Supplementary Fig. 1. Dimedone labels reversibly oxidized Cys residues in vitro and in cells. (a) Purified recombinant PTP1B (1-321) was incubated under the indicated conditions for 30 min. Reaction mixtures were then subjected to in vitro phosphatase assays using pNPP as substrate, either immediately (post-oxidation) or after an additional incubation with DTT (10 mM) and catalase (5 U) (recovered activity). Relative phosphatase activities are shown in the graph, setting the activity of DTT-treated PTP1B as 1. Representative data from 2 experiments are shown. Error bars indicate SD (n = 3 technical replicates). (b) PTP1B was incubated with or without 5 mM dimedone under the indicated conditions for 5 min, followed by SDS-PAGE and immunoblotting with anti-dimedone Cys and anti-PTP1B antibodies. Representative data from 3 experiments are shown. (c) Purified PTP1B was labeled with dimedone (5 mM) for 5 min under the indicated conditions or after pre-incubation with the indicated concentrations of...
DTT or H₂O₂, followed by immunoblotting with anti-dimedone-Cys and anti-PTP1B antibodies. Representative data from 2 experiments are shown. (d) Serum-starved Swiss 3T3 cells were treated with PDGF-BB (50 ng ml⁻¹) or H₂O₂ (1 mM) for 10 min. Cells were fixed in the presence or absence of dimedone (5 mM) for 5 min, and subjected to immunostaining with anti-dimedone-Cys antibodies (green). Representative images are shown for each condition from one of 2 independent experiments. The graph shows the average intensities of the anti-dimedone Cys signal subtracted by background signal in the images, relative to the values in untreated control cells (n = 10 images). Error bars represent SD. ***P<0.0001, ns: not significant, ANOVA with Bonferroni/Dunn’s post-hoc test. (e) Serum-starved Swiss 3T3 cells were treated with H₂O₂ (1 mM or 50 mM) for 10 min. Cells were then fixed in the presence of dimedone (5 mM) for 5 min, and subjected to immunostaining with anti-dimedone Cys antibodies (green). Representative images are shown for each condition from one of 2 independent experiments. The graph shows the average intensities of the anti-dimedone Cys signal with the background signal in each image subtracted, relative to the values in untreated control cells (n = 10 images). Error bars represent SD. ***P<0.0001 to control cells, ANOVA with Bonferroni/Dunn’s post-hoc test. (f) Serum-starved Swiss 3T3 cells were treated with PDGF-BB (50 ng ml⁻¹) or H₂O₂ (1 mM) for 10 min, followed by treatment with dimedone (5 mM) for 30 min. Lysates were immunoblotted with anti-dimedone-Cys and anti-SHP2 antibodies. Representative images are shown from one of 2 independent experiments. (g) Serum-starved Ptpn11fl/fl MEFs expressing CRE-ER<sup>Tam</sup> treated with or without 4-hydroxytamoxifen (4OHT) were stimulated with PDGF-BB (50 ng ml⁻¹) or H₂O₂ (1 mM) for 10 min. Cells were fixed in the presence of dimedone, and immunostained with anti-dimedone Cys antibodies (green). Representative images are shown for each condition from one of 2 independent experiments. Lysates from Ptpn11fl/fl MEFs were immunoblotted with anti-SHP2 and anti-ERK2 antibodies. The graph shows the average intensities of the anti-dimenedonylated cysteine signal, with the background signal in each image subtracted, relative to the values in untreated cells (n = 10 images). Error bars represent SD. ns: not significant, ANOVA with Bonferroni/Dunn’s post-hoc test. Scale bars: 50 μm.
