H-Ras or ErbB-2-induced transformation in Prdx1+/−/−/−/MEFs compared with Prdx1+/+/−/−/MEFs (Table 1): 5 nM WM reduced Prdx1+/+/−/−/−/H−/−/RasV12MEFs colony number by <4%, 10 nM WM by 2.3%. However, 5 nM WM decreased Prdx1+/−/−/−/−/H−/−/RasV12MEFs colony numbers by 46% and (P<0.001) 10 nM WM by 67%. Similarly, 10 μM Ly decreased Prdx1+/+/−/−/−/H−/−/RasV12MEFs colony number by 61% and in Prdx1+/+/−/−/−/H−/−/RasV12MEFs by 73%. However, 10 μM Ly decreased colony number of Prdx1+/+/−/−/−/ErbB−/−/−/neuTMEFs by only 53%, whereas in Prdx1+/+/−/−/−/ErbB−/−/−/neuTMEFs by 75%. Moreover, 25 μM Ly decreased colony formation equally in Prdx1+/−/−/−/−/ErbB−/−/−/neuTMEFs and Prdx1+/−/−/−/−/ErbB−/−/−/−/neuTMEFs to 4–5%.

**Discussion**

Compared with Prdxs, catalase peroxidase activity decomposes H2O2 with a >1000-fold higher Km (Chae et al, 1994a; Loewen et al, 2004) and therefore eliminates H2O2 with much higher efficiency. Thus, we cannot expect that Prdx1 scavenges H2O2 in considerable amounts, as shown in Figure 1A and Supplementary Figure S1A. Yet, Prdx1′s peroxidase activity is important in preventing H-RasV12 (Figure 1B) or ErbB-2/neuT (Supplementary Figure S5A) induced transformation. Similar to the catalase knockout mice, all other currently published Prdxs knockout mice, show phenotypically no increased susceptibility to transformation (Ho et al, 1997). Therefore, Prdx1 may have a unique function amongst cellular peroxidases in tumour suppression, by affecting signalling directly through physical interaction with target enzymes. This was shown for c-Abl tyrosine kinase (Wen and VanEtten, 1997) and c-Jun terminal kinase (Kim et al, 2006). As described here for PTEN (Figure 3B), oxidative stress induced Prdx1 to dissociate, which in case of c-Abl (Neumann et al, 1998) and JNK (Kim et al, 2006) resulted in kinase activation. The Prdx1:PTEN heterodimer is most likely formed in a 1:1 molar ratio, as (1) the Prdx1 preservation of PTEN lipid phosphatase activity under oxidative stress is achieved by a 1:1 (mol:mol) ratio of Prdx1 and PTEN and could not be further increased by excess of Prdx1 (Figure 4B) and (2) Prdx11510/1725, which is unable to form dimeric structures (Chae et al, 1994b), bound to PTEN and did not dissociate under H2O2 treatment (Figure 3C). Therefore, these data provide compelling evidence that Prdx1 may bind PTEN as a monomer.

We further propose that the Prdx1:PTEN interaction is essential for protecting PTEN from oxidation-induced inactivation (Figure 4C), as Prdx1 C- and N-terminal peptide

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**Table 1** Prdx1+/− MEFs have a higher sensitivity to PI3K inhibitors in H-Ras or ErbB-2 induced transformation

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<th>Wortmannin (nm)</th>
<th>H-Ras colony number in %</th>
<th>Ly294002 (μM)</th>
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<th>ErbB-2/neuT colony number in %</th>
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<td>3.2 ± 4.1</td>
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Prdx1+/− MEFs and Prdx1+/+/− MEFs were retrovirally infected with constructs carrying genes for H-RasV12 or ErbB-2/neuT (ErbB-2Val644Glu) and treated after plating in soft agar either with 10 or 25 μM Ly294002 every 2 days or Wortmannin 5 or 10 nM every day. Colonies were counted after 21 days. Decrease in colony formation (%) relates to untreated of the same genotype (%). This experiment is representative of three independent studies with two different sets of MEF clones from Prdx1 littermates.
replica, which were designed based on our mapping studies and computer modelling to block PTEN docking sites on Prdx1 (Figure 3F and G; Supplementary Figure S3E), resulted after H2O2 treatment in a 47% reduction of PTEN activity. This was comparable to PTEN activity after H2O2 treatment in the absence of Prdx1 (50%) (Figure 4C). The Prdx1 N terminus, which includes Cys51, may have an important function in regulating the H2O2-induced dissociation of the Prdx1:PTEN complex, as Cys51 when replaced by a serine enhances Prdx1 binding to PTEN (Figure 3D). X-ray crystallography of Prdxs suggested significant conformational changes, such as unwinding of the active site N-terminal helix, to form disulfides, as in reduced Prdxs, the sulfur atoms of the N- and C-terminal conserved cysteine residues are too far apart to react with each other (Schröder et al., 2000; Matsumura et al., 2008). Therefore, in the Prdx1:PTEN complex, increased H2O2 may oxidize Cys51 (Prdx1), thus, promoting unwinding of the PTEN-binding conformation of Prdx1 consequently inducing dissociation.

