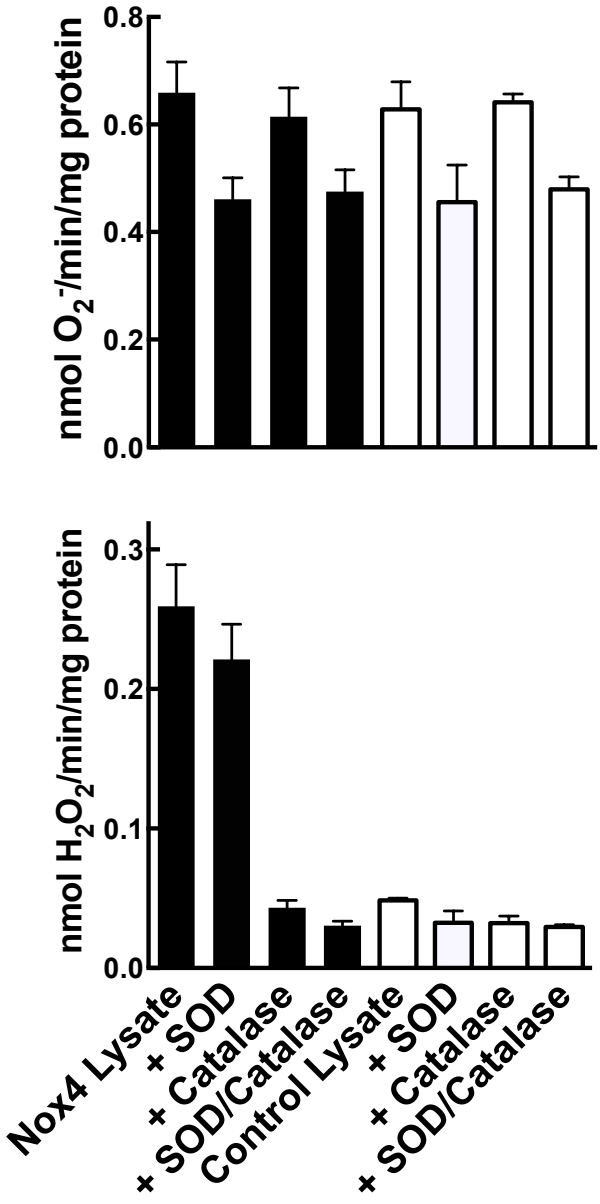
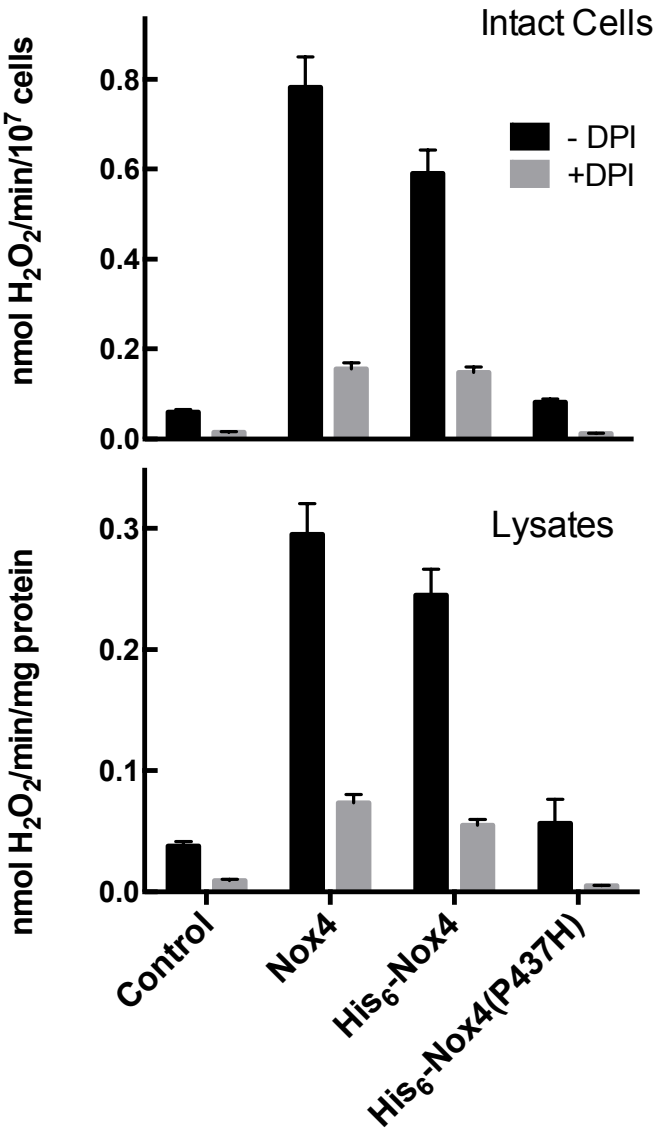
# Supplemental Fig. 1