Supplementary Fig. 2. Specific detection of oxidized SHP2 by dimedone-PLA. (a) Serum-starved Swiss 3T3 cells were stimulated with PDGF-BB (50 ng ml$^{-1}$), EGF (50 ng ml$^{-1}$), or H$_2$O$_2$ (1 mM) for 10 min, fixed in the presence of dimedone (5 mM) for 5 min, and subjected to dimedone-PLA (gray). Representative images are shown for each condition from one of 3 independent experiments. The graph shows the average number of PLA signals per cell ($n = 6$ images for each condition, 5-20 cells in an image), relative to unstimulated control cells (normalized to 1). Error bars represent SD. ***$P<0.0001$, ANOVA with Bonferroni/Dunn’s post-hoc test. (b) Serum-starved Swiss 3T3 cells were stimulated with PDGF-BB (50 ng ml$^{-1}$) or H$_2$O$_2$ (1 mM) for 10 min, fixed in the presence or absence of dimedone for 5 min, and subjected to dimedone-PLA (gray) with or without either primary or secondary antibody. Representative images are shown for each condition from one of 2 independent experiments. The graph shows the average number of PLA signals per cell ($n = 6$ images for each condition, 5-20 cells in an image) relative to control cells without stimulation. Error bars represent SD. ***$P<0.0001$, ANOVA with Bonferroni/Dunn’s post-hoc test. (c) 4-OHT-treated, serum-starved Ptpn11$^{fl/fl}$ MEFs expressing Cre-ER$^{Tam}$ with re-expression of either wild-type SHP2 (WT SHP2) or C459E SHP2 (SHP2$^{C459E}$) were stimulated with PDGF-BB (50 ng ml$^{-1}$) or H$_2$O$_2$ (1 mM) for 10 min. Cells were fixed in the presence of dimedone, and subjected to dimedone-PLA (gray). Representative images are shown for each condition from one of 2 independent experiments. The graph shows the average number of PLA signals per cell ($n = 6$ images for each condition, 5-20 cells in an image) relative to control cells without stimulation. Error bars represent SD. ***$P<0.0001$, ANOVA with Bonferroni/Dunn’s post-hoc test. (d) Lysates from Ptpn11$^{fl/fl}$ MEFs with or without 4-OHT treatment, and MEFs treated with 4-OHT re-expressing either WT SHP2 or SHP2$^{C459E}$ were immunoblotted with indicated antibodies. Representative immunoblots are shown from one of 2 experiments. (e) Serum-starved Swiss 3T3 cells, pre-treated with NAC (10 mM, 15 min) or BSO (2.5 mM, 16 h), were stimulated with PDGF-BB (50 ng ml$^{-1}$) or H$_2$O$_2$ (1 mM) for 10 min, fixed in the presence of dimedone for 5 min, and subjected to dimedone-PLA (gray). Representative images are shown for each condition from one of 2 independent experiments. The graph shows the average number of PLA signals per cell ($n = 6$ images for each condition, 5-20 cells in an image) relative to unstimulated control cells. Error bars represent SD. ***$P<0.0001$, ANOVA with Bonferroni/Dunn’s post-hoc test. Scale bars: 50 μm.
Supplementary Fig. 3. Dimedone-PLA detects oxidized PTP1B. (a) Serum-starved Swiss 3T3 cells were treated with PDGF-BB (50 ng ml⁻¹) or H₂O₂ (1 mM) for 10 min, fixed in the presence of dimedone (5 mM) for 5 min, and subjected to dimedone-PLA for PTP1B (gray). Representative images are shown for each condition from one of 2 independent experiments. The graph shows the average number of PLA signals per cell (n = 6 images for each condition, 5-20 cells in an image), relative to untreated control cells (set to 1). Error bars represent SD. ***P<0.0001, ns: not significant, ANOVA with Bonferroni/Dunn’s post-hoc test. Scale bar: 50 µm. (b) Serum-starved HepG2 cells were treated with insulin (25 nM) for the indicated times. Lysates were immunoblotted with anti-AKT pT308 and anti-AKT1 antibodies. Representative images are shown for each condition from one of 2 independent experiments. (c) Lysates from HepG2 cells expressing control shRNA or shRNA targeting human PTPN1 (shPTPN1) with or without stable expression of mouse PTP1B (WT mPTP1B) or C215S PTP1B (mPTP1B_C215S) were immunoblotted with anti-mouse PTP1B, anti-PTP1B antibodies that recognize both mouse and human PTP1B, and anti-calnexin antibodies. Representative immunoblots are shown from one of 2 independent experiments. (d) HepG2 cells expressing control shRNA or shPTPN1 with or without stable expression of WT mPTP1B or mPTP1B_C215S were immunostained with anti-mouse PTP1B antibodies (green). Nuclei were visualized with DAPI (blue). Representative images are shown for each condition from one of 2 independent experiments. Scale bar: 10 µm.
