

Supplementary Materials for Novel p47^{phox}-Related Organizers Regulate Localized NADPH Oxidase 1 (Nox1) Activity

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Fig. S1. Myc-tagged transfected organizer subunits are expressed in comparable amounts in reconstituted HEK293 cells.

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Fig. S3. The presence of Tks5 single SH3 domain inactivating mutations (M1 to M5), or the TksM1M2 double mutation did not affect the ability of Tks5 to support Nox1-dependent ROS generation and to bind NoxA1.

Fig. S4. Tks4-specific siRNA efficiently knocks down Tks4 protein at 72 and 96 hours and significantly reduces the ability of DLD1 cells to degrade ECM.

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Fig. S6. In DLD1 cells, Nox1 and phalloidin- and Nox1 and cortactin-positive structures are ventral organelles that have the characteristics of invadopodia.

Fig. S7. NoxO1-GFP does not localize to invadopodia in DLD1 cells and invadopodia formation in NoxO1-transfected cells is greatly reduced.

Figure S1

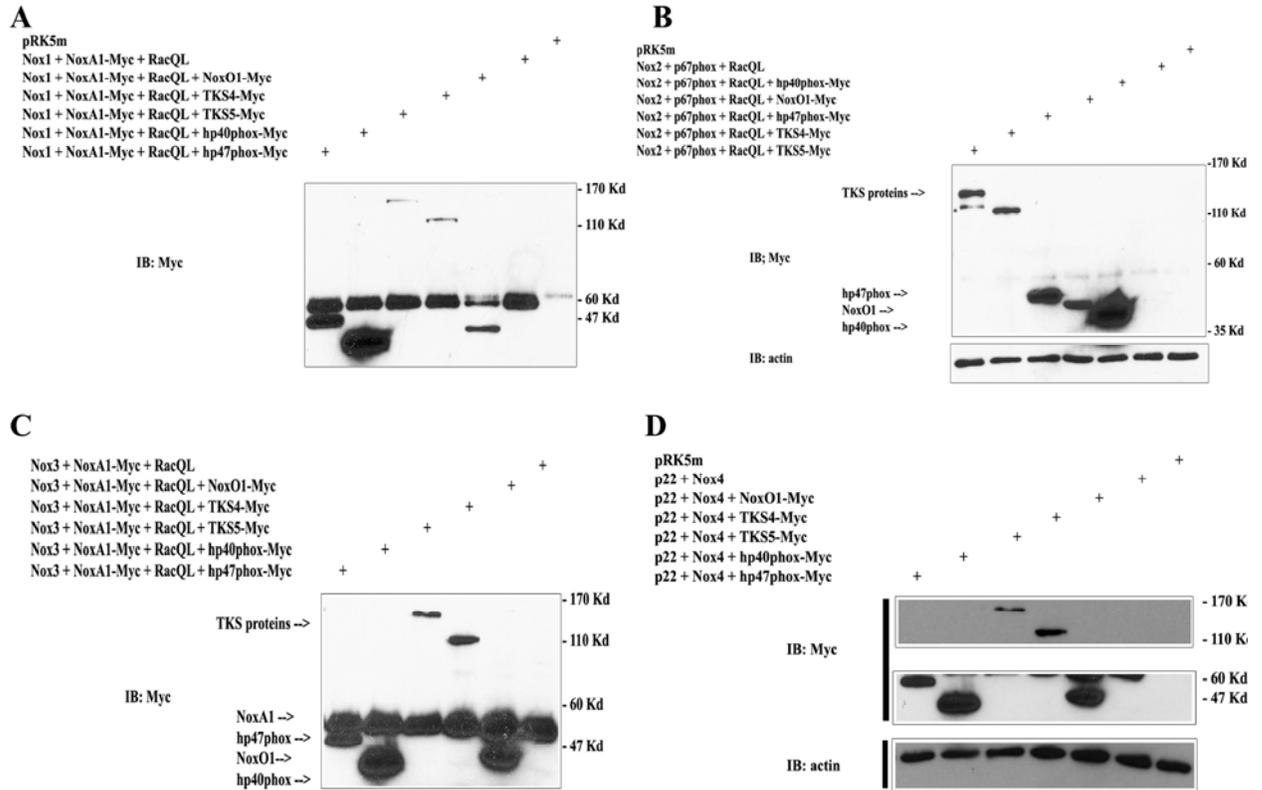


Fig. S1: Myc-tagged transfected organizer subunits are expressed in comparable amounts in reconstituted HEK293 cells. (A), (B), (C) and (D) HEK293 cells were transfected as indicated with expression vectors for different Myc-tagged organizer subunits and with all the known components required for Nox1-, Nox2-, Nox3- and Nox4-dependent ROS generation in (A), (B), (C) and (D) respectively. After 24hrs, the abundance of Myc-tagged proteins was assessed by Western blot using Myc antibody.

