

INTERACTION BETWEEN P22^{PHOX} AND NOX4 IN THE ENDOPLASMIC RETICULUM SUGGESTS A UNIQUE MECHANISM OF NADPH OXIDASE COMPLEX FORMATION

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1 **ABSTRACT**

2 The p22^{phox} protein is an essential component of the phagocytic- and inner ear NADPH oxidases but its
3 relationship to other Nox proteins is less clear. We have studied the role of p22^{phox} in the TGF-β1-
4 stimulated H₂O₂ production of primary human and murine fibroblasts. TGF-β1 induced H₂O₂ release of
5 the examined cells, and the response was dependent on the expression of both Nox4 and p22^{phox}.
6 Interestingly, the p22^{phox} protein was present in the absence of any detectable Nox/Duox expression, and
7 the p22^{phox} level was unaffected by TGF-β1. On the other hand, Nox4 expression was dependent on the
8 presence of p22^{phox}, establishing an asymmetrical relationship between the two proteins. Nox4 and p22^{phox}
9 proteins localized to the endoplasmic reticulum and their distribution was unaffected by TGF-β1. We used
10 a chemically induced protein dimerization method to study the orientation of p22^{phox} and Nox4 in the
11 endoplasmic reticulum membrane. This technique is based on the rapamycin-mediated heterodimerization
12 of the mammalian FRB domain with the FK506 binding protein. The results of these experiments suggest
13 that the enzyme complex produces H₂O₂ into the lumen of the endoplasmic reticulum, indicating that
14 Nox4 contributes to the development of the oxidative milieu within this organelle.

15

16 **KEYWORDS** : Reactive oxygen species / NADPH oxidase / Nox4 / Hydrogen peroxide / p22^{phox}

17

18 **ABBREVIATIONS:**

19

20 Nox4: NADPH oxidase 4

21 Nox2: NADPH oxidase 2

22 Duox: dual oxidase

23 TGF-β1: transforming growth factor β1

24 ROS: reactive oxygen species

25 HPF: human pulmonary fibroblast

26 TTF: tail-tip fibroblast

27 FKBP: FK506 binding protein

28 ERO1: ER oxidoreductin 1

1 INTRODUCTION

2
3 Regulated production of reactive oxygen species (ROS) is now considered an essential component
4 of maintaining homeostasis of live organisms [22,29]. Among ROS, hydrogen peroxide (H_2O_2) has
5 emerged as a particularly important molecule with pleiotropic functions that include roles in host defense,
6 thyroid hormone production, synthesis of the extracellular matrix and signal transduction[22]. H_2O_2 is
7 produced at different intracellular sites as a byproduct of various biochemical pathways, but regulated
8 production of H_2O_2 is mediated by members of the Nox/Duox family of NADPH oxidases[16,22]. Nox2,
9 the prototypic enzyme of this family, was originally identified in phagocytes, whereas non-phagocytic
10 Nox/Duox isoforms are expressed and function in a wide variety of cells and tissues.

11 Currently, the most intensively studied, dedicated ROS source is Nox4, which was originally
12 identified in kidney epithelial cells[15,33]. Subsequent studies revealed that the expression of Nox4 is not
13 restricted to the kidney, but other cells including endothelial and alveolar epithelial cells, osteoclasts,
14 cardiomyocytes and activated fibroblasts also contain the enzyme[3]. The physiological function of Nox4
15 remains unclear. Recent studies on knockout animals suggest that ROS production by Nox4 is often
16 protective in different disease models[31,42], although pathogenic roles were also described for the
17 enzyme[18,20].

18 We know little about the regulation of Nox4. Several data suggest that the activity of Nox4 is
19 regulated at the transcriptional level[7], but acute, hitherto unknown regulatory mechanisms might also
20 exist. Nox/Duox proteins often interact with other partners to form fully active enzyme complexes[7].
21 Genetic evidence proves that p22^{phox}, which was originally identified as a component of the phagocytic
22 oxidase, also supports the biological activity of Nox3[25]. Furthermore, in heterologous expression
23 systems, the activity of both Nox4 and Nox1 was stimulated by the co-expression of p22^{phox}[1] [19,24]. In
24 the majority of the Nox4-related studies, the activity and localization of the enzyme were analyzed in
25 heterologous expression systems. However, it remained unknown, whether the activity of Nox4 is also
26 dependent on p22^{phox} in cells where these proteins are naturally co-expressed. Using genetically-modified
27 cell models we demonstrate that endogenously expressed p22^{phox} is an essential component of the Nox4
28 enzyme complex, and we demonstrate an asymmetrical relationship between the two proteins: the level of
29 p22^{phox} is not influenced by Nox4 expression; however the stability of Nox4 is dependent on the presence
30 of p22^{phox}. We also show that the Nox4-p22^{phox} complex localizes to the endoplasmic reticulum, and their
31 membrane topology is compatible with ROS being released into the lumen of the endoplasmic reticulum.

32

1 **EXPERIMENTAL PROCEDURES**

3 **Materials**

4 The Alexa488 and 568-labeled goat monoclonal anti-mouse and polyclonal anti-rabbit Fab-
5 antibodies were obtained from Life Technologies. The monoclonal anti-V5 antibody was purchased from
6 AbD Serotec. The 16G7, IgG1-type monoclonal anti-p22^{phox} antibody was previously characterized by the
7 authors[8]. The monoclonal anti-AU1 was obtained from Covance (AFC-130P), while the rabbit
8 polyclonal was ordered from Abcam (ab3401). The monoclonal β -actin and all other chemicals were
9 purchased from Sigma-Aldrich unless otherwise stated. Rapamycin was a kind gift of Péter Várnai. The
10 anti-Nox4 antibody was obtained from Novus Biologicals.

11 **Cell culture**

12 HeLa wt (ATCC: CCL-2™), dermal fibroblast (BJ, ATCC: CRL-2522™) and HEK293 FS cells
13 were grown in DMEM (Lonza) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin and
14 50 μ g/mL streptomycin (Sigma-Aldrich). Human pulmonary fibroblasts (HPF, PromoCell, C-12360) were
15 grown in fibroblast medium (PromoCell) supplemented with 2% FBS, 5 ng/mL Basic Fibroblast Growth
16 Factor and 5 μ g/mL insulin. Nox4-overexpressing HEK 293 FS cells were described in our previous
17 report[12]. The fibroblasts of p22^{phox}-mutant (*nmf333*)[25] and Nox4 knockout (3FAFyh) mice were
18 generated from tail tips of 8-weeks-old animals. After 30 min collagenase digestion of the tail tips, cells
19 were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin for several days. Before
20 TGF- β 1 treatment, we serum.deprived the cells in the presence of 0.05% serum. Cells were treated with
21 TGF- β 1 (R&D Systems, Minneapolis, MN) for 24 h in the absence of serum.

22 **Animals**

23 Nox4-deficient mice (strain 3FAFyh), obtained from the Biology Division of the Oak Ridge
24 National Laboratory (Oak Ridge, TN), were identified within a group of homozygous-viable, radiation-
25 induced albino mice that lack the *Tyrosinase* (*Tyr*) color coat gene[30]. *Nox4* and *Tyr* are adjacent gene
26 sequences located on the q-arm (c-locus) of chromosome 7. Southern blots probed with the full-length
27 Nox4 cDNA confirmed the absence of the entire *Nox4* genomic sequence. PCR-based analysis was used
28 to determine the boundaries of the mutated *Nox4-Tyr* locus, which revealed a deletion ~2100 kilobases in
29 length. Most of the deleted segment encompassed a region upstream of Nox4 that includes a cluster of
30 olfactory-vomer nasal receptor genes and pseudogenes (*Vmn2r-70* through *Vmn2r-79*) and folate
31 hydrolase (*Folh1*), followed by *Nox4* and *Tyr*. Intact *Gmr5* and *Ctsc* genomic sequences were detected
32 immediately distal to this deleted sequence.

