Supporting Information

Conrad et al. 10.1073/pnas.1007909107

SI Materials and Methods

PTP Activity Assay. The Src optimal peptide (AEEEIYGEFEAKKK) was subjected to in vitro phosphorylation with a mixture of [γ-^{32}P] ATP (Amersham Biosciences) and nonradioactive ATP by the recombinant c-Src protein (Upstate). To separate the nonincorporated ATPs from the peptide, the labeling reaction was loaded on a column with AG1 × 8 beads (BioRad). Radiolabeled peptide was dried in a "speed-vac" centrifuge before being redissolved in assay buffer [25 mM imidazole (pH 7.4), 0.1 mg/mL BSA, and 10 mM DL-DTT (DTT)].

After 60 h of 4-hydroxytamoxifen (Tam) treatment, cells were washed twice in ice-cold PBS before lysis in degassed protein tyrosine phosphatase (PTP)-assay lysis buffer (0.5% Triton X-100, 0.5% deoxycholic acid, 150 mM NaCl, and 20 mM Tris, pH 7.5) supplemented with 1 mM benzamidine and 1% Trasylol. After removal of cell debris by centrifugation, aliquots were mixed with degassed assay buffer with and without 10 mM DTT. After addition of the labeled substrate the samples were left at 30 °C for 8 min before the reaction was terminated by addition of charcoal mixture (0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH_2PO_4, and 4% vol/vol Norit A) and centrifuged. The supernatant was transferred to a scintillation tube with 5 mL of Ecoscint A (National Diagnostics) and the amounts of radioactivity determined in a 2000CA, Tri-Carb liquid scintillation analyzer (Perkin Elmer).

In-Gel Assay of PTP Oxidation. ***Labeling of substrate.*** One milligram of Poly(Glu_4Tyr)n (Sigma Aldrich) was labeled with [γ-^{32}P]ATP overnight at 30 °C in kinase buffer [50 mM Hepes (pH 7.4), 2 mM DTT, 20 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 mM sodium orthovanadate, 0.5 μg recombinant Src kinase (Upstate), 0.2 mM ATP, and 20 μL [γ-^{32}P]ATP (Perkin-Elmer Applied Biosystems, NEG002H)]. Peptides were precipitated with 20% trichloroacetic acid on ice for 30 min and centrifuged at 15,000 × *g* before being dissolved in 2 M Tris base. The peptides were loaded onto a 10-mL Sephadex G50 column, and 0.5 mL fractions were eluted with 20 mM imidazole, pH 7.2. The radioactive fractions were saved at −80 °C.

Cell lysis and in-gel peptide dephosphorylation. Cells were lysed in degassed lysis buffer [20 mM Tris (pH 7.5), 1% Nonidet P-40, 10% glycerol, and 1 μg/mL aprotinin] with and without 50 mM iodoacetic acid (Sigma Aldrich). After centrifugation and removal of cell debris, total cell lysate and immunoprecipitated T cell protein tyrosine phosphatase (TC-PTP) or unspecific mouse IgG_1 negative control (1 μg/IP) was boiled with sample buffer. The samples were loaded on a 10% acrylamide gel containing 2 × 10^5 cpm/mL ^{32}P-poly(Glu_4Tyr)n. The gel was incubated with 50 mM Tris/HCl (pH 8.0) added with 20% isopropanol for 90 min for fixation and SDS removal. The isopropanol was removed by two washing steps (30 min each) with 50 mM Tris/HCl (pH 8.0), 0.3% β-mercaptoethanol solution. The proteins were then renatured (50 mM Tris/HCl (pH 8.0), 0.3% β-mercaptoethanol, 1 mm EDTA, and 6 M guanidine-HCl) for 2 × 40 min and finally reduced and dephosphorylated with denaturation buffer supplemented with 4 mM DTT overnight. The gels were washed three times with distilled water before they were dried in an air gel dryer (Bio-Rad) and exposed to film at −80 °C.