Figure S2

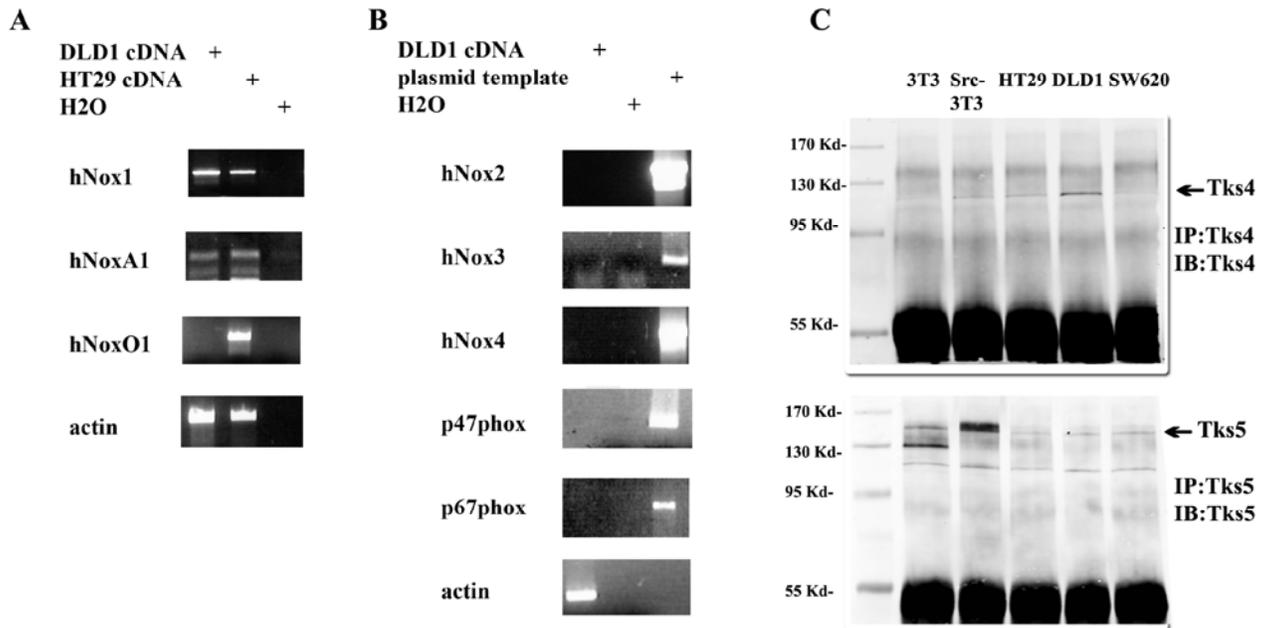


Fig. S2: DLD1 cells only express the Nox1 member of the NADPH oxidase family, the NoxA1 activator, and Tks4 (and small amounts of Tks5) as members of the p47^{phox} organizer superfamily. (A) and (B) RT-PCR was performed to detect the expression of several members of NADPH oxidase family (Nox1-4) and different members of the p47^{phox} organizer superfamily (NoxO1, p47^{phox} and p40^{phox}) along with NoxA1 activator. cDNA from human HT29 colon cancer cells in (A) and expression plasmids in (B) were used as positive controls for primer sets. (C) Several colon cancer cell line protein lysates were screened for Tks4 (upper panel) or Tks5 (lower panel) using Tks4- or Tks5-specific antibody for immunoprecipitation, followed by Western blot. Protein lysates from 3T3 and Src-3T3 were used as negative or positive controls respectively.