33 p22^{phox}-mutant (*nmf333*) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).
34 Animals were maintained on a standard diet and given water ad libitum.

1

2 **DNA constructs and transfection**

3 The p22^{phox}-containing plasmids were generated by PCR based amplification of p22^{phox} ORF from
4 human renal cDNA (Applied Biosystems/Ambion, Austin, TX, USA) with a forward primer pair
5 containing a 5' NheI and 3' BamHI sites followed by a V5-epitope-encoding sequence to frame into
6 pcDNA3.1 vector. The p22^{phox}-AU1 epitope-tagged version was created with site-directed mutagenesis
7 (QuikChange II Site-Directed Mutagenesis Kit, Agilent) by using primers containing the AU1-coding
8 sequence. The V5-tagged Nox4 pcDNA3.1 plasmid was available in our lab, and it was used for further
9 modification with different tags. N-terminal AU1 tag was introduced by site-directed mutagenesis. CFP-
10 FRB-HA-tag was amplified with NheI-BglII restriction sites by PCR from a pEGFP-based vector of P.
11 Várnai [37] and ligated to the N-terminal site of Nox4. The p22^{phox}-FRB-HA-CFP was generated by PCR
12 amplification from the previous vector, with SalI-MfeI restriction sites to ligate into the C-terminal end of
13 p22^{phox}. The FRB-YFP plasmid was received from P. Várnai's lab[37].

14 Vectors encoding HyPer (cytosolic) described by Belousov et al.[4] were purchased from Evrogen
15 (Moscow, Russia). The targeted versions of HyPer were formerly cloned and described by Enyedi *et*
16 *al.*[13]. All constructs have been verified by DNA sequencing.

17 Plasmids were transfected by using Lipofectamine LTX (Life Technologies) or with Neon
18 Transfection System (Life Technologies) in the stage of 60-70% confluency. For gene silencing we
19 applied standard siRNA treatment: we transfected 60-80% confluent, adherent cells with 25 pM specific
20 or scrambled siRNA with Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's
21 instructions. After transfection, the cells were incubated for further 2-3 days.

22 **Immunocytochemistry**

23 Cells were plated on coverslips were fixed in 4% paraformaldehyde in PBS, and then washed and
24 quenched for 10 min in 100 mM glycine in PBS. After four times washing in PBS, cells were
25 permeabilized in 1% BSA and 0.1% Triton X-100 in PBS for 20 min. We blocked the cells for 1 hour in
26 3% BSA in PBS. Cells were subsequently incubated with primary antibody in 3% BSA-containing PBS
27 for 1 or 2 hours, then washed in PBS several times. The secondary antibody was used for 1 hour in 3%
28 BSA in PBS then washed in PBS again. After the final washing steps the cells were mounted with Mowiol
29 4–88 antifade reagent [Tris (pH 8.5) and glycerol-based polyvinyl alcohol 4–88].

30

31 **Western blot analysis**

32 In Western Blot experiments, cells were washed once with cold PBS, then lysed in RIPA buffer
33 (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Na-deoxycholate, 1% Triton-X) enriched with proteinase

1 inhibitor cocktail (Roche Life Science) then were spun at 13400 rpm for 10 min. After that, the
2 supernatant was combined with 4×Laemmli sample buffer [0.005% Bromophenol blue, 4% SDS, 20%
3 glycerol, 0.1 M Tris (pH 6.8)] then loaded without boiling on 12% SDS–polyacrylamide gels. After
4 electrophoresis, the gels were blotted onto nitrocellulose membranes, blocked in 5% milk powder
5 containing PBS for 1 hour or overnight. The primary antibody was incubated for 1 hour in 3% BSA-
6 containing PBS at room temperature. The binding was visualized by peroxidase-coupled goat anti-mouse
7 or anti-rabbit IgG (GE Healthcare), using the enhanced chemiluminescence method (Millipore).

9 **Fluorescent intensity measurements and confocal microscopy**

10 Immunostained cell images were collected on a Zeiss LSM710 confocal laser scanning
11 microscope equipped with a 63x1.4 oil immersion numerical aperture plan Aplan objective (Zeiss).
12 Images were acquired from optical slices of 1–2 μm thickness. Alexa488 immunofluorescence detection
13 involved excitation with a 488 nm argon laser, while in case of Alexa568 the 543 nm helium/neon laser
14 was applied. Emissions were collected using a 500-530 nm band-pass filter and Alexa488 and a 560 nm
15 long-pass filter for Alexa568. Image analysis was performed using Zen software (Zeiss). The ratiometric
16 measurements of HyPer were performed on an inverted microscope (Axio Observer, Zeiss) equipped with
17 40x1.4 oil-immersion objective (Fluar, Zeiss) and a Cascade II. camera (Photometrics, Tucson, AZ).
18 Excitation wavelengths were set by a random-access monochromator connected to a xenon arc lamp
19 (DeltaRAM, Photon Technology International, Birmingham, NJ). The excitation wavelengths of HyPer
20 are 490 and 420 nm combined with a 505 nm dichroic filter and a 525/36 nm emission filter set. Data
21 acquisition was handled by Metafluor software (Molecular Devices, Downingtown, PA). Ratios were
22 calculated upon background fluorescence subtraction. We applied FKBP12-FRB system as a chemically
23 inducible translocation assay with rapamycin as an inducer. Within a cell, after administration of 300nM
24 rapamycin, the molecule first binds to FKBP12 (12-kDa FK506-binding protein) and only then the
25 FKBP12–rapamycin complex binds to FBR, which is the FKBP and rapamycin binding domain of TOR
26 kinase[28,37]. BJ fibroblasts were plated in six-well dishes at 60% confluence and cotransfected the CFP-
27 FKBP12-Nox4 or p22phox-FKBP12-CFP with FRB-YFP together with electroporation. Kinetic
28 measurements were performed at cell chamber (AttoFluor, Life Technologies) containing coverslips at
29 room temperature in 1 ml HEPES-buffered medium (H-medium), where stimuli were added in 0.1 ml of
30 H-medium. Images were acquired every 5-10 seconds for a period of 15 min.

32 **In situ hybridization**

33 Animals were sacrificed by decapitation, the kidneys were removed and frozen on dry ice. 12 μm -
34 thick sections were cut in a cryostat (Leica Microsystems GmbH, Wetzlar, Germany) and mounted onto

1 positively charged Superfrost Plus slides (Life Technologies Magyarország Kft, Budapest, Hungary). To
2 create templates for probe synthesis, the full coding sequence of the mouse p22^{phox} cDNA and a DNA
3 fragment composed of the last 300 bps of the murine Nox4 coding sequence were subcloned into PCR 4.0
4 TOPO vector (Life Technologies). Hybridizations were performed with 10⁶ cpm/slide of the [35S]UTP-
5 labeled (Per-Form Hungaria Kft, Budapest, Hungary) antisense and sense riboprobes, prepared according
6 to the MAXIscriptT7/T3 Transcription Kit (Life Technologies), overnight at 55°C. Next day the slides
7 were washed, dehydrated and dipped into NTB nuclear track emulsion (Carestream Health Deutschland
8 GmbH, Stuttgart, Germany) for 3 days. Emulsion-coated slides were developed using Kodak Dektol
9 developer and Fixer (Sigma-Aldrich Kft, Budapest, Hungary). Sections were counter-stained with 0.5%
10 Giemsa solution (Sigma), air dried and coverslipped using Cytoseal 60 mounting medium (Stephens
11 Scientific, Riverdale, NJ, USA).