Over-oxidation of Prdx1 Cys51 occurs during catalysis and correlates with increasing amounts of Trx (Yang et al., 2002). The reduction of Cys51 by Trx inhibits the folding back of Cys51 into its pocket thereby exposing it to further oxidation by H2O2. The binding of PTEN to Prdx1 could have similar effects on Prdx1, thereby inhibiting sufficient reduction of Cys51 by Trx and decreasing Prdx1 peroxidase activity (Figure 4C and D). Alternatively, as it has been shown that Trx binds PTEN and inactivates its lipid phosphates activity, it could be that under our experimental conditions Trx is depleted from the Prdx1 scavenging system by binding PTEN (Meuillet et al., 2004). This seems unlikely though, as Trx was added in approximately 300-fold molar excess, which should provide a large Trx pool to reduce oxidized Prdx1 proteins.

We defined a novel interaction site of Prdx1 with PTEN in the C2 domain of PTEN (Figure 3F; Supplementary Figure S3D and E), which together with the N-terminal PTD, are both required for enzyme activity. Surface plasmon resonance analysis revealed that the C2 domain is essential for high-affinity membrane binding of PTEN (Das et al., 2003). Our data support this finding, as it has been shown recently through chemical proteomic strategy using cleavable lipid baits, that Prdx1 is a potential novel phosphoinositides-binding protein (Pasquali et al., 2007). Prdx1 may, therefore, stabilize PTEN binding to the plasma membrane, which in a recent analysis using single-molecule TIRF microscopy,
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lasts only for a few hundred milliseconds (Vazquez et al., 2006). Such short time of membrane binding could explain why we (Figure 2B, C, E and F; Supplementary Figure S2B and D) and others see only small amounts of PTEN oxidized after cell exposure to H2O2 or PDGF (Kwon et al., 2004). PDGF-induced H2O2 stems either from metabolized superoxide released by NADPH oxidases (NOXs) or the mitochondria and is considered a signalling molecule (Rhee, 2006). As discussed earlier (Leslie et al., 2003), such finding may be attributed to the proximity of NOXs complexes and PIP3. Over-expression of Nox1, in PDGF-stimulated NIH 3T3 cells rapidly increases PIP3 levels, in contrast to PI3K activity, suggesting that Nox1-induced superoxide enhances PIP3 levels by inactivating PTEN rather than activating PI3K. Expression of a peroxidase inactive Prdx2 leads to a rapid increase of PIP3 levels in growth factor stimulated cells, which is reversed by Prdx2wt (Kwon et al., 2004). In our hands however, Prdx2 did not bind PTEN, as we were unable to detect an interaction of Prdx2 and PTEN by immunoprecipitation (data not shown).

PTEN lipid phosphatase activity has an important function in tumour suppression (Tolkacheva and Chan, 2000; Koul et al., 2002) and Akt1 ablation protects (MMTV)-ErbB2/neu, MMTV-polyoma middle T and MMTV-v-H-Ras mice from breast cancer initiation (Skeen et al., 2006; Maroulakou et al., 2007). Here, we show that loss of Prdx1 increased basal, H2O2 and PDGF-induced Akt activity (Figure 2A, D and G) and Prdx1 peroxidase activity was essential in suppressing H-Rasv12 and ErbB-2/NeuT-induced transformation (Figure 1B; Supplementary Figure S5A). Along those lines, Prdx1 ablation accelerated mammary tumorigenesis in MMTV-v-H-Ras mice and increased levels of pAktSer473 compared with PTEN oxidation will be important in future work.