Figure S3

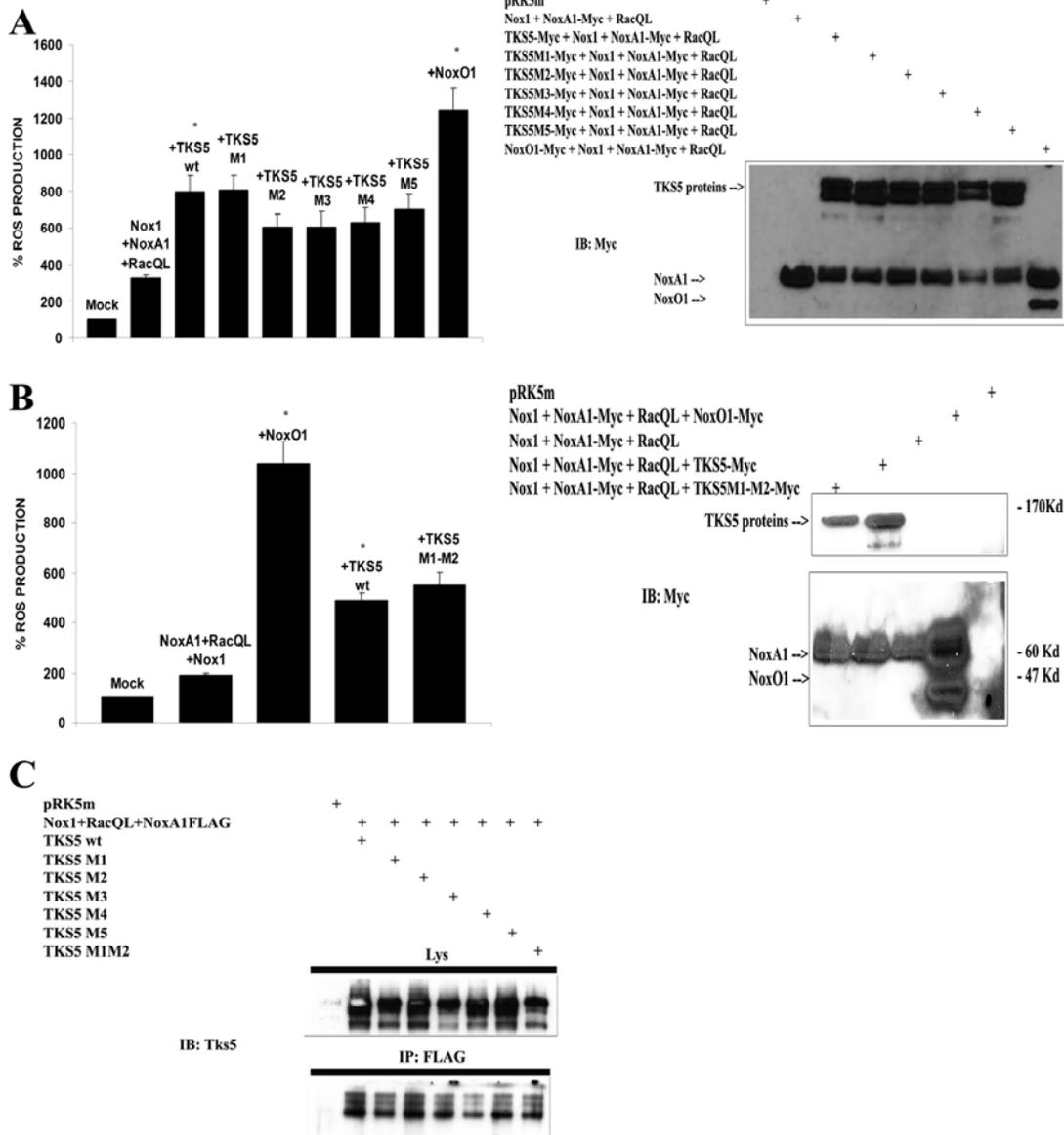


Fig. S3: The presence of Tks5 single SH3 domain inactivating mutations (M1 to M5), or the TksM1M2 double mutation did not affect the ability of Tks5 to support Nox1-dependent ROS generation and to bind NoxA1. (A) HEK293 cells were transfected as indicated with Myc-tagged Tks5 mutants (M1 to M5), Tks5 wild-type or NoxO1, and with other components required for Nox1 activity (Nox1, NoxA1, RacQL). After 24hrs, ROS production was monitored by CL-assay (left panel), while presence of transfected Myc-tagged adaptors was determined by Western blot using Myc antibody