oxPTP-Antibody–Based Analyses of PTP Oxidation. ***Analyses of cellular SHP-2 oxidation.*** Cells were lysed with degassed lysis buffer [20 mM Tris (pH 7.5), 1% Nonidet P-40, 10% glycerol, and 1 μg/mL aprotinin] with and without 50 mM iodoacetic acid at room temperature for 20 min in the dark. After removal of cell debris, SHP-2 was immunoprecipitated with 1 μg antibody, then washed once with lysis buffer and twice with 20 mM Hepes. Oxidized cysteines were reduced with 100 mM DTT on ice for 30 min, followed by three washing steps with Hepes. One-hour incubation with 100 μM pervanadate on ice was performed to oxidize the reduced cysteines to sulfonic acid. Samples were boiled with sample buffer and loaded onto a 10% acrylamide gel. Oxidized SHP-2 was detected with the oxPTP antibody. To ensure equal loading, membrane was also probed with SHP-2 antibody.

Analyses of PTP oxidation of recombinant PTPs. Experiments were carried out as previously described (1), with some minor modifications. In brief, 0.5 μg purified GST-epitope-tagged TC-PTP was allowed to bind to glutathione-Sepharose in assay buffer (20 mM Tris/HCl, pH 7.5) for 1 h in a head-over-head tumbler. All steps were carried out at 4 °C. Then, the bound protein was reduced with 50 mM DTT for 1 h in 500 μL degassed assay buffer. After three washing steps, the samples were treated with the indicated H_2O_2 and 15-OOH-eicosatetraenoic acid (15-HPETE) concentrations for 30 min. The negative control—not treated with H_2O_2 and 15-HPETE—was immediately treated with 100 mM iodoacetic acid to alkylate all thiol groups, whereas the positive control was left untreated. Subsequently, the samples were washed two times and treated with 40 mM iodoacetic acid for 30 min. After alkylation, the samples were washed three times and reduced with 50 mM DTT for 30 min. Before pervanadate treatment, DTT was removed by two washing steps. Pervanadate was prepared by mixing 940 μL 20 mM Hepes, 10 μL 100 mM vanadate (heated to 95 °C for 3 min and allowed to cool on ice), and 50 μL 97 mM H_2O_2. From this solution 10 μL was mixed with 90 μL 20 mM Hepes and added to the sample for 60 min to convert all nonalkylated cysteine residues into the sulfonic acid form, which is recognized by the oxPTP-specific antibody (1, 2). After pervanadate treatment, the samples were boiled for 5 min in 40 μL sample loading buffer and subjected to SDS/PAGE and immunoblotting with both the oxPTP antibody and a GST-tag-specific antibody to determine loading.

Measurement of Lipid Peroxidation. Cells were loaded with 2 μM BODIPY 581/591 ^{11}C (Invitrogen) for 60 min to detect cellular lipid peroxidation. Channels used to detect BODIPY emission were FL1-H at 530 nm and FL2-H at 585 nm.

Analyses of PTP Oxidation by Labeling Reduced Cysteines with Iodoacetyl-Biotin. The experimental procedure was performed essentially as described by Chen et al. (3), with some modifications. The cells were lysed for 20 min on ice in the dark in degassed lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, and 20 mM Tris-HCl, pH 5) containing 1 mM EZ-link Iodoacetyl-PEG-2-Biotin (Thermo Scientific) and 1 μg/mL aprotinin. The low pH of the lysis buffer creates a preferable binding of PTPs rather than other cysteine-containing proteins. After removal of cell debris precipitation of biotinylated proteins was performed with streptavidin-agarose (Sigma Life Science). Samples were separated on a 10% acrylamide gel and probed for PTP-LAR. Total cell lysates were also analyzed to ensure equal protein content.

Analyses of PDGF Receptor Expression and Phosphorylation. To achieve maximum deletion of Gpx4, cells were treated for 48–60 h with Tam. In some experiments, *N*-acetyl-cysteine (NAC, 5 mM),