Materials and methods

Reagents

All chemicals were purchased from Sigma Aldrich unless otherwise indicated. Ly294002, WM, antibodies against PTEN (138G6), Akt (11-E7), Akt-phosphothreonine 473 (193H12), Akt-phosphoxygen (308 (244P)), phospho-Akt substrate (11087E) and HA-tag were purchased from Cell Signaling; Antibodies recognizing PTEN (A2B1), PTEN (N-19) and Actin (C-11) were purchased from Santa Cruz; antibodies against Prdx1 and Prdx1-So2/-So3 were purchased from Abcam; Ras antibody from Oncogene; ErbB-2 antibody from Biosource. HA-conjugated agarose beads were purchased from Roche. Amplex Red reagent: Molecular Probes. DMEM, PBS, Glutamax, NEAA, Pen/Strep, Sodium Pyruvate, DMEM without Phenol Red, PBS and PDGF stem from Invitrogen; GST-Sepharose from PharmaCia. 6His–PTEN was expressed in Escherichia coli BL21 from the fusion vector pProEx Htp PTEN and purified by standard protocols.

Retrovirus

The retrovirus was generated after conducting a transient transfection of retroviral constructs into a 293T/17 (ATCC) ecotropic-packaging cell line. Retroviral infection of MEFs was carried out for 4–6 h in the presence of 8 μg/ml polybrene. An antibiotic selection was carried out 24 h later until all uninfected cells were eliminated and stable polyclonal cell lines were generated.

Generation of MEFs

MEFs were generated from Prdx1+/−/− littermate matings in a C57BL/6J129Sv background. Primary MEFs were harvested from E13.5 embryos as described earlier (Neumann et al., 2003). Primary Prdx1+ and PTEN MEFs (passages 3–4) were immortalized by retroviral infection with pBabe-hygro DD p53 expressing dominant-negative p53 (Hahn et al., 2002) and selected with 150 μg/ml hygromycin for 14 days.

Amplex red assay

In total, 35,000 MEFs were plated (n = 6) in 24-well plates (Costar 24) coated with fibronectin. On the day of the assay, cells were washed with PBS and repleted with serum-free and phenol red-free DMEM medium. Amplex Red reagent and horse radish peroxidase (HRP) were prepared as recommended by the manufacturer (HRP 0.2 U/ml). MEFs were incubated with Amplex Red/HRP solution at 37 °C before the first reading (T0) at 540 nm on a plate reader (Molecular Devices SpectraMax MS). Plates were kept in an incubator between readings. The resulting fluorescence (FLU) was compared with FLU from MEFs under same conditions as a control.

Soft agar assays

P53DD-immortalized MEFs (Prdx1−/−, Prdx1+/−/−) were infected with retrovirus containing either Prdx1wt or Prdx1C172S/S185 both subcloned into pQCX1-puro (Clontech) and selected 10 days in puromycin 2 μg/ml. Some clones were further infected with a retrovirus containing either H-RasV12 (pBabe-puro H-RasV12) (Yu et al., 2001) or wild-type ErbB-2 (pBabe-puro ErbB-2) (Yu et al., 2001) and selected in 5 μg/ml puromycin for another 5 days. MEFs were then resuspended in DMEM containing 0.53% agarose (Difco) and plated onto a layer of 0.7% agarose-containing medium in 3.5-cm plates in duplicate (10,000 cells per plate). Colonies were counted after 14–21 days.
Akt activity and PTEN oxidation

MEFs (1.8 × 10^4) were plated on 10-cm plates and serum-starved (0.25% FBS) for 48 h. Plates were then incubated with H_2O_2 (±100 μM) or WM) as a control. Akt and PTEN protein analysis were done as described earlier (Leslie et al., 2008) with slight modifications. Bacterial protein lysis buffer (0.1 M Tris pH 7.4/4°C, 150 mM NaCl, 0.5 mM EDTA, 0.25 mM EGTA, 200 mM NaCl, 10% Glycerol; 50 mM NaF; 100 mM NaVO_4; 40 mM β-Glycerophosphate) was degassed with Argon for 20 min before adding 1% TritonX-100; 2 mM PMSF; 5 mg/ml Aprotinin; N-ethylmaleimide 40 mM; and bovine catalase 100 μg/ml. Plates were transferred into an anaerobic/argon cabinet and were washed twice with cold degassed PBS. Cells were then scraped into lysis buffer and lysed on ice for 20 min before conducting quantitative analysis as described above. A measure of 65 μg of protein were analysed under non-reducing conditions with a 7.5% SDS–PAGE.

Co-immunoprecipitation

PTEN WT (Addgene) was subcloned into a pCGL vector (Clontech) and N-terminally tagged with Myc epitope. Prdx1 WT and Prdx1 C185S/172D (generated by site-specific mutagenesis) were subcloned into a pCAG vector and N-terminally tagged with an HA epitope. All constructs were transfected into 293T/17 (ATCC) cells using Fugene transfection reagent (Roche). Cells were harvested 72 h later for protein lysis under anaerobic conditions (as described above). A measure of 1000 μg of protein was immunoprecipitated using anti-HA affinity matrix overnight at 4°C before washing four times with degassed lysis buffer before analysis on 4–12% gradient SDS–PAGE. Proteins were detected as described above.