12
13

14 **Amplex Red assay for extracellular H₂O₂ level quantification**

15 The extracellular H₂O₂ levels were measured with Amplex Red method (Life Technologies).
16 Adherent confluent cells were incubated in the presence of 50µM Amplex red and 0.1 U/ml horseradish
17 peroxidase in H-medium. After 40 min incubation at 37°C, resorufin fluorescence was measured at 590
18 nm.

19 **Statistics**

20 Data are presented as mean ± S.E.M unless otherwise stated. Statistical analyses were performed
21 using Sigmaplot 13.0 software for Windows (Systat Software Inc.). For estimating the significance of
22 differences Student's t-test or Mann-Whitney-U test was used.

23

24 **RESULTS**

25

26 **TGF-β1-induced H₂O₂ production of primary fibroblasts is dependent on Nox4- and p22^{phox}** 27 **expression**

28

29 Since TGF-β1 was described to stimulate Nox4-dependent H₂O₂ production [23] and this response seems
30 to have multiple roles in TGF-β1 signaling [9] [6,10], we decided to study the role of p22^{phox} in primary
31 human and mouse fibroblasts stimulated by TGF-β1. As shown in Fig. 1, treatment of human pulmonary
32 (HPF) or foreskin-derived (BJ) fibroblasts with TGF-β1 for 24 h lead to increased H₂O₂ production.
33 Experiments using Nox4-specific siRNAs confirmed that Nox4 was responsible for the increased ROS
34 output (Figs. 1A and 1B).

1 The participation of Nox4 in TGF- β 1-stimulated H₂O₂ release has not yet been confirmed by
2 experiment on gene-deficient cells, thus we prepared tail-tip fibroblasts (TTFs) from Nox4 knockout and
3 wild-type animals and studied the effect of TGF- β 1 on the H₂O₂ output of the cells. TGF- β 1 effectively
4 stimulated the H₂O₂ production of wild-type cells, but Nox4-deficient fibroblasts failed to exhibit
5 enhanced ROS production (Fig. 2A). This result clearly suggested the essential role of Nox4 in the ROS
6 response. Next, we wanted to assess the importance of p22^{phox} in the TGF- β 1-stimulated H₂O₂ response.
7 To achieve this, we prepared TTFs from the *nmf333 strain* mice that carry a mutation in the *CYBA*
8 gene[25], leading to instability of the encoded protein. This mutation was described to result in a
9 decreased Nox2-mediated ROS production in neutrophil granulocytes[25]. Fig. 2B shows that TGF- β 1-
10 treated p22^{phox}-deficient TTFs released significantly less H₂O₂ than cells prepared from wild-type
11 littermates. This experiment proved that endogenously expressed p22^{phox} supports the activity of Nox4.

12

13 **The expression of p22^{phox} in primary fibroblasts is independent of Nox4**

14

15 According to our current understanding, Nox4 is mainly regulated at the level of gene transcription, and
16 the stimulatory effect of TGF- β 1 is a consequence of increased Nox4 expression[7]. We wanted to know,
17 whether TGF- β 1 also stimulates the expression of p22^{phox} that proved to be an essential partner of Nox4 in
18 previous experiments. After exposure to TGF- β 1 for 24 h, we observed the induction of Nox4 mRNA
19 expression in both HPFs and BJ fibroblasts (Fig. 3A). Interestingly, the expression of p22^{phox} mRNA in
20 the same cell types was unaffected by TGF- β 1 (Fig. 3A). In further experiments, we analyzed the p22^{phox}
21 protein content of control- and TGF- β 1-stimulated HPFs and BJ fibroblasts. These experiments revealed
22 that the p22^{phox} protein is already expressed in unstimulated cells, and its level remains constant following
23 TGF- β 1 treatment (Fig. 3B). We also studied p22^{phox} expression levels in a cell line where Nox4 was
24 heterologously expressed [12]. Fig. 3C (right panel) shows a marked increase in H₂O₂ production by
25 Nox4-expressing cells, whereas the p22^{phox} content of the Nox4-expressing and parent cell line was
26 essentially the same (Fig. 3C, left panel). In subsequent experiments, we compared the p22^{phox} expression
27 of wild-type and Nox4-deficient TTFs. As shown in Fig. 3D, the p22^{phox} protein was present in the genetic
28 absence of Nox4 and its level was unaffected by TGF- β 1. Altogether, the results of the above-described
29 experiments suggested that the expression of p22^{phox} is independent of Nox4, and complex formation with
30 Nox4 is not required for p22^{phox} stabilization.

31

32 **Nox4 is stabilized by p22^{phox} in the kidney**

33

1 Next, we wanted to examine whether the presence of p22^{phox} is required for the expression of the Nox4
2 protein or it regulates Nox4 activity by other means. Since we could not detect Nox4 at the protein level in
3 TGF-β1-stimulated fibroblasts, we decided to study the interaction between the two proteins in the kidney
4 that shows the highest Nox4 level among mammalian organs[15]. First, we checked whether Nox4 and
5 p22^{phox} are expressed in the same region of the kidney. *In situ* hybridization experiments confirmed that
6 Nox4 and p22^{phox} mRNAs are both present in proximal tubules epithelial cells (Figs. 4A-D). Next, we
7 studied the p22^{phox} content of kidney lysates from wild-type, p22^{phox} mutant (*nmf333*) and Nox4-deficient
8 animals. As shown in Fig. 4E the level of p22^{phox} was unaffected in the absence of Nox4, whereas p22^{phox}
9 mutation resulted in reduced protein level. When the expression of Nox4 was analyzed in the same
10 samples we found Nox4 to be absent in both p22^{phox} mutant and Nox4-deficient kidney lysates (Fig. 4F).
11 This observation suggests that p22^{phox} is essential for the stabilization of Nox4 in kidney epithelial cells.

12

13 **Nox4 and p22^{phox} localize to the endoplasmic reticulum of primary fibroblasts**

14

15 Since our previous experiments revealed an intimate relationship between p22^{phox} and Nox4, we wanted to
16 determine the intracellular localization of p22^{phox} in primary fibroblasts. Unfortunately, none of the tested
17 p22^{phox}-specific antibodies gave specific labeling; thus, we added a V5 epitope to the C-terminus of
18 p22^{phox} and studied the distribution of the labeled protein. Fig. 5A shows that p22^{phox}-V5 localized to the
19 endoplasmic reticulum in BJ fibroblasts. This staining pattern was not due to the modification of the C-
20 terminal part of p22^{phox} since introducing a different (AU1) tag into the protein did not change the
21 characteristic localization pattern (data not shown). As we demonstrated in earlier experiments, Nox4 was
22 absent in unstimulated primary fibroblasts. Therefore, it was possible that localization of p22^{phox} to the ER
23 did not represent the final position of the protein in the Nox4 enzyme complex. We, therefore, tested
24 whether the subcellular distribution of p22^{phox} changes upon TGF-β1 stimulation, e.g. under conditions
25 when Nox4 becomes induced. As shown in Fig. 5A, TGF-β1 treatment did not modify the localization of
26 p22^{phox} that remained in the endoplasmic reticulum. When an epitope-labeled version Nox4 was
27 introduced into fibroblasts by heterologous expression, it was also detected in the ER, where it co-
28 localized with BiP (Fig. 5B). These observations suggested that the physiological location of the p22^{phox}-
29 Nox4 complex in primary fibroblasts is the ER.