(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 250 μM), AA861 (0.5 μM), MK886 (10 μM), indomethacin (200 μM), AG1296 (10 μM), or diphenyliodonium (DPI, 100 μM) were included. Cells were routinely stimulated with PDGF-BB (10 ng/mL final concentration) for 3 min at 37 °C in starve medium (DMEM supplemented with 100 μg/mL BSA). After stimulation, cells were washed twice with ice-cold PBS and lysed in lysis buffer [0.5% Triton X-100, 0.5% sodium deoxycholate salt/deoxycholic acid, 150 mM NaCl, 20 mM Tris (pH 7.5), 10 mM EDTA, and 30 mM Na-pyro-Px10H_2O $NaPO_3$ (pH 7.5)] supplemented with 200 μM Na_3VO_4 and 1% Trasylol for 15 min on ice. Cell debris was removed by centrifugation for 20 min at 4 °C, 10,000 × *g*. Equal amounts of total protein were separated on an 8% SDS-polyacrylamide gel, transferred via semidry transfer to a Hybond C super membrane (Amersham Biosciences), and blocked in 5% BSA in Tris-buffered saline before being subjected to immunoblotting using the antibodies given below.

For enrichment of PDGF β-receptor, cell lysates were incubated with lectin agarose (Sigma-Aldrich, L-1394) overnight at 4 °C. After three washing steps with lysis buffer, beads were boiled in loading buffer, and supernatants were subjected to immunoblotting.

To monitor in vivo activity of PDGF receptor targeting PTPs, cells were stimulated with 10 ng/mL PDGF-BB for 3 min. Ligand was removed by washing with 37 °C PBS, and PDGF receptor inhibitor AG1296 was added. Cells were subsequently lysed after different times, and PDGF β-receptor phosphorylation was analyzed as described above.

PDGF-BB–Induced Cytoskeletal Changes. Cells were plated on coverslips and treated for 60 h with Tam. The PDGF β-receptor inhibitor AG1296 (10 μM) was added 12 h before PDGF-BB stimulation. After washing with prewarmed PBS, cells were stimulated for 3 min with PDGF-BB in starve medium (DMEM plus 100 μg/mL BSA). Subsequently, cells were washed again with PBS and fixed with 4% paraformaldehyde in PBS for 5 min at room temperature. Fixed cells were washed three times for 5 min with PBS and permeabilized in 0.1% Triton X-100 in PBS for 5 min, and unspecific binding was blocked with 10% FCS in PBS for 1 h. Cells were stained with phalloidin solution (#P5282; phalloidin-FITC, dissolved in methanol, 20 μg/mL final concentration; Sigma-Aldrich) in a humidified chamber for 45 min in the dark. After staining, coverslips were washed three times with PBS/0.1% Tween 20, and then nuclei were counterstained with DAPI and washed again with PBS. Coverslips were mounted in DakoCytomation Fluorescent Mounting Medium. Cells were photographed by a Hamamatsu ORCA CCD digital camera using QED Imaging System software using a Zeiss Axioplan2 microscope.

Analysis of PDGF-BB–Induced PLCγ-1 Activation. In brief, 10^5 cells were seeded per well of 12-well plates. The next day, the medium was replaced with serum-free M199 medium (Invitrogen) containing 1 mg/mL BSA. Twenty-four hours later, 4 μCi of [H-3]-myo-inositol (myo-[2-^{3}H] inositol with PT6-271 stabilizer; TRK911, GE Healthcare) was added per well, and incubation continued for another 24 h. Thereafter, the medium was replaced with 1 mL fresh M199/BSA medium per well, and 10 μL 1 M LiCl was added. After 15 min incubation, stimulation with 10 ng/mL PDGF-BB was carried out at 37 °C for 30 min, or cells were left unstimulated. The medium was removed, 1 mL 10% trichloroacetic acid was added, and precipitation allowed for 10 min with occasional agitation. The supernatant was transferred into a 15-mL tube and extracted four times with 2 mL water-saturated diethyl-ether. The water phase was then neutralized by addition of 20 μL 3 M Tris-base, supplemented to 4 mL with water, and applied to a 1.5-mL AG1-X8 ion-exchange column (200–400 mesh, formate form; Bio-Rad), which had been prewashed with 2 mL H_2O twice. The column was washed consecutively with 2 mL H_2O and with 5 mL 60 mM ammonium formate, 5 mM Na-tetraborate. The inositol phosphates were then eluted directly into a scintillation vial by adding 5 × 2 mL 1 M ammonium formate, 0.1 M formic acid. The eluate was mixed with 9 mL Flo-Scint IV scintillator (Perkin Elmer), and the samples were measured in a scintillation counter.