GST-pull down

BL2I (DE3) were transformed and cultured overnight with pGEX-4T-2 (Amersham) ligated earlier with Prdx1. At absorbance of 0.6–0.8 at 600 nm, GST–Prdx1 expression was induced by the addition of IPTG (200 μM). Bacterial pellets were re-suspended using fusion protein buffer containing 140 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 1 mM DTT, 50 mM PMSF, 5 mM benzamidine hydrochloride hydrate and 3 μg aprotinin. Lysate was then sonicated and proteins were solubilized by the addition of Triton X-100 (2%). After incubation of supernatant with glutathione affinity matrix, the matrix was washed consecutively with 500 mM NaCl, 50 mM Na_2HPO_4, 100 μM NaF, 100 mM NaVO_4; 40 mM β-Glycerophosphate; 50 mM NaF; 100 mM NaVO_4; 40 mM β-Glycerophosphate was degassed with Argon for 20 min before adding 1% TritonX-100; 2 mM PMSF; 5 mg/ml Aprotinin; N-ethylmaleimide 40 mM; and bovine catalase 100 μg/ml. Plates were transferred into an anaerobic/argon cabinet and were washed twice with cold degassed PBS. Cells were then scraped into lysis buffer and lysed on ice for 20 min before conducting quantitative analysis as described above. A measure of 65 μg of protein were analysed under non-reducing conditions with a 7.5% SDS–PAGE.

PTEN Plasmids

Different lengths of cDNA fragments encoding human PTEN amino acids from 1–403 were subcloned from pSG5–HA–PTEN WT (Addgene#10750), pSG5–HA–PTEN C185S/172D (±10745), pSG5–HA–PTEN WT (±10740), pSG5–HA–PTEN C185S/172D (±10766), pSG5–HA–PTEN 185–373 (±10724), pGSJX2–PTEN +30 to +45 (±10739) (Ramassamy et al, 1999) at restriction sites BamH I/EcoRI. A double-stranded oligonucleotide encoding the Myc-tag sequence was inserted 5′ of the PTEN to generate Myc–PTEN. Inserts tagged with Myc tag were cloned into pQCGX to generate the corresponding pQCGX–Myc–PTEN plasmids; pQCGX–Myc–PTEN WT, or pQCGX–Myc–PTEN 185–245 were generated by PCR mutation and confirmed after DNA sequencing. Prdx1 N- and C-terminal truncation mutants were generated by Bal31 nuclease digest, fill-in with T4-polymerase and subcloned into pCGN-HA.

Recombinant Prdx1 activity assay

Prdx1 peroxidase activity was studied by standard Trx/Trx reductase (TrxR)/NADPH-coupled spectrophotometric assay as described (Chae et al., 1994b). Briefly, NADPH oxidation coupled to the reduced H_2O_2 was monitored at 37°C as a decrease in A_340 using a SpectraMax M5 plate reader spectrophotometer. The reaction was initiated by addition of the H_2O_2 (500 μM) as a substrate into a reaction mixture containing 50 mM Hepes (pH 7.0), 0.15 U TrxR, 18 μM Trx, 25 μM NADPH and 680 nM Prdx1 (Sigma). The effect of PTEN on Prdx1 activity was studied by titration of reaction mixture with indicated increasing amount of PTEN (0.25–1.25 PTEN/Prdx1 mol/mol ratio). All experimental data were normalised to initial (before addition of substrate) absorbency and fitted with linear regression (Sigma Plot 10, Systat Software, Inc., San Jose, CA). Quality of fit was controlled by regression coefficient (R^2 > 0.9).

PTEN inositol phosphatase activity

Myc–PTEN was expressed in 293T/17 cells in the presence or absence of HA–Prdx1. Cells were lysed as described above in phosphatase inhibitors free lysis buffer. PTEN proteins were immunoprecipitated from lysates with anti-PTEN (Santa Cruz) or/and as described above. IPs were then washed twice as described (Schwartzbauer and Robbins, 2000), followed by a final wash in PTEN enzyme reaction buffer (10 mM Hepes, 150 mM NaCl, 10 mM DTT pH 7.2). The phosphatase reactions were done following manufacturer’s instructions (Echelon Biosciences). Briefly, the immunoprecipitated PTEN proteins were incubated with PTEN enzyme reaction buffer for an appropriate amount of time at 37°C in a 96-well plate coated with Pl(3,4,5)P_3. After removal of PTEN proteins from plates, Pl(3,4,5)P_3 was determined according to the manufacturer’s instructions. Absorbance was measured at 450 nm in a microplate reader, and Pl(4,5)P_3 produced was calculated from a standard curve.