(right panel). One representative experiment of three separate experiments is shown and results of CL-assays are given as mean of triplicates \pm S.D. * $p < 0.001$. (B) HEK293 cells were transfected as indicated with Myc-tagged adaptors (NoxO1, Tks5 wild-type or Tks5M1M2), and with all the components required for Nox1 signaling. After 24hrs, ROS generation was measured by CL-assay (left panel), while expression of transfected Myc-tagged adaptors was confirmed by Western blot using Myc antibody (right panel). One representative experiment of three separate experiments is shown and results of CL-assays are given as mean of triplicates \pm S.D. * $p < 0.001$ compared to condition with no organizers. (C) HEK293 cells were transfected as indicated with empty vector, Nox1, RacQL, Flag-tagged NoxA1, and Myc-tagged Tks5 mutants (M1 to M5 and M1M2). After 24hrs, cells were lysed and immunoprecipitation was carried out using anti-Flag antibody. Interaction of Tks5 with NoxA1 was analyzed using anti-Tks5-specific antibody. One representative experiment is shown of three separate experiments.

Figure S4

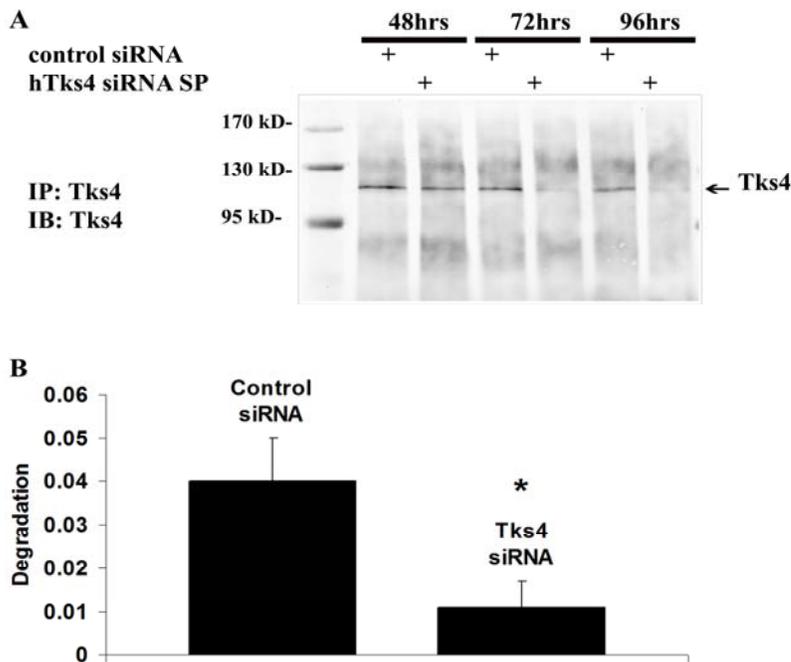


Fig. S4: Tks4-specific siRNA efficiently knocks down Tks4 protein at 72 and 96hrs and significantly reduces the ability of DLD1 cells to degrade ECM. (A) DLD1 cells were transfected with 50nM of Tks4-specific or control siRNA and protein lysates were collected at 48, 72 and 96hrs after transfection. Tks4 knockdown was analyzed by immunoprecipitation followed by Western blot using Tks4 antibody. One representative experiment of three separate experiments is shown. (B) Quantification of experiment illustrated in Figure 4D. Quantification of gelatin degradation activity was performed on at least 10 randomly chosen fields, representing a minimum of 300 total cells scored per experimental point. Quantification of the degradation area per field was performed using ImageJ software, and the percent of degraded area per field normalized to the number of cells on this field. Error bars represent SEM. * $p < 0.02$.