To confirm the activity assays, immunoblotting of lysates obtained from cells that had been stimulated with 10 ng/mL PDGF-BB for 30 min was carried out using a phospho-specific PLCγ-1 antibody (pPLCγ-1; #2821, Cell Signaling Technology). For quantification of expression levels, a PLCγ-1 antibody was used at 1:2,000 final dilution (sc-61, Santa Cruz, Biotechnology).

Antibodies. Gpx4 expression levels were studied using a monoclonal peptide antibody raised against murine Gpx4 as previously described (4). Further antibodies used in this study were as follows: PDGF β-receptor (described in ref. 5, 1 μg/mL), pTyr (pY99, sc 7020, Santa Cruz, Biotechnology, 1 μg/mL), SHP-2 (sc-280, Santa Cruz, Biotechnology, 1 μg/mL), β-actin (#A5441, clone AC-14, Sigma-Aldrich, 1:5,000), pY751, pY771, pY1009 (2 μg/mL, described in ref. 6; pY751 and pY771 were used together with pY1021 blocking peptide to reduce unspecific binding, 1:400 of 1 mM), pY1021 (ab16868, Abcam, 1 μg/mL), Akt (#9272, 1:1,000), p-Akt (#9271, Ser-473, 1:1,000), Erk (#9102, 1:1,000), p44/42 (#9101, 1:1,000), all Cell Signaling Technologies, TC-PTP (MM-0018, Medimabs), LAR (rabbit serum, kind gift from Wiljan Hendriks, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands), and oxPTP antibody (MAB2844, R&D Systems, 1 μg/mL). Secondary antibodies against rabbit (#NA934V) or mouse (#NA931V) were purchased from GE Healthcare U.K. Limited. Detection was achieved using the Amersham ECL Western Blotting Detection Reagents (# RPN2106, GE Healthcare U.K. Limited).

1. Groen A, et al. (2005) Differential oxidation of protein-tyrosine phosphatases. *J Biol Chem* 280:10298–10304.
2. Persson C, et al. (2004) Preferential oxidation of the second phosphatase domain of receptor-like PTP-alpha revealed by an antibody against oxidized protein tyrosine phosphatases. *Proc Natl Acad Sci USA* 101:1886–1891.
3. Chen K, Kirber MT, Xiao H, Yang Y, Keaney JF, Jr (2008) Regulation of ROS signal transduction by NADPH oxidase 4 localization. *J Cell Biol* 181:1129–1139.
4. Seiler A, et al. (2008) Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell Metab* 8:237–248.
5. Karlsson S, et al. (2006) Loss of T-cell protein tyrosine phosphatase induces recycling of the platelet-derived growth factor (PDGF) beta-receptor but not the PDGF alpha-receptor. *Mol Biol Cell* 17:4846–4855.
6. Persson C, et al. (2004) Site-selective regulation of platelet-derived growth factor beta receptor tyrosine phosphorylation by T-cell protein tyrosine phosphatase. *Mol Cell Biol* 24:2190–2201.