30

31 **Orientation of p22^{phox} and Nox4 in the endoplasmic reticulum membrane**

32

33 H₂O₂ production by Nox4 in TGF-β1-stimulated fibroblasts is readily detected in the extracellular space,
34 although the enzyme complex localizes to an intracellular compartment. We, therefore, became interested

1 in determining the orientation of the Nox4-p22^{phox} complex in the ER membrane. To study the orientation
2 of p22^{phox}, we applied a chemically induced protein dimerization technique, which is based on the
3 rapamycin-induced heterodimerization of the mammalian FRB domain with the FK506 binding
4 protein[28,37]. We coupled FRB along with the Cyan Fluorescent Protein (CFP) to the C-terminus of
5 p22^{phox} and FKB12 was labeled with the Yellow Fluorescent Protein (YFP). Fig. 6A shows that the FRB-
6 CFP labeled p22^{phox} localized to the ER, whereas the FKB12-YFP protein was cytosolic (Fig. 6B). After
7 the addition of rapamycin the FKB12-YFP protein rapidly relocated to the ER indicating that the
8 dimerization event occurred on the cytosolic surface of the ER (Fig. 6D). In other experiments, we
9 introduced the FRB-CFP tag to N-terminus of Nox4. This Nox construct also located to ER and following
10 the addition of rapamycin, the YFP-linked FKBP12 showed colocalization with Nox4 (Fig. 6H). Based on
11 these experiments, the orientations of p22^{phox} and Nox4 are compatible with ROS release into the lumen of
12 the endoplasmic reticulum (Figs. 6I and J).

13

14

15 **DISCUSSION**

16

17 Nox4 is currently the most intensively studied regulated source of hydrogen peroxide. Although
18 Nox4 was originally identified in the kidney [15,33], Nox4-dependent production of ROS is now
19 recognized in several other tissues and cells. The great majority of Nox4-related studies aimed to identify
20 the physiological and pathological roles of Nox4-derived H₂O₂. Thus, we still know little about the protein
21 interactions and regulation of Nox4 [7,34]. The intracellular localization of Nox4 is also obscure since the
22 enzyme was detected at several different intracellular locations [5,17,21,39,41].

23 In this work, we characterized the expression and function of p22^{phox} in primary fibroblasts and
24 kidney, i.e. at locations where Nox4 is also endogenously expressed. Our experiments revealed an
25 interesting asymmetrical relationship between p22^{phox} and Nox4. We found p22^{phox} to be expressed in the
26 absence of Nox4 in both fibroblasts and the kidney, but the presence of p22^{phox} was required for the
27 activity of Nox4. Since Nox4 protein was not detected in kidney lysates of p22^{phox} mutant animals, we can
28 conclude that p22^{phox} is required for the stabilization of Nox4.

29 p22^{phox} was first identified as an essential membrane component of the phagocytic oxidase, where
30 together with Nox2 they form the cytochrome b₅₅₈ complex[26,32]. According to our current view of the
31 phagocytic oxidase, cytochrome b₅₅₈ catalyzes the final steps of electron transfer during superoxide
32 production. P22^{phox} was also described as an essential component of an NADPH oxidase expressed in
33 vascular smooth muscle cells, but the identity of the partner Nox isoform was unknown at the time [36].
34 Genetic evidence supports the cooperation between p22^{phox} and Nox3, as mice with a mutant p22^{phox} gene

1 exhibit a vestibular defect that is similar to the one observed in Nox3-deficient animals[25]. In
2 heterologous expression models, p22^{phox} was also found to interact with and support the activity of
3 Nox4[1,24] [19], however, our data obtained from experiments on p22^{phox}-mutant fibroblasts provide
4 genetic evidence for the importance of p22^{phox} in the stabilization of endogenously expressed Nox4. The
5 *nmf333* mutation results in a Y121H amino acid substitution in p22^{phox}[25]. When the effect of this amino
6 acid change on Nox4 function was tested in a heterologous expression system, the stimulatory effect of the
7 mutant p22^{phox} protein was indistinguishable from the effect of its wild-type counterpart[38]. In a different
8 study on Nox4-transfected HEK293 cells, the expression level of Nox4 was not affected by silencing
9 p22^{phox} [19]. The difference between these data and our results obtained from experiments on primary
10 cells emphasizes the importance of cautious interpretation of data derived from heterologous expression
11 systems.

12 In phagocytic cells, the presence of p22^{phox} is essential for the stabilization of Nox2, and p22^{phox}
13 protein is detected only in the presence of Nox2, thus the simultaneous expression of both proteins is
14 necessary for the appearance of the cytochrome b558 complex[27,35]. Stabilization of the p22^{phox} protein
15 by Nox4 was also reported in HEK293 cells transfected with epitope-tagged Nox4- and p22^{phox} constructs
16 [1]. However, several of our observations suggest that the symmetrical relationship between p22^{phox} and
17 Nox2 is not characteristic of the Nox4 system. First, we detected p22^{phox} by Western blot in human
18 pulmonary and foreskin fibroblasts that did not express any Nox proteins. TGF-β1 treatment of these cells
19 boosted the expression of Nox4 and consequent ROS production, but the p22^{phox} content of TGF-β1-
20 treated cells did not differ from that of control, unstimulated cells. Furthermore, p22^{phox} was also detected
21 in Nox4-deficient TTFs, confirming that the presence of Nox4 is not required for the stabilization of
22 p22^{phox}. It will be interesting to study whether this asymmetrical relationship between the two proteins is a
23 unique feature of the Nox4 system or other Nox proteins (Nox1 and Nox3) behave similarly in cells where
24 they are endogenously expressed.

25 It is noteworthy that we could not study the effect of p22^{phox} on Nox4 expression in TGF-β1-
26 stimulated fibroblasts because we were unable to detect Nox4 at the protein level in these cells. When we
27 compared the p22^{phox} content of primary fibroblasts and neutrophil granulocytes, we observed a much
28 higher p22^{phox} content in neutrophils than in fibroblasts (Fig. S1). Since Nox proteins are supposed to form
29 a 1:1 complex with p22^{phox}, the p22^{phox} content of fibroblasts may limit the maximum of Nox4 expression.
30 Apparently, this expression level is too low to be captured by anti-Nox4 antibodies which were tested
31 during the course of our studies. This observation cautions for the critical interpretation of experiments
32 where Nox4 is detected at the protein level by Western blot analysis.

33 The subcellular localization of Nox enzymes is one of the most important issues in the Nox/Duox
34 research field. To know the specific intracellular sites of Noxes is critical because the highly reactive

1 species produced by these enzymes unlikely travel a long distance before exerting their effects; thus their
2 intracellular localization is a major determinant of their affected targets[40]. In previous works Nox4 was
3 detected at various intracellular locations including the plasma membrane, nucleus, mitochondria,
4 endoplasmic reticulum, and focal adhesions [5,17,21,39,41]. It is possible that the subcellular distribution
5 of Nox4 is cell type-specific, however, the lack of specific antibodies might also explain the observed
6 differences. According to our results, p22^{phox} resides in the endoplasmic reticulum of primary fibroblasts
7 and this localization persists during myofibroblastic differentiation, e.g. when Nox4 becomes expressed in
8 the cells. The localization of p22^{phox} likely defines the site of the Nox4-p22^{phox} complex, which in the case
9 of TGF- β 1-stimulated fibroblasts, appears to be the ER. Although we could not locate p22^{phox} in the
10 murine kidney by immunostaining, cell fractionation experiments suggest that protein – along with Nox4
11 – is enriched in the microsomal fraction of the cells (data not shown).