Figure S5

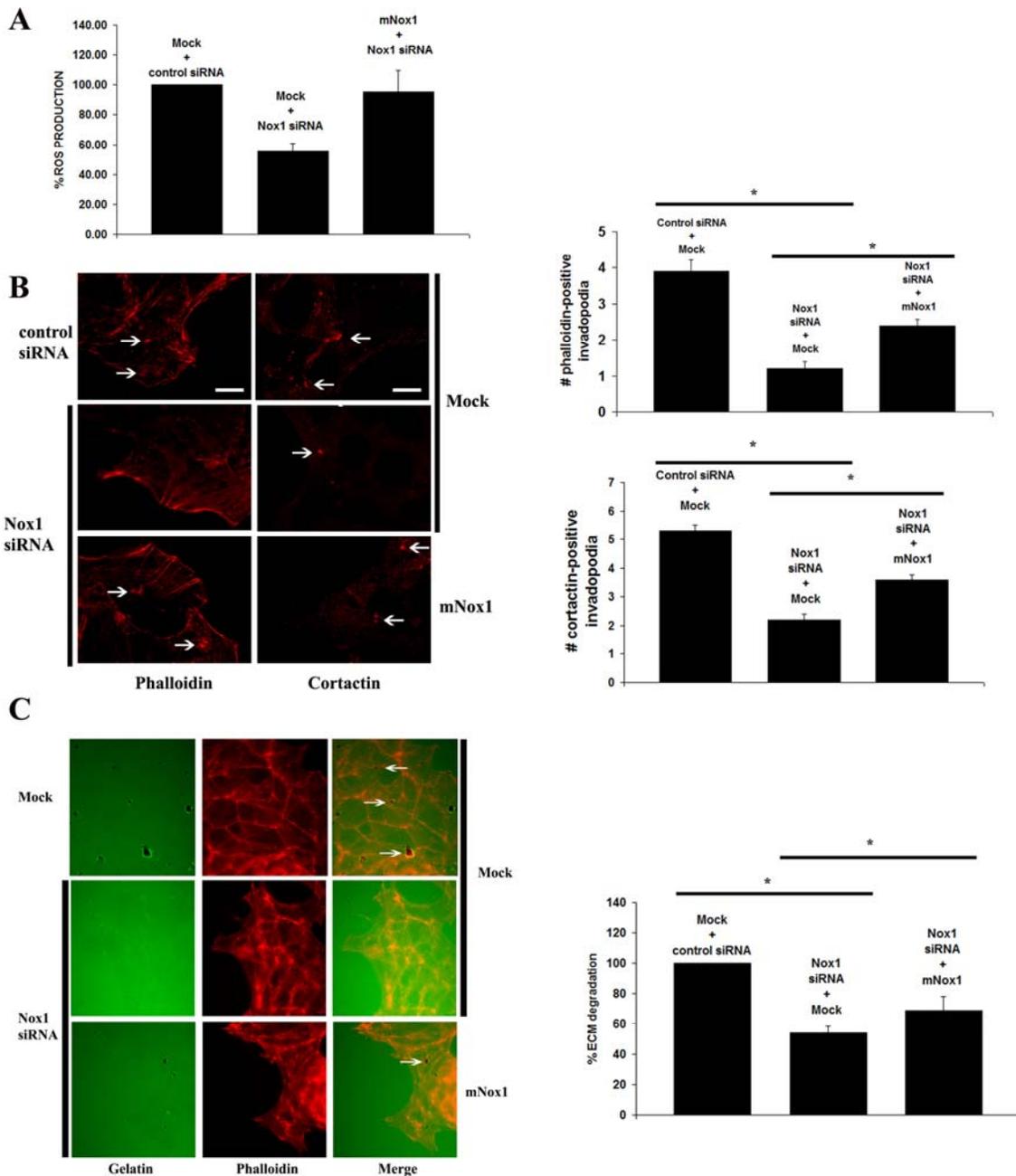
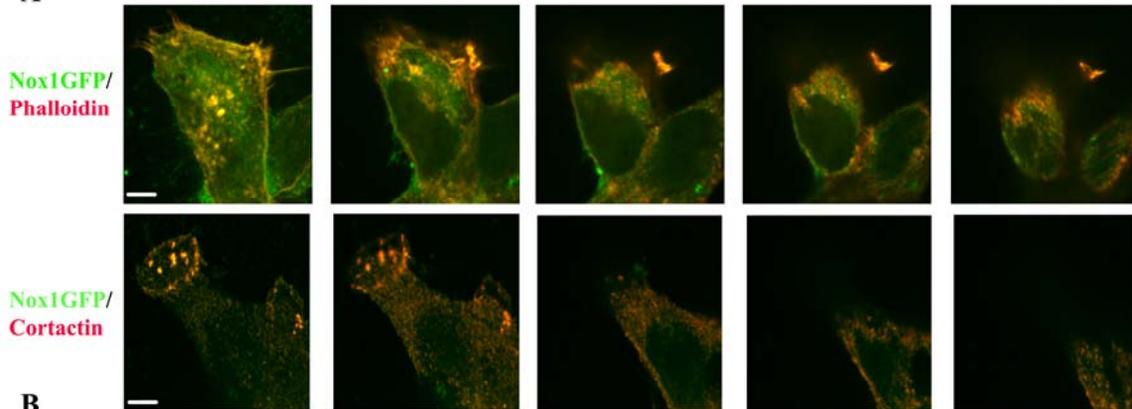