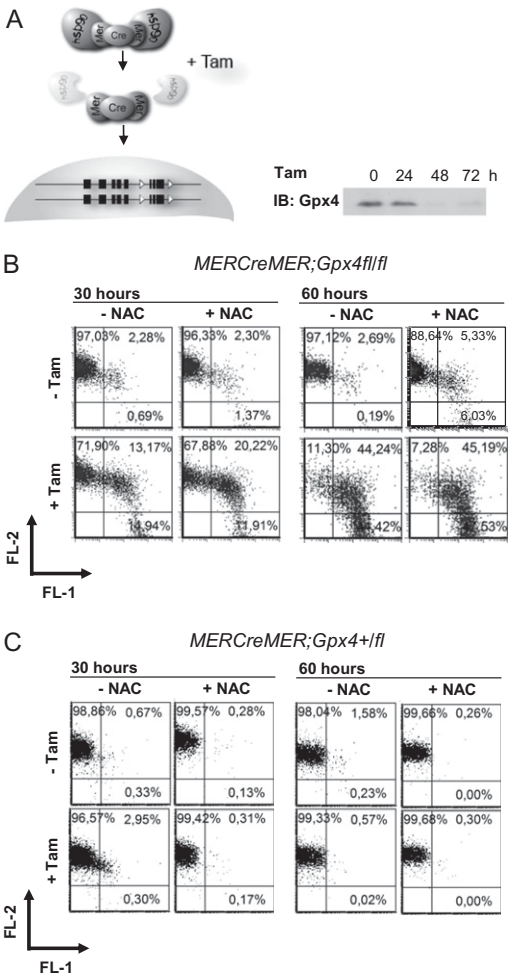

Fig. S1. 4-Hydroxytamoxifen (Tam)–induced MERCreMER-mediated deletion of Gpx4. (*A*) *MERCreMER;Gpx4*$^{fl/fl}$ cells carry two loxP-flanked *Gpx4* alleles (4). Addition of Tam to fibroblasts stably expressing MERCreMer leads to Cre-mediated removal of *Gpx4* in *Gpx4*$^{fl/fl}$ cells. Immunoblotting showed down-regulation of Gpx4 protein 48 h after addition of Tam. (*B*) Lipid peroxidation, as measured by BODIPY 581/591 ^{11}C staining (FL-1 oxidized; FL-2 reduced) was apparent already 30 h after Tam addition to *MerCREMer;Gpx4*$^{fl/fl}$ cells. Lipid peroxidation was not significantly affected by NAC. (*C*) No lipid peroxidation was formed by Tam in the control *MERCreMER;Gpx4*$^{+/fl}$ cells.

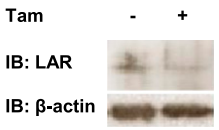

Fig. S2. Increased oxidation of LAR in *Gpx4*$^{-/-}$ cells. Analyses of LAR oxidation, by labeling of reduced LAR with 1 mM EZ-link Iodoacetyl-PEG-2-Biotin, revealed lower levels of reduced LAR in *Gpx4*$^{-/-}$ cells (*Upper*). Equal input material was determined by actin immunoblotting of total cell lysates from wild-type and *Gpx4*$^{-/-}$ cells (*Lower*).

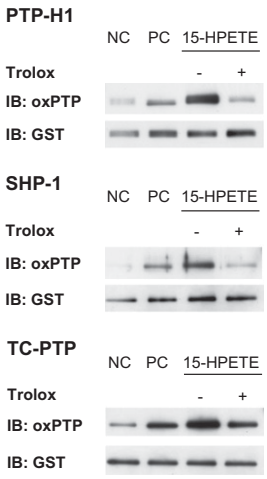

Fig. S3. Trolox prevents 15-HPETE–induced PTP oxidation. 15-HPETE–induced in vitro oxidation of PTP-H1, SHP-1, and TC-PTP was analyzed by oxPTP immunoblotting as in Fig. 1*D* of main text. Addition of the Trolox (100 μM) prevented PTP oxidation induced by 15-HPETE (1 μM).

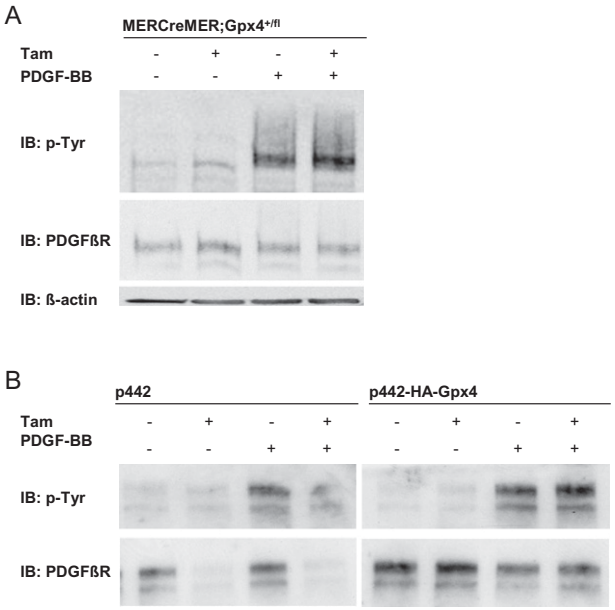

Fig. S4. Tam does not affect PDGF β-receptor expression or phosphorylation in control cells or in *MERCreMER;Gpx4*$^{fl/fl}$ cells transfected with *Gpx4*. (*A*) *MERCreMER;Gpx4*$^{+/fl}$ control cells did not show increased ligand-induced PDGF β-receptor phosphorylation or Tam-induced changes in PDGF β-receptor levels. (*B*) Lentivirus-mediated add-back of HA-tagged wild-type Gpx4 in *Gpx4*$^{-/-}$ cells prevented Tam-induced PDGF β-receptor down-regulation and hyperphosphorylation. Empty virus (p442) was used as negative control.