12 According to the current topological model of p22^{phox}, which was described in phagocytic cells,
13 the protein has two transmembrane domains with both termini facing the cytosol. Our data obtained by
14 rapamycin-induced dimerization technique suggests that p22^{phox} resides in the ER membrane in a similar
15 fashion, that is N- and C-termini located on the cytoplasmic side. This membrane topology would be
16 compatible with H₂O₂ being released into the ER lumen. The ER lumen is characterized by a highly
17 oxidative environment, which is probably due to the activity of the protein folding machinery. The
18 oxidized state of the ER of primary fibroblasts was readily detected by an ER-targeted, redox-sensitive
19 protein sensor, Hyper (data not shown). However, the already oxidized sensor would unlikely capture the
20 presence of “extra” oxidants in the ER lumen. On the other hand, Nox4-derived H₂O₂ production by TGF-
21 β 1-activated cells was readily detected in the extracellular space, confirming earlier observations, where
22 Nox4 was described to produce mainly H₂O₂ [11]. At the first sight, the localization of Nox4 in the ER
23 does not seem to be compatible with ROS release to the extracellular space, however, the intimate
24 relationship between the ER and the plasma membrane can explain the extracellular detection of Nox4-
25 produced H₂O₂.

26 The function of Nox4 in TGF- β 1-stimulated fibroblasts is still incompletely understood. In a
27 previous study, where RNAi was used to suppress Nox4 activity, the enzyme was found to promote
28 myofibroblastic differentiation [9]. On the other hand, experiments on Nox4 knockout mice did not
29 support a role of Nox4 in the development of kidney fibrosis [2] and we did not observe altered fibroblast-
30 myofibroblast transition in Nox4-deficient TTFs (data not shown). In more recent studies on vascular
31 smooth muscle cells, Nox4 activity was found to have a role in TGF- β 1-induced palladin expression [23]
32 and focal adhesion formation [10]. This latter response seems to be dependent on the interaction of Hic-5
33 and HSP27, indicating a complex role for Nox4-derived ROS in the organization of cytoskeletal responses
34 [14].

1 The formation and regulation of the redox environment in the ER is very complex and remains
2 partially unexplored. According to our current understanding of the ER redox machinery, the major
3 source of H₂O₂ in the ER is ERO1 which produces H₂O₂ during the oxidation of PDI. Thus, the production
4 of H₂O₂ in the ER is essentially thought to be a byproduct of oxidative protein folding. The addition of a
5 dedicated H₂O₂ source to this scene appears to indicate redundancy, however, the redox milieu of the ER
6 is unlikely homogenous, and it is possible that different ROS sources are compartmentalized within the
7 organelle. Hopefully, the future identification of Nox4 targets will improve our understanding of the
8 Nox4-p22^{phox} complex role in ER redox homeostasis.

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18 **CONFLICT OF INTEREST**

20 The authors declare that they have no conflict of interest.

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16 LEGENDS TO FIGURES

17 **Figure 1.**

18 **TGF- β 1 stimulates H₂O₂ production in human primary dermal and pulmonary fibroblasts.**

19 Extracellular H₂O₂ production by adherent human pulmonary (HPF, **A**) and foreskin (BJ, **B**) fibroblasts,
20 was determined by Amplex Red assay in the presence of 50 μ M Amplex Red and 0.1 U/ml horseradish
21 peroxidase in H-medium. After 40 min incubation at 37°C, resorufin fluorescence was measured at 590
22 nm. HPF. Cells were treated with Nox4 or control (scrambled) siRNA for 24 h and were stimulated with
23 5 ng/mL TGF- β 1 in serum-free medium for 24 h. Bars with SEM represent mean values of 3 independent
24 experiments. ** P<0.001 * P<0.002 in Mann-Whitney U test or t-test.

25
26

27 **Figure 2.**

28 **TGF- β 1-stimulated H₂O₂ production by wild-type, NOX4- and p22^{phox}- deficient tail tip fibroblasts.**

29 Serum-depleted, adherent, tail tip fibroblasts (TTFs) were treated with 5 ng/mL TGF- β 1 for 24 h.
30 Extracellular H₂O₂ production was measured by the Amplex Red assay in Nox4-deficient (**A**) and p22^{phox}-
31 mutant cells (**B**). Bars with SEM represents mean values in 3 independent experiments. ** P<0.001, *
32 P<0.002 in t-test

33

34 **Figure 3.**

35 **P22^{phox} mRNA and protein expression in primary fibroblasts and Nox4-transfected HEK293 cells**

1 (A). Nox4 and p22^{phox} mRNA expression levels of control and TGF-β1-induced HPFs, and BJ fibroblasts
2 were determined after a 24 h treatment with TGF-β1. Relative expressions are expressed, where the non-
3 induced expressions are defined as 1. (B). Western blot analysis of the p22^{phox} protein content of control
4 and TGF-β1-stimulated HPFs and BJ fibroblasts. (C). Western blot analysis of the p22^{phox} protein content
5 in lysates of Nox4-expressing and control (untransfected) HEK293 FS cells. The right panel shows the
6 H₂O₂ output of Nox4-expressing and control cells, assessed by the Amplex Red assay. Western blot
7 experiments yielded essentially the same result in at least three separate experiments. Mean values and
8 SEM are calculated from 3 independent experiments* P<0.005 in Paired t-test. (D). p22^{phox} protein
9 expression of unstimulated and TGF-β1-induced TTFs, isolated from Nox4 knockout animals.

10
11 **Figure 4.**

12 **Nox4 and p22^{phox} expression in the kidneys of wild-type, Nox4-knockout, and p22^{phox}-deficient mice**
13 (A-D) *In situ* hybridization for Nox4 and p22^{phox} mRNAs in mouse kidney. (A, C: antisense probes, B, D:
14 sense probes, PT: proximal tubule, DT: distal tubule). (E) Analysis of p22^{phox} expression by Western blot
15 in kidney lysates from wild-type, p22^{phox} mutant (*nmf333*) and Nox4-deficient animals. (F) Nox4 protein
16 detection by Western blot in kidney lysates from wild-type, p22^{phox} mutant (*nmf333*) and Nox4-deficient
17 animals.

18
19 **Figure 5.**

20 **Subcellular localization of Nox4 and p22^{phox} in BJ fibroblasts**
21 Representative confocal images of permeabilized BJ cells expressing V5-tagged p22^{phox} (A) and Nox4 (B)
22 compared to endogenous BiP. Fibroblasts were transiently transfected then induced with TGF-β1 or left
23 untreated after overnight serum depletion. The cells were fixed and immunostained with anti-V5 and anti-
24 BiP antibody. Bars represent 10 μm.

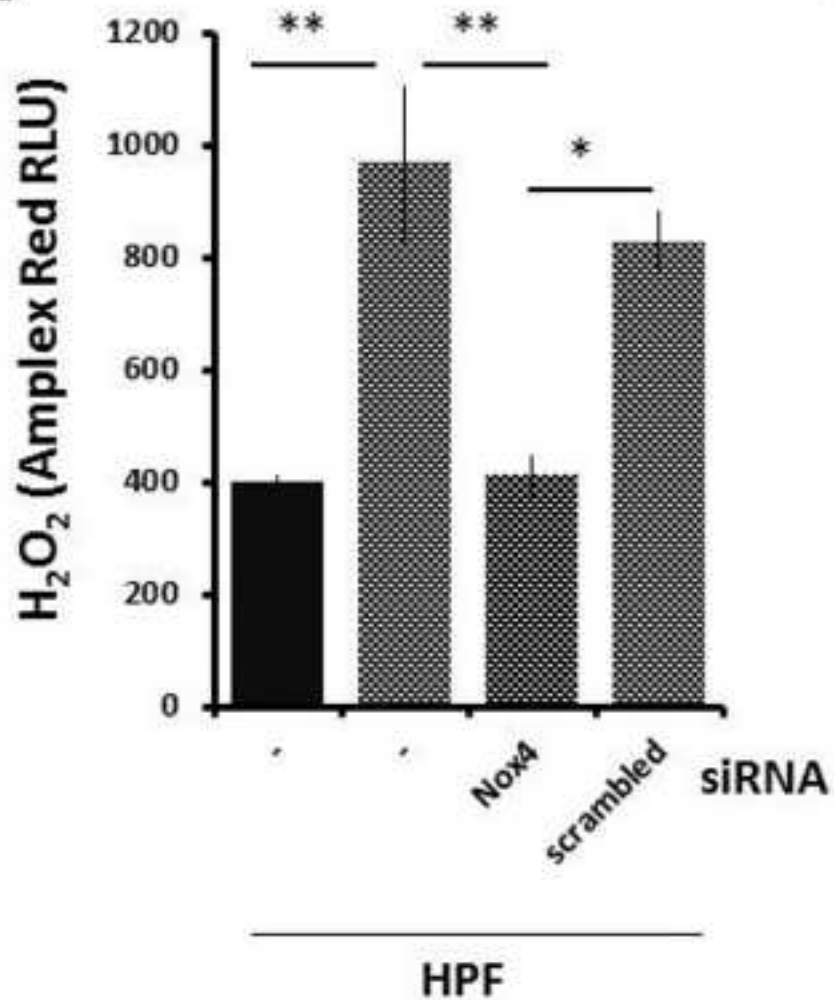
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26
27 **Figure 6.**