Fig. S5: Nox1-specific siRNAs efficiently block ROS generation, invadopodia formation, and ECM degradation in DLD1 cells and this effect can be rescued by overexpression of murine Nox1 (mNox1). (A) DLD1 cells were transfected with 50nM of Nox1-specific or control siRNAs and with empty vector or mNox1. After 48hrs, ROS generation was measured by CL-assay. One representative experiment from two separate

experiments is shown and results of CL-assays are given as mean of triplicates \pm S.D. * $p < 0.001$. (B) DLD1 cells were plated on glass coverslips and after 24hrs cells were transfected as indicated with control or Nox1-specific siRNAs, and with empty vector or mNox1 expression vector. After 48hrs the cells were fixed in PFA4% and stained with Alexa-Fluor-568 phalloidin (first column) or cortactin antibody, followed by Alexa-Fluor 568-conjugated secondary antibody (second column) and visualized by confocal microscopy (100X) (left panels). White arrows indicate phalloidin- or cortactin-positive structures. Scale bars, 5 μ m. One representative picture from two separate experiments is shown. Quantification from two independent experiments is shown in the right panels: the number of phalloidin positive-structures (upper panel) or cortactin positive-structures (lower panel) was counted and averaged from 15 images for each experiment. Error bars represent SEM. * $p < 0.02$. (C) DLD1 cells were transfected as indicated with Nox1-specific or control siRNA, and with mNox1 or empty vector. 24hrs later, cells were trypsinized and plated on FITC-labeled gelatin-coated coverslips, and after 48hrs the cells were fixed in 4%PFA, stained with Alexa-Fluor-568 phalloidin (in red) and visualized by epifluorescence microscopy (40X). In the merge, the white arrows indicate areas in which cells (in red) degrade the ECM (in green). Scale bars, 45 μ m. One representative image from two separate experiments is shown. Quantification from two independent biological experiments is shown in the right panel: For each experiment, the total degradation area was obtained as sum of degradation areas calculated using Metamorph software from 15 images and reported as percentage (mock set as 100%). In the graph, error bars represent SEM. * $p < 0.05$ (Mann-Whitney U test)

Figure S6

A



B

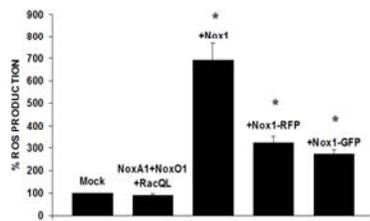


Fig. S6: In DLD1 cells, Nox1 and phalloidin- and Nox1 and cortactin-positive structures are ventral organelles that have the characteristics of invadopodia. (A) Z-stacks with 0.2 μm steps of the merged images shown in Fig. 5A and B. Scale bars, 5 μm . (B) RFP-tagged and GFP-tagged Nox1 support ROS production, albeit to a lesser extent than untagged Nox1. HEK293 cells were transfected with NoxA1, NoxO1, and RacQL and with expression vectors for Nox1, Nox1-RFP, Nox1-GFP, or empty vector. After 24hrs, ROS generation was measured by CL-assay. One representative experiment from three separate experiments is shown and results of CL-assays are given as mean of triplicates \pm S.D. * $p < 0.001$ compared to condition with no organizers.