**Supplemental Figure 1.** Determination of Superoxide and Hydrogen Peroxide levels in lysates from control and Nox4-expressing HEK294 cells. Lysates (0.42 mg) from HEK293 cells stably expressing Nox4 (filled bars) or control HEK293

cells (open bars) were supplemented with FAD, $NADP^+$ and glucose 6-phosphate, as in Materials and Methods. Upper panel. The rate of cytochrome *c* reduction was monitored. Vehicle alone, SOD (SOD, 300 U/ml), catalase (125 U/ml), or SOD plus catalase were included in the assay as indicated. Lower panel. Hydrogen peroxide production was measured by Amplex Red oxidation, measuring its absorption increase at 572 nm, also without or with added SOD, catalase or both as in the upper panel. The values shown are the mean ± S.E.M. of three determinations made using one preparation, and is representative of three experiments carried out using separate preparations.

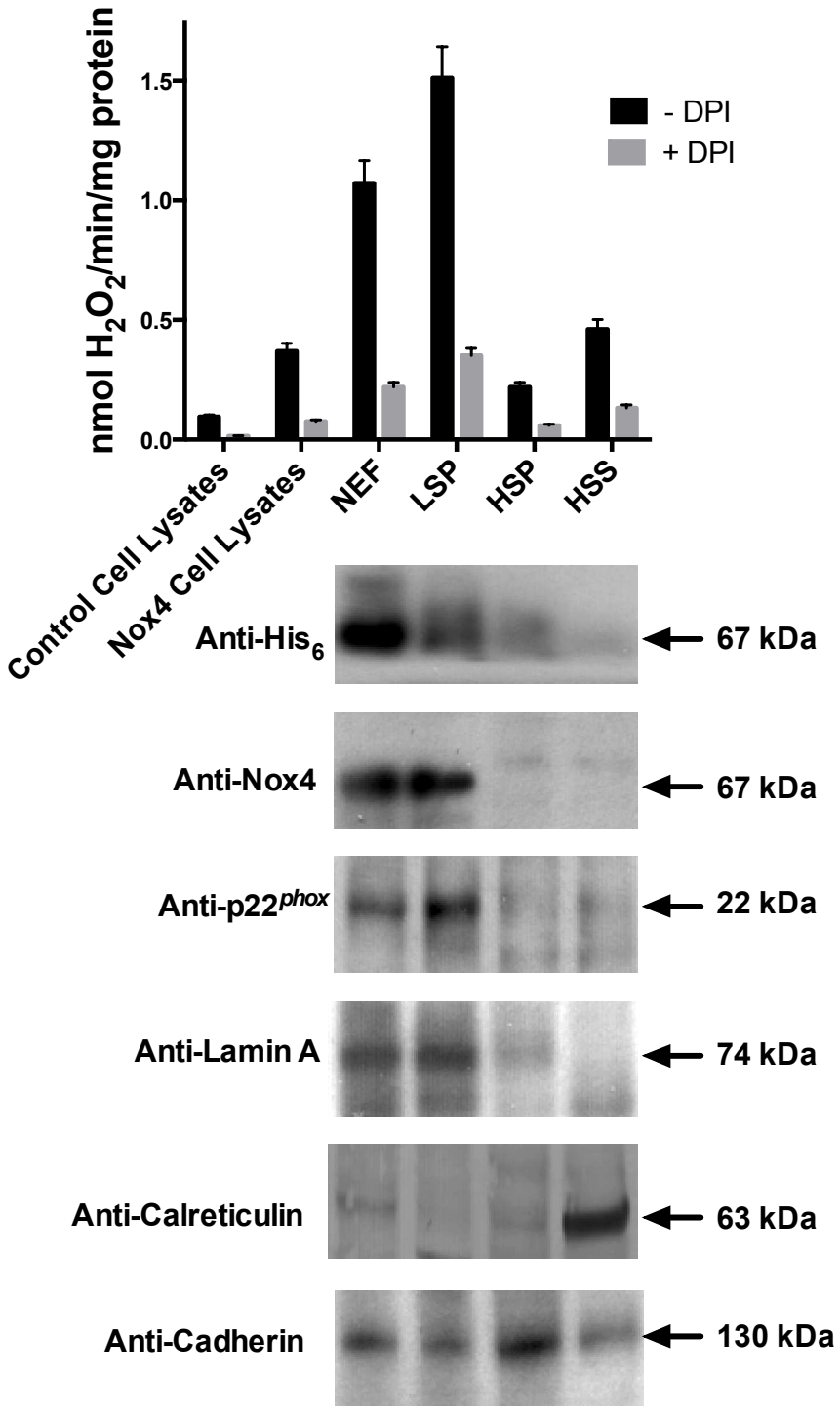
## Supplemental Fig. 2




**Supplemental Figure 2.** Hydrogen peroxide generation by cells transiently expressing Nox4 or $His_6$-Nox4. Amplex Red oxidation was measured as in supplemental Figure 1 in intact transiently transfected cells (upper panel) and their cell lysates (lower panel) expressing native Nox4, $His_6$-Nox4, or the inactive

$His_6$-Nox4(P437H), as described in Materials and Methods. The experiment was carried out in the absence (filled bars) or presence (gray bars) of 20 μM diphenylene iodonium. Values represents the mean ± S.E.M. of three determinations from one set of transfections, and is representative of 3 experiments repeated using three different sets of transfected cells.

# Supplemental Figure 3



**Supplemental Figure 3.** Subcellular fractionation of $His_6$-Nox4 in transfected HEK293 cells. Transfected cells were disrupted by homogenization and fractionated as described in Materials and Methods into a nucleus-enriched fraction (NEF), a low-speed pellet (LSP), a high-speed pellet (HSP) and a high-speed supernatant (HSS). In the lower panel, 20 μg of each fraction was electrophoresed on SDS-PAGE and immunoblotted with antibodies to the $His_6$, Nox4, $p22^{phox}$, Lamin A, calreticulin and cadherin as indicated. In the upper panel, cell lysates and the above fractions were evaluated for NADPH-dependent Amplex Red oxidation using fluorescence change as in Materials and Methods, in the absence (black bars) and presence (grey bars) of 20 μM DPI. Control lysate from non-transfected HEK293 cells showed low activity. Activity values represent the mean ± S.E.M. of three determinations using one set of transfected cells, and is representative of three transfection experiments. Western blotting is representative of two experiments using different transfections.