28 **Translocation of FKBP12 domain upon heterodimerization with FRB in dermal fibroblasts.**
29 The rapamycin-induced heterodimerization of the mammalian FRB domain with FK506 binding protein
30 12 (FKBP12) can be followed in confocal images. The p22^{phox}-FRB-CFP (A) or CFP-FRB-Nox4 (B) and
31 YFP-linker-FKBP12 were cotransfected in BJ fibroblasts. After 24h incubation, the cells were seeded in
32 cell chamber with H-medium in the confocal microscope. Administration of 300 nM rapamycin, induced
33 the translocalization of the FKBP12 to reach the spatially available FRB domain. Bars represent 10 μm.
34 The schematic structure of the p22^{phox} and Nox4 is shown in panels C and D.

- 1 **Figure S1**
- 2 **Comparison of the p22^{phox} content of neutrophils and primary fibroblasts**
- 3 Western blot analysis of the p22^{phox} protein content of different numbers of neutrophils (PMN) human
- 4 pulmonary (HPF) and dermal (BJ) fibroblasts.

Figure 1.

A



B

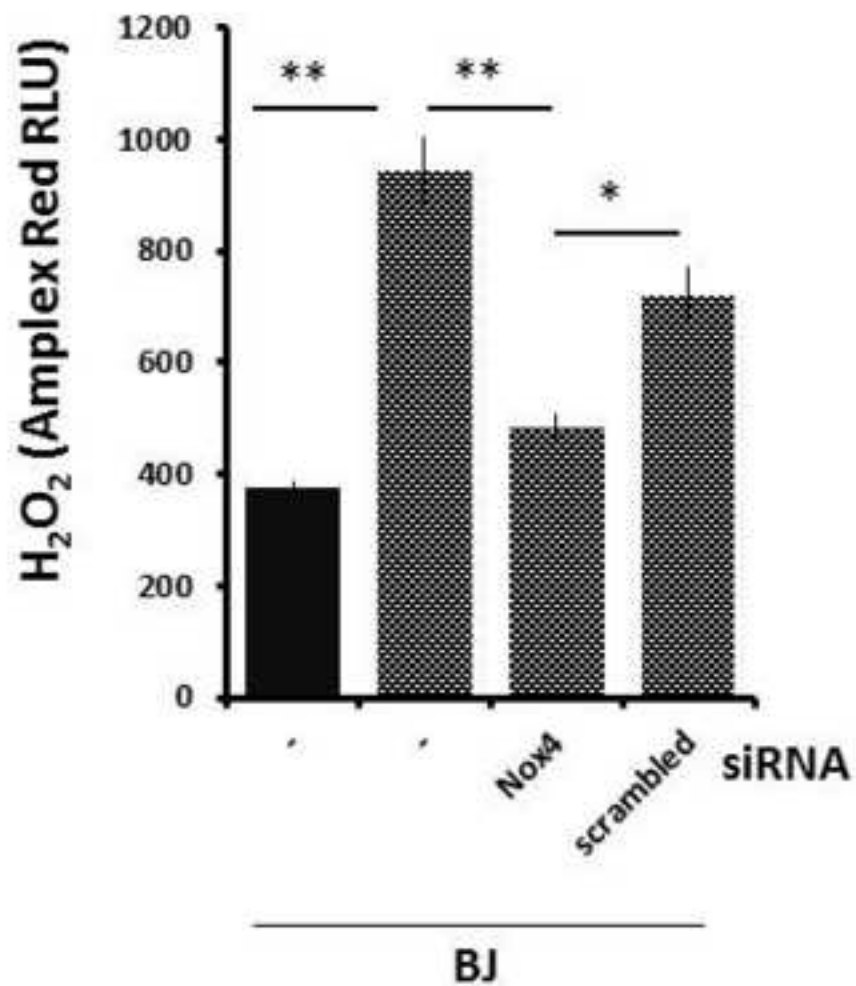


Figure 2.

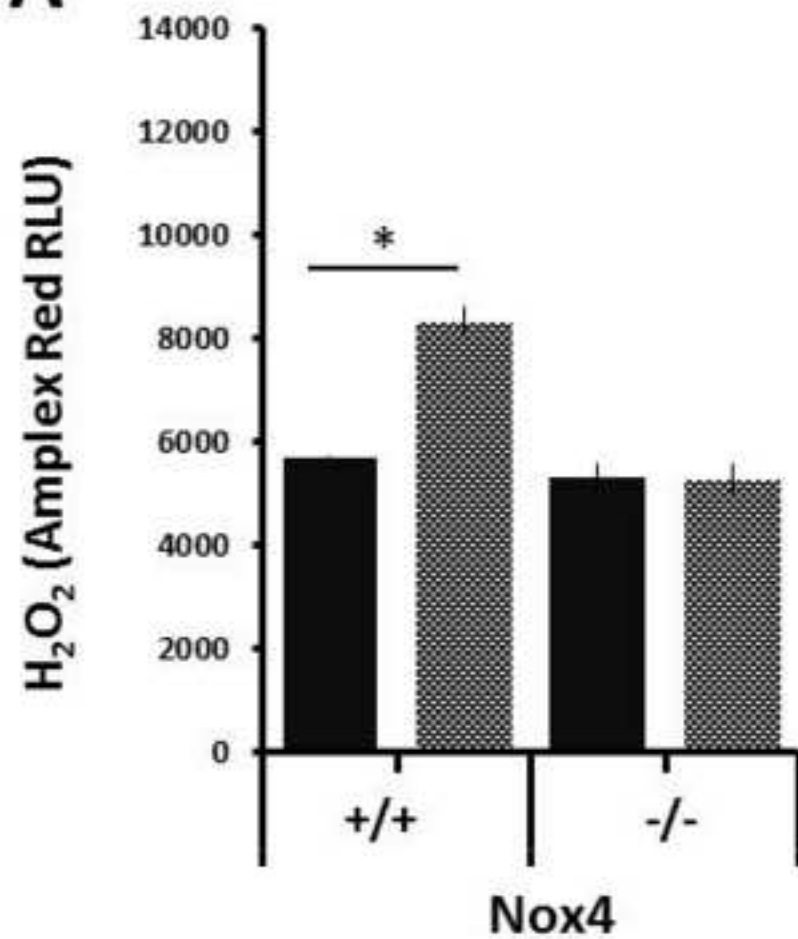
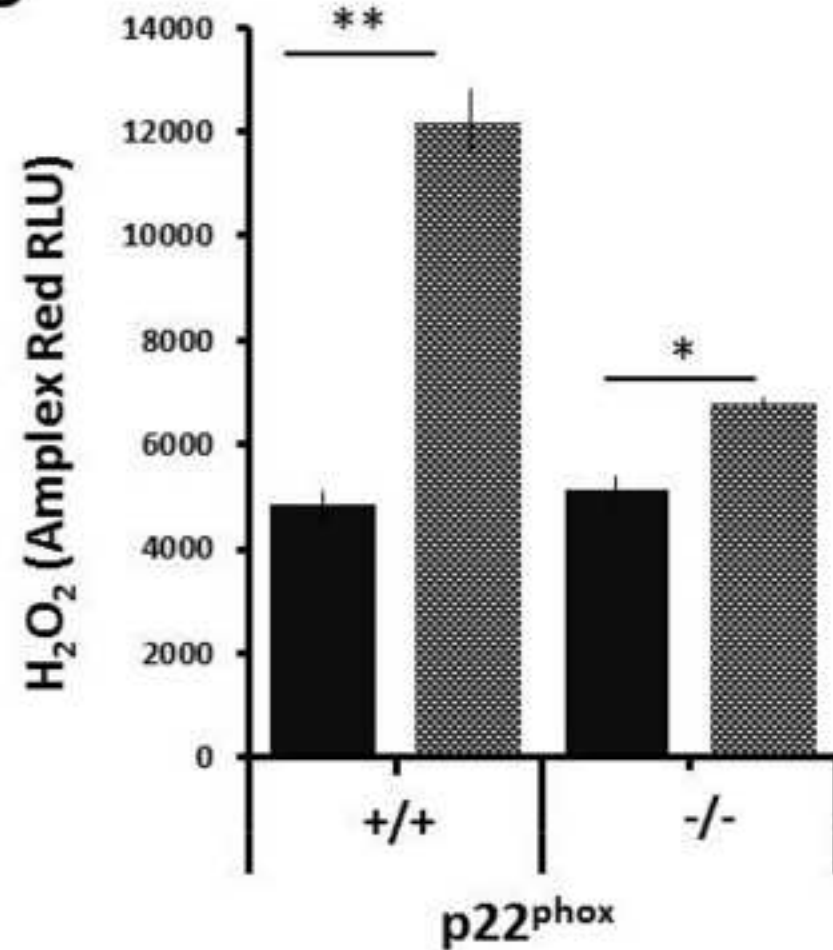
A**B**