Recombinant PTen inositol phosphatase activity

A measure of 20 pmol purified recombinant human His–PTEN was incubated with different amounts of Prdx1 (Sigma Aldrich) and 500 μM H_2O_2 in 20 mM Tris–Cl (pH 7.4) buffer at room temperature for 10 min. H_2O_2 was removed by Bio-Gel P-6 chromatography columns (Bio-Rad), following the manufacturer’s instructions. The protein mixtures were then loaded onto PTEN activity assay plates as described above (Echelon Biosciences). Equal amount of PTEN was confirmed by western blot with the PTEN antibody. For Prdx1 peptide interference, 20 pmol purified recombinant human His–PTEN was pre-incubated with 20-fold excess amount of peptides on ice in 20 mM Tris–Cl (pH 7.4) buffer (for Peptide 1 or scramble peptide 1) or 0.2 mM Tris–Cl (pH 7.4) buffer (peptide 2 or scramble peptide 2) for 1 h, the unbound peptides were removed by Microcon Ultrafiltr YM-10 filters following the manufacturer’s instructions. The recovered PTEN–peptide mixtures were then incubated with 20 pmols Prdx1 (Sigma Aldrich) and 500 μM H_2O_2 in 20 mM Tris–Cl (pH 7.4) buffer at room temperature for 10 min and further processed as described above. Peptide 1: MSSGNAKIGHPAPNFKATA VM; scrambled peptide 1: IKKSWKFGPGQPTSAPKQSKEV; Peptide 2: MUSSGNAKIGHPAPNFKATA VM; scrambled peptide 2: IKKSWKFGPGQPTSAPKQSKEV; all peptides had purity > 75% and were purchased from Genescript, NJ.

PTEN/MEFs experiments

An optimal sequence for knock down of Prdx1 by sh (5′-GCTGAGGATTGGAGTCTTA-3′) was identified from the TRC consortium (http://www.broad.mit.edu) and was subcloned into pMKO1 retroviral vector by AgeI/EcoRI digest. PTEN+/− MEFS were infected with retroviral supernatant containing either pMKO1–Prdx1 shRNA or pMKO1 EV, and selected in puromycin 2 μg/ml (for 1 week). GFP–PTEN was subcloned from pGFP–C2–PTEN (Leslie et al., 2000) into pQCXIP at restriction sites of AgeI/EcoRI. PTEN+/−/MEFS or PTEN−/−/shPrdx1+MEFS were then transduced with GFP–PTEN retrovirus and selected in 6 μg/ml puromycin. Infection and selection with retrovirus expressing either H-Ras+/− or ErbB2−/−euT was then challenged by 10 μg/ml puromycin.

MMTV-v-H-Ras mice lacking Prdx1

MMTV-v-H-Ras mice (Sinn et al., 1987) were purchased from Charles River. Prdx1+/−/MMTV-v-H-Ras mice were intercrossed to generate Prdx1−/−/MMTV-v-H-Ras and Prdx1+/−/MMTV-v-H-Ras mice. Mice were monitored twice a week for tumour appearance. Mouse mammary glands were isolated from age-matched 16-week old female MMTV-v-H-ras/Prdx1−/− and MMTV-v-H-ras/Prdx1+/− (n = 3) mice following earlier described procedures (Fujitawa et al., 2005). Tumours were processed as described above.
Supplementary data
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

Acknowledgements
We especially thank Drs Yusuf Hannun, Peter Sicinski, Alex Toker, Steve Rosenzweig and Scott Eiben for fruitful discussions. We thank Ningfei An and Joe Blumer for assistance with GST-pull down experiments and Joseph Moore for editorial assistance. We thank Dr Rick Van Etten for sharing the Prdx1-constructs and knockout mice and Vic Stambolic for providing PTEN-/-MEFs. We also thank Dr William Sellers for sharing the PTEN wild-type construct through Addgene. This work was supported by grants from the NIEHS-K22 ES012985, ACS-IRG-97-219-05, Claudia Adams Barr Award-DFCI All (CAN), and Abney research Foundation-MUSC (JC) and (AK).

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