# Supplemental Figure 4

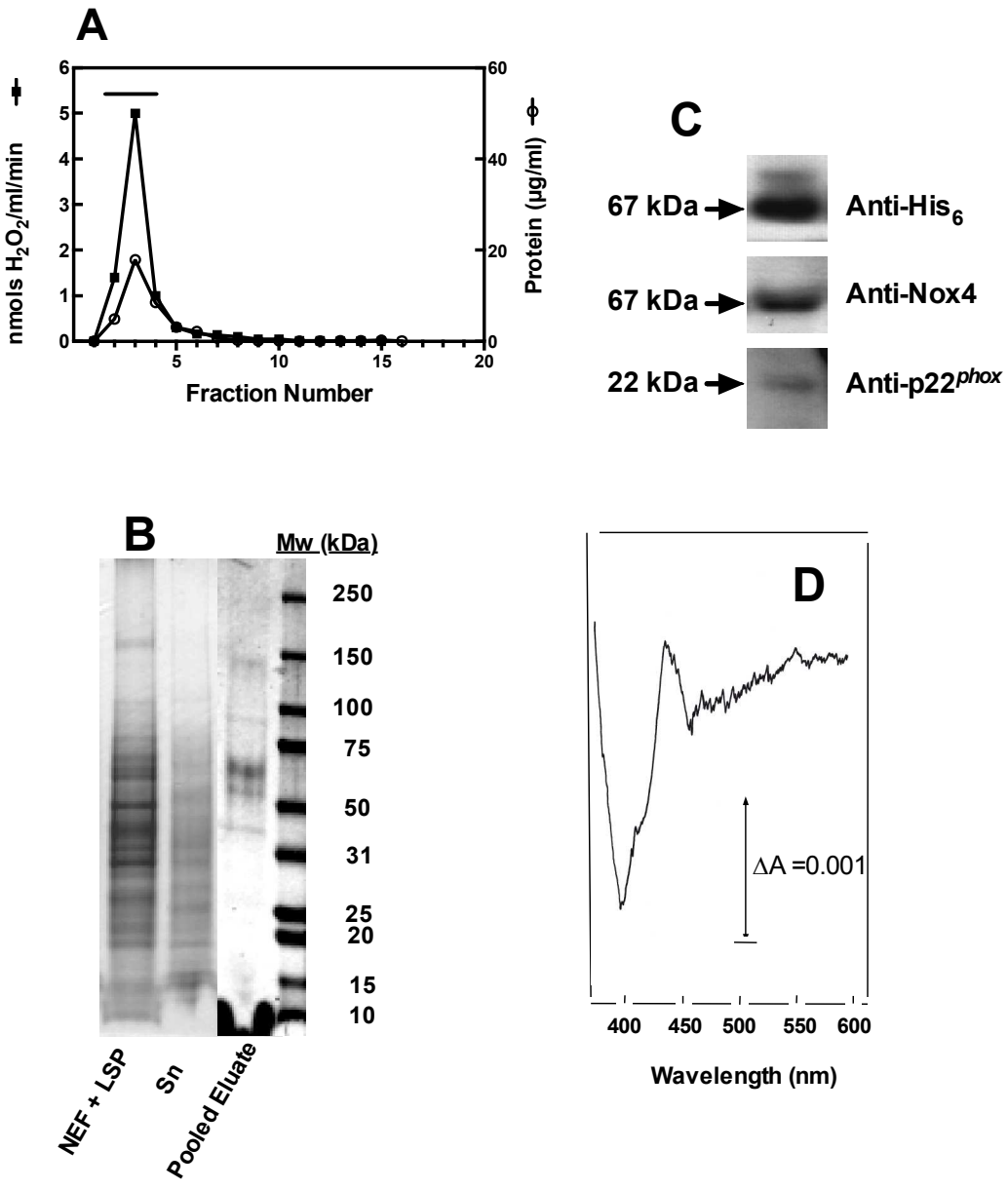



**Figure 4 Supplemental.** Partial purification of $His_6$-Nox4. Panel A. After cell lysis, NEF and LSP fractions were pooled and solubilized as described in "Materials and Methods". After centrifugation (105,000 x g x 60 min, 4°C), the supernatant (Sn) was applied to a $Ni^{2+}$-beads column, washed and eluted with an

imidazole step gradient as described in Materials and Methods, with aliquots of each fraction assayed for NADPH-dependent Amplex Red oxidation measured by fluorescence (filled squares) or for protein (open circles). The three eluted fractions showing high activity (horizontal bar) were pooled, concentrated and used for further characterization. Panel B. The combined NEF + LSP fraction, Sn fraction, and pooled column eluate were chromatographed on 4-15% SDS-PAGE, and protein was stained with colloidal blue. The most prominent band corresponds to ~67 kDa, the theoretical molecular weight of $His_6$-Nox4. Panel C. The pooled eluate was transferred to an Imobilon PVDF membrane after SDS-PAGE (4-15%) and Western blotting was performed using antibodies to $His_6$, Nox4 and $p22^{phox}$ as indicated. Shown also are molecular weights of staining bands, estimated based on molecular weight standards seen in the right lane of panel B. Panel D. The eluate was dialyzed, the oxidized-minus-reduced difference spectrum was obtained, and the heme content of the partially purified material was calculated all as described in Materials and Methods. The experiment shown is representative of five.