Supplementary Fig. 4. Spatio-temporal dynamics of SHP2 oxidation. (a) Serum-starved Swiss 3T3 cells were stimulated with EGF (50 ng ml\(^{-1}\)) for the indicated times, fixed in the presence of dimedone, and subjected to dimedone-PLA. Representative images are shown for each condition from one of 2 independent experiments. The graph shows the average number of PLA signals per cell (\(n = 6\) images for each condition, 5-20 cells in an image) relative to unstimulated control cells (normalized to 1). Error bars represent SD. Scale bar: 50 \(\mu\)m. (b) Serum-starved Swiss 3T3 cells were stimulated with PDGF (50 ng ml\(^{-1}\)) for the indicated times, fixed, and subjected to immunostaining with anti-PDGFR\(\beta\) antibodies
without permeabilization to visualize surface PDGFRβ. Representative images are shown for each condition from one of 2 independent experiments. The graph shows the average fluorescence intensities of the surface PDGFRβ signal with the background signal in the image subtracted, relative to the values in untreated cells \( (n = 10 \text{ images}) \). Error bars represent SD. Scale bar: 50 μm. (c) Serum-starved Swiss 3T3 cells were treated with 50 ng ml\(^{-1}\) PDGF-BB for 2.5 min (see Fig. 2b). Median distances of the centers of mass between punctate signals of ox-SHP2 and of the nearest indicated marker signal, or median distances of between punctate signals of indicated marker themselves, were obtained by object-based image analysis. Box-whisker plots show the 25\(^{th}\)-75\(^{th}\) percentiles (boxes) and the 5\(^{th}\)-95\(^{th}\) percentiles (whiskers) of median distances \( (n = 50 \text{ cells}) \). ***\(P<0.0001\), ns: not significant, unpaired \(t\) test (PDGFRβ and RAB5) or unpaired Welch’s \(t\) test (CHC and EEA1), as appropriate. (d) Swiss 3T3 cells expressing EGFP-fused RAB5, RAB7, RAB9, or RAB11 were serum-starved, stimulated with PDGF-BB (50 ng ml\(^{-1}\)) for 10 min, and then fixed in the presence of dimedone. Dimedone-PLA (magenta) and anti-GFP staining (green) are shown. Representative images are shown for each condition from one of 2 independent experiments. Scale bar: 10 μm. (e) Serum-starved Swiss 3T3 cells were stimulated with PDGF-BB (50 ng ml\(^{-1}\)) for 5 min, fixed, subjected to PLA with anti-ERK2 and anti-pERK1/2 antibodies (magenta), and co-stained with the anti-RAB5 antibody (green). Representative images are shown for each condition from one of 2 independent experiments. Scale bar: 10 μm. (f) Serum-starved Swiss 3T3 cells were treated with PDGF-BB (50 ng ml\(^{-1}\)) for 5 min and immunostained with anti-RAB5 (gray or green) and anti-EEA1 (gray or magenta) antibodies. Nuclei were labeled with DAPI (blue). Representative images are shown from a single experiment. Scale bar: 10 μm. (g) Serum-starved Swiss 3T3 cells were treated with H\(_2\)O\(_2\) (1 mM) for 10 min and subjected to dimedone-PLA (magenta), followed by immunostaining with anti-RAB5 antibody. Nuclei were labeled with DAPI (blue). Representative images are shown from one of 2 independent experiments. Scale bar: 10 μm.
**Supplementary Fig. 5.** Involvement of endocytosis in SHP2 oxidation. (a) Serum-starved Swiss 3T3 cells were pre-treated with Dynasore (50 μM) (top panels) or Pitstop® 2 (25 μM) (bottom panels) for 30 min, stimulated with PDGF-BB (50 ng ml⁻¹) or H₂O₂ (1 mM) for 10 min in the presence of the inhibitor, and then subjected to dimedone-PLA (gray). Representative images are shown for each condition from one of 3 independent experiments. Graphs show the average number of PLA signals per cell (n = 6 images for each condition, 5-20 cells in an image), relative to unstimulated control cells (set to 1). Error bars represent SD. ***P<0.0001, ns: not significant, ANOVA with Bonferroni/Dunn’s post-hoc test. (b) Lysates from Swiss 3T3 cells expressing EGFP-fused wild-type dynamin 2 (WT Dynamin2) or dominant-negative dynamin 2 (dynamin2^K44A) (see Fig. 3d) were immunoblotted with the indicated antibodies. Arrows indicate exogenous (EGFP-fused) and endogenous dynamin, respectively. Representative immunoblots are shown from one of 2 experiments. (c) Serum-starved Swiss 3T3 cells were pre-treated with Dynasore (50 μM) or Pitstop® 2 (25 μM) for 30 min, and stimulated with PDGF-BB (50 ng ml⁻¹) for the indicated times. Lysates were subjected to immunoblotting with the indicated antibodies. Representative immunoblots from one of 3 experiments are shown. (d) Serum-starved Swiss 3T3 cells expressing EGFP, EGFP-WT dynamin2 or EGFP-K44A dynamin2 were stimulated with PDGF-BB (50 ng ml⁻¹) for 5 min. Cells were stained with an anti-pTyr (gray or magenta) antibody cocktail. EGFP is shown in green; nuclei are stained with DAPI. Representative images are shown for each condition from one of 2 independent experiments. Arrows indicate cells expressing EGFP or EGFP-dynamin2. Scale bars: 50 μm.