Figure 3.

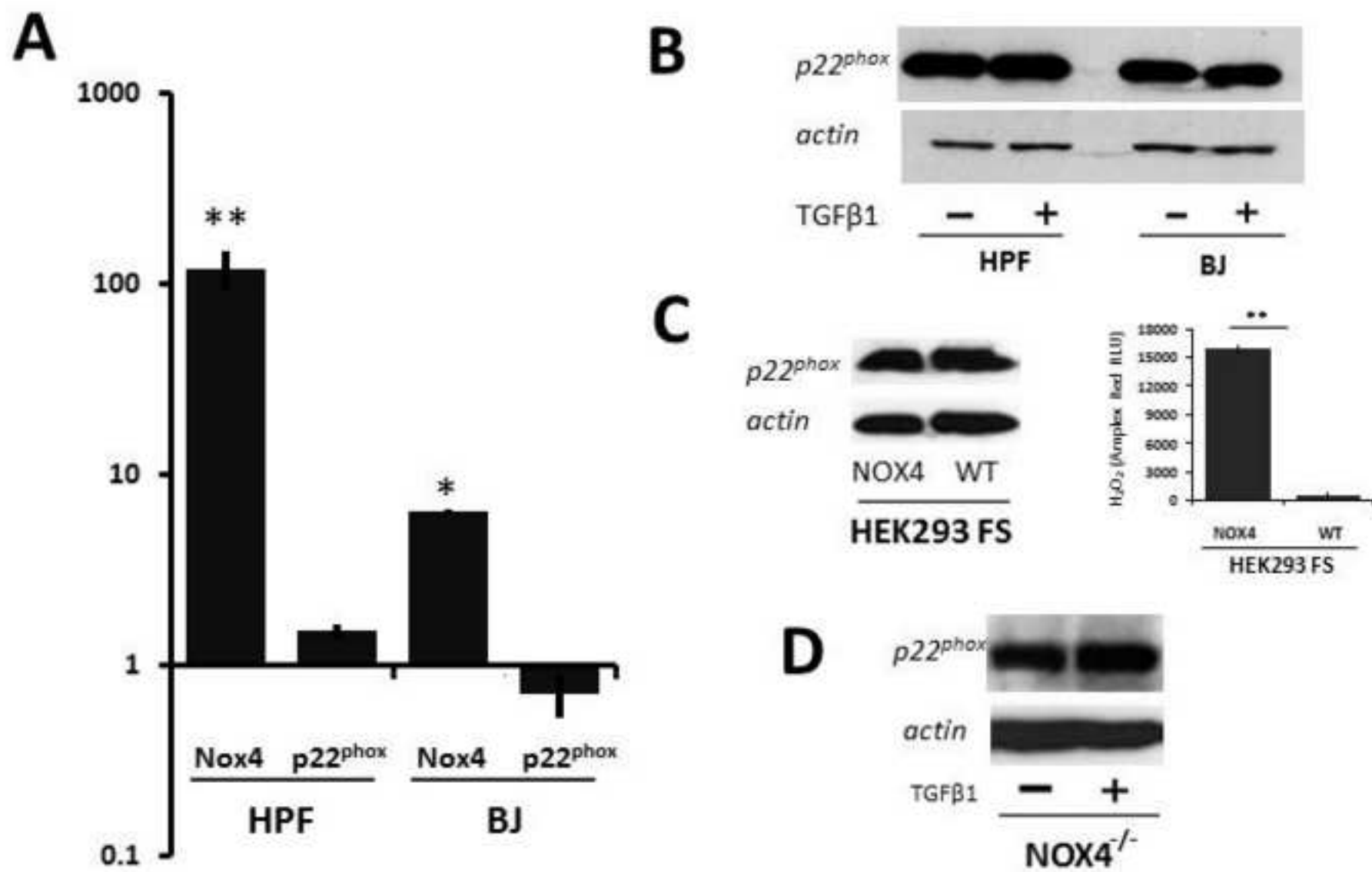


Figure 4

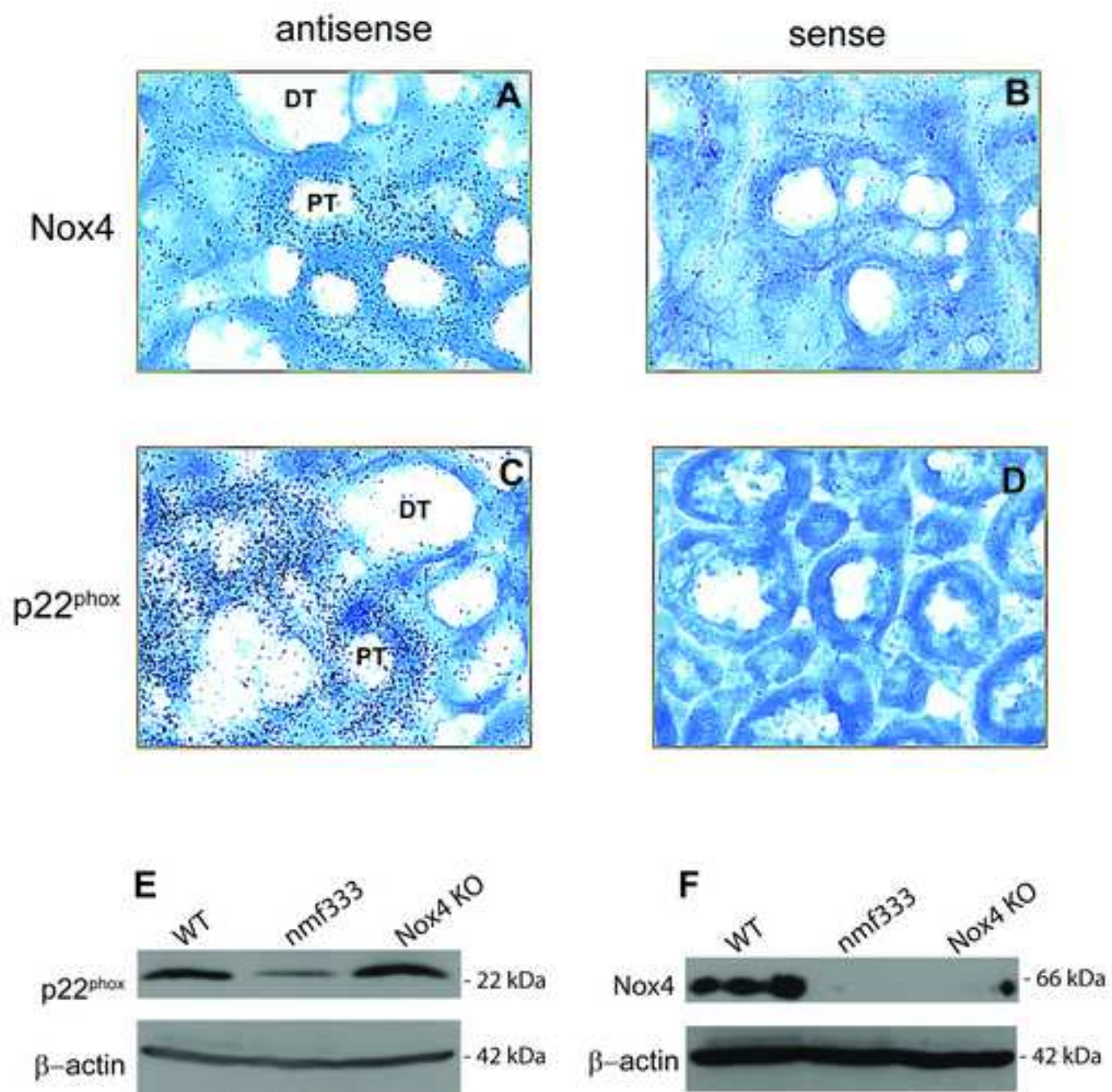


Figure 5.