Figure S7

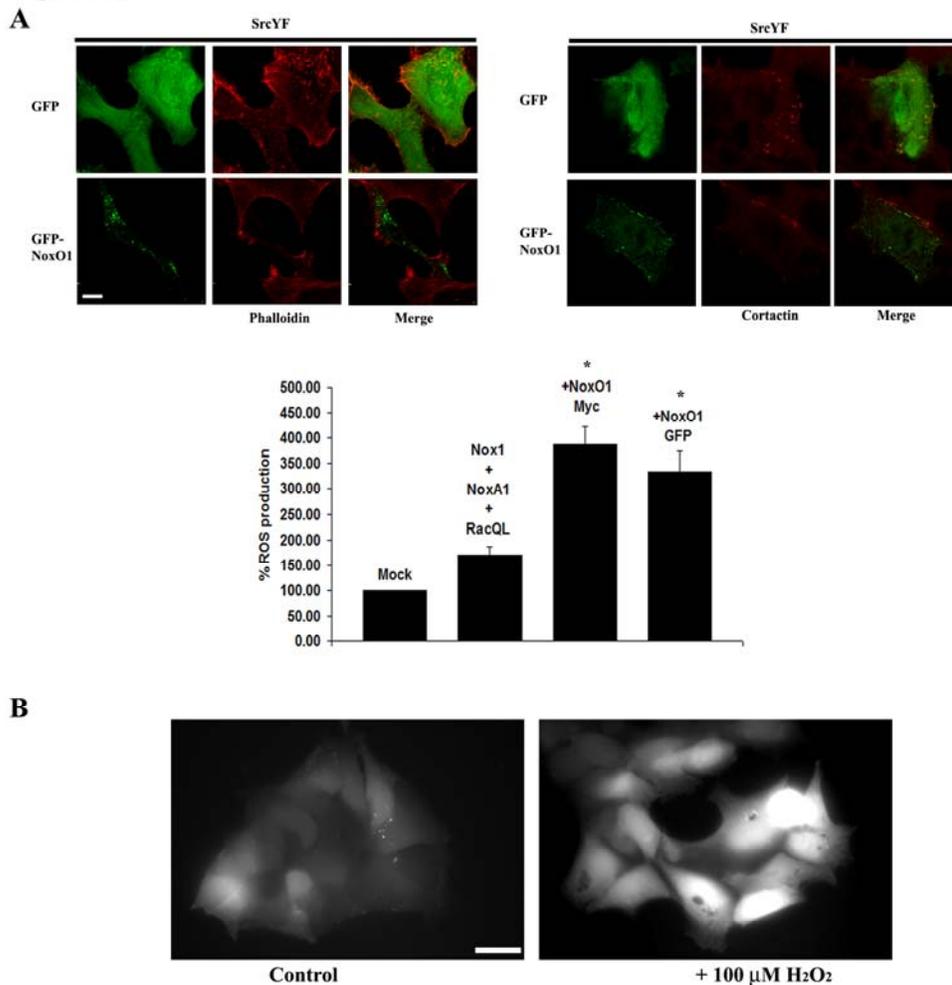


Fig. S7: NoxO1-GFP does not localize to invadopodia in DLD1 cells and invadopodia formation in NoxO1-transfected cells is greatly reduced. (A) DLD1 cells were plated on glass coverslips and after 24hrs cells were transfected with active SrcYF and GFP-tagged NoxO1 or GFP empty vector. After 48hrs, cells were fixed in PFA4% and stained with Alexa-Fluor-568 phalloidin (left panel, in red) or cortactin antibody, followed by Alexa-Fluor 568-conjugated secondary antibody (middle panel, in red) and visualized by confocal microscopy (100X). The merge in both panels indicates that NoxO1-GFP does not localize to invadopodia in these cells and that invadopodia formation in NoxO1-transfected cells is greatly reduced. Scale bars, 5 μ m. One representative picture from three separate experiments is shown. In the lower panel, it is shown that NoxO1-GFP construct used in previous experiments is able to support Nox1-dependent ROS generation. HEK293 cells were transfected as indicated with all the components required for Nox1-dependent ROS generation and with Myc-tagged or GFP-

tagged NoxO1. After 24hrs, ROS production was monitored by CL-assay. One representative experiment of three is shown and results of CL-assays are given as mean of triplicates \pm S.D. * $p < 0.001$ compared to condition with no organizers. (B) ROS-sensitive probe PY1-AM specifically stains DLD1 cells incubated with 100 μ M H₂O₂. DLD1 cells were plated on glass coverslips and, after 24hrs, treated with 5 μ M of PY1-AM in HBSS for 30 minutes at 37C. Cells were washed with PBS, incubated with water (control) or 100 μ M H₂O₂ for 20 min at 37C, and then visualized by epifluorescence microscopy (40X). The fluorescence intensity was greatly increased in the presence of H₂O₂. Scale bars, 45 μ m. One representative picture from two separate experiments is shown.