Supplementary Fig. 6. PDGF-evoked SHP2 oxidation requires H₂O₂ and PI3K activity but not mitochondrial ROS. (a) Lysates from Swiss 3T3 cells stably expressing EGFP or Flag-tagged cytoplasmic catalase were immunoblotted with the indicated antibodies (see Fig. 4a). Representative immunoblots from one of 2 experiments are shown. (b) Serum-starved Swiss 3T3 cells were pre-treated with MitoQ (1 μM) for 1 h, stimulated with PDGF-BB (50 ng ml⁻¹) or H₂O₂ (1 mM) for 10 min, fixed in the presence of dimedone, and subjected to dimedone-PLA (gray). Representative images are shown for each condition from one of 2 independent experiments. Graphs show the average number of PLA signals per cell (n = 6 images for each condition, 5-15 cells in an image), relative to unstimulated cells. Error bars represent SD.
***P<0.0001, ns: not significant, ANOVA with Bonferroni/Dunn’s post-hoc test. (c) Serum-starved Swiss 3T3 cells were pre-treated with LY294002 (20 μM) or BKM120 (20 μM) for 1 h, stimulated with PDGF-BB (50 ng ml⁻¹) for 10 min. Lysates were subjected to immunoblotting with anti-AKT pT308 and anti-AKT antibodies. Representative immunoblots from one of 2 experiments are shown. (d) Serum-starved Swiss 3T3 cells were pre-treated with LY294002 (20 μM) or BKM120 (20 μM) for 1 h, stimulated with PDGF-BB (50 ng ml⁻¹) or H₂O₂ (1 mM), fixed in the presence of dimedone, and subjected to dimedone-PLA (gray). Representative images are shown for each condition from one of 2 independent experiments. The graph shows the average number of PLA signals per cell (n = 6 images for each condition, 10-25 cells in an image), relative to unstimulated cells. Error bars represent SD. ***P<0.0001, ns: not significant, ANOVA with Bonferroni/Dunn’s post-hoc test. Scale bars: 50 μm.
**Supplementary Fig. 7.** NOX complexes co-localize with oxidized SHP2. (a) Anti-NOX antibodies are specific. Lysates from primary murine dermal fibroblasts (MDFs) from wild type (WT) or Nox1,2,4 triple-KO (TKO) animals were subjected to immunoblotting with the indicated antibodies. Representative immunoblots are shown from one of 2 experiments. (b) WT- or TKO-MDFs were immunostained with the indicated anti-NOX antibodies (gray and green). Nuclei and actin cytoskeleton were stained with DAPI (blue) and phalloidin (magenta), respectively. Representative images are shown for each condition from one of 2 independent experiments. Scale bar: 50 μm. (c) Serum-starved Swiss 3T3 cells were treated with PDGF-BB (50 ng ml⁻¹) for 10 min. The median distances of centers of mass between punctate signals of ox-SHP2 and the nearest indicated marker signal, or median distances of between punctate signals of the indicated markers themselves, were obtained by object-based image analysis. Also see Fig. 3c. Top panel shows comparisons between ox-SHP2-to-marker and marker-to marker distances at 10 min after PDGF stimulation. Note that although all of the inter-marker distances are less than the intra-marker distances, the inter-marker distances between the ox-SHP2/NOX2 and ox-SHP2/NOX3 comparisons are far too large to indicate co-localization (compare distance scale on y-axis). Bottom panel shows data from the same experiment indicating ox-SHP2-to-marker distances before and after PDGF-BB (10 min) stimulation. Box-whisker plots with the 25th-75th percentiles (boxes) and the 5th-95th percentiles (whiskers) of the median distances (n = 50 cells each), ***P<0.0001, unpaired t test or unpaired Welch’s t test, as appropriate. (d) Serum-starved Swiss 3T3 cells were stimulated with PDGF-BB (50 ng ml⁻¹) for 10 min, and subjected to dimedone-PLA labeling of