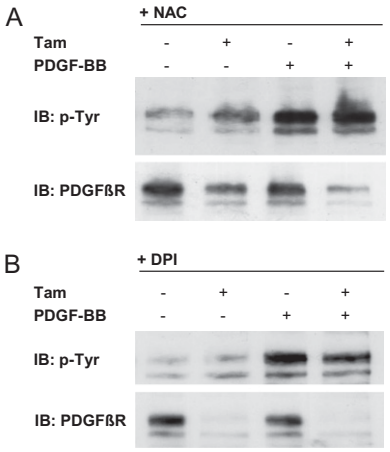

Fig. S5. Lipid peroxide-regulated PDGF β-receptor signaling is not affected by NAC or DPI. Neither the antioxidant NAC (5 mM) (*A*) nor the NADPH oxidase inhibitor DPI (100 μM) (*B*) affected PDGF β-receptor expression or phosphorylation in *Gpx4*$^{-/-}$ cells.

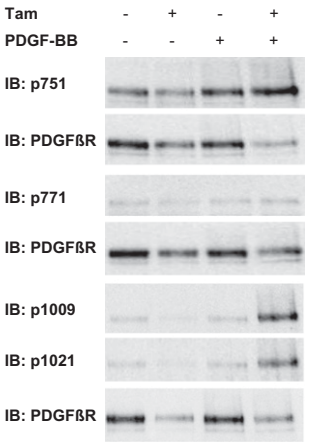

Fig. S6. Gpx4 deletion causes site-specific alterations in PDGF β-receptor phosphorylation. Site-specific analyses of PDGF β-receptor phosphorylation demonstrated that the phosphorylation of sites 1009 and 1021 was more strongly affected by Gpx4 deletion than the phosphorylation of sites 751 and 771.

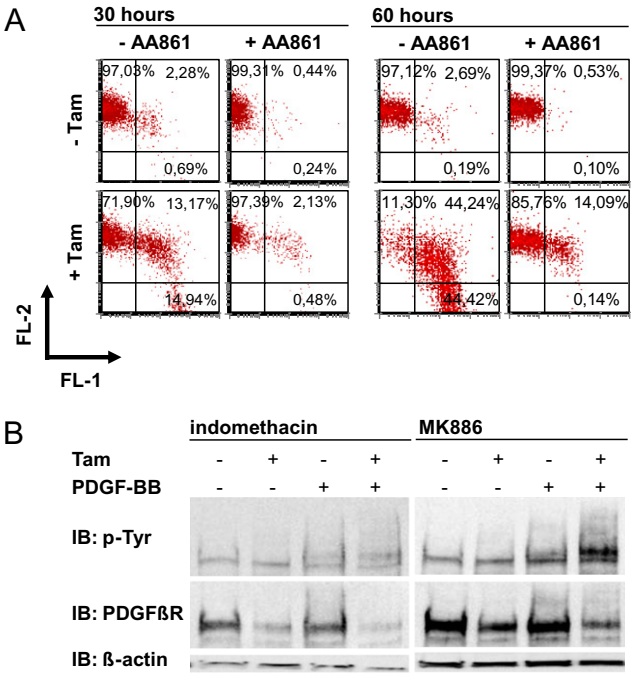

Fig. S7. Differential impact of AA861, indomethacin, and MK886 on effects induced by deletion of Gpx4. (*A*) The lipoxygenase inhibitor AA861 prevented lipid peroxidation in *Gpx4*$^{-/-}$ cells, as measured by BODIPY 581/591 ^{11}C staining (FL-1 oxidized; FL-2 reduced). (*B*) Indomethacin and MK866 did not affect PDGF β-receptor phosphorylation or expression in *Gpx4*$^{-/-}$ cells.