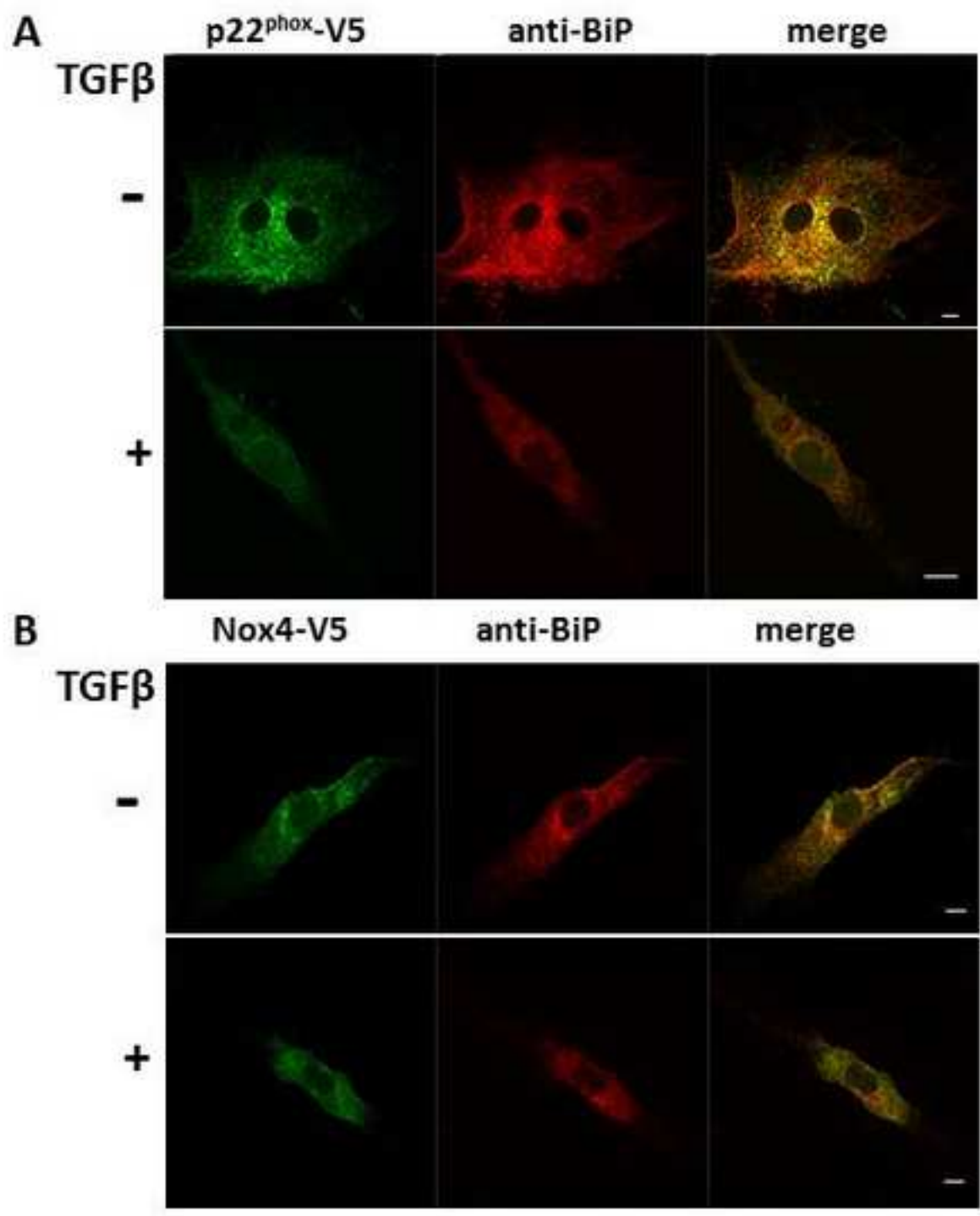
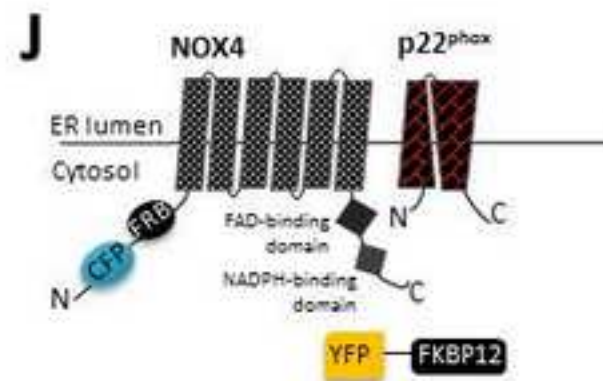
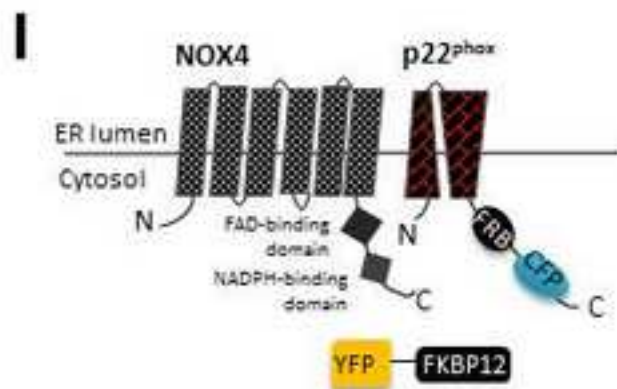