ox-SHP2 (magenta) followed by immunolabeling with either anti-NOX1 or anti-NOX4 antibodies (green). Representative semi-super resolution microscopic images (AiryScan) are shown from one of 2 independent experiments. Higher magnification images of the boxed regions are shown at the bottom. Scale bars: 5 μm. (e) Swiss 3T3 cells were immunostained with anti-NOX1 or anti-NOX4 (green) and anti-EEA1 or anti-calnexin (ER marker) (magenta) antibodies. Nuclei were stained with DAPI (blue). Representative images for each condition from one of 2 independent experiments are shown. Scale bar: 10 μm. (f) Serum-starved Swiss 3T3 fibroblasts were stimulated with EGF (50 ng ml⁻¹) for 5 min, and fixed in the presence of dimedone. Co-staining of dimedone-PLA labeling ox-SHP2 (magenta) and indicated antibodies (green) is shown. Representative images are shown for each condition from one of 2 independent experiments. Nuclei were stained with DAPI (blue). Scale bar: 10 μm.
Supplementary Fig. 8. PDGF-evoked redoxosome formation. (a) Serum-starved Swiss 3T3 cells were stimulated with or without PDGF-BB (50 ng ml\(^{-1}\)) for 5 min, immunostained with anti-RAB5 (magenta) and anti-NOX4 (green) antibodies and visualized by semi-super resolution microscopy (AiryScan). Higher magnification images of the boxed regions are shown at the bottom. Representative images are shown for each condition from one of 2 independent experiments. Scale bar: 10 µm. Graphs show Pearson’s or Manders’ coefficients for co-localization of RAB5 and NOX1 (left) or RAB5 and NOX4 (right) before and after PDGF-BB stimulation (\(n = 20\) ROIs from 10 cells). ***\(P<0.0001\), ns: not significant, Unpaired two-tailed \(t\) test or Welch’s \(t\) test, as appropriate. Error bars represent SD. (b) Serum-starved Swiss 3T3 cells expressing plasma membrane-targeted EGFP (EGFP-tk) or RAB5-fused EGFP (EGFP-RAB5) were subjected to live-cell time-lapse imaging, and fluorescence intensity was
monitored at 1 min intervals. PDGF-BB (50 ng ml⁻¹) was added, as indicated, after the third scan. Graph shows the relative fluorescence intensities of individual cells (n = 7 cells each from individual time-lapse experiments), setting the average of the first 3 time-points to 1. Error bars represent SEM. (c) Serum-starved WT- or TKO-MDFs expressing HyPer3-RAB5 were subjected to live-cell time-lapse imaging, and fluorescence intensity was monitored. Graph shows the relative fluorescence intensities of individual cells (n = 5 cells each from individual time-lapse experiments) before and 5 min after PDGF-BB stimulation, with unstimulated cell values normalized to 1. P value was calculated with two-tailed Welch’s t test. Error bars represent SD.
Supplementary Fig. 9. NOX activity is necessary for PDGF signaling. (a) Serum-starved WT- or TKO-MDFs were stimulated with PDGF-BB (50 ng ml\(^{-1}\)) for the indicated times. Lysates were subjected to immunoblotting with the indicated antibodies. Representative immunoblots are shown from one of 4 experiments. (b) Graph shows the relative amount of PDGFR\(\beta\) (compared to ERK2) at various times following PDGF addition, as obtained from immunoblots, with the level at time 0 normalized to 1. Data are means ±SD (\(n = 4\)). (c) Relative phosphorylation of PDGFR\(\beta\) on the indicated tyrosine residues compared to total PDGFR\(\beta\) at the indicated times, as obtained from immunoblots. For each residue, the value in WT-MDF at 10 min is set as 1. Data are means ±SD (\(n = 4\)). P values were calculated using paired two-tailed t tests.
Supplementary Fig. 10. Uncropped immunoblot images presented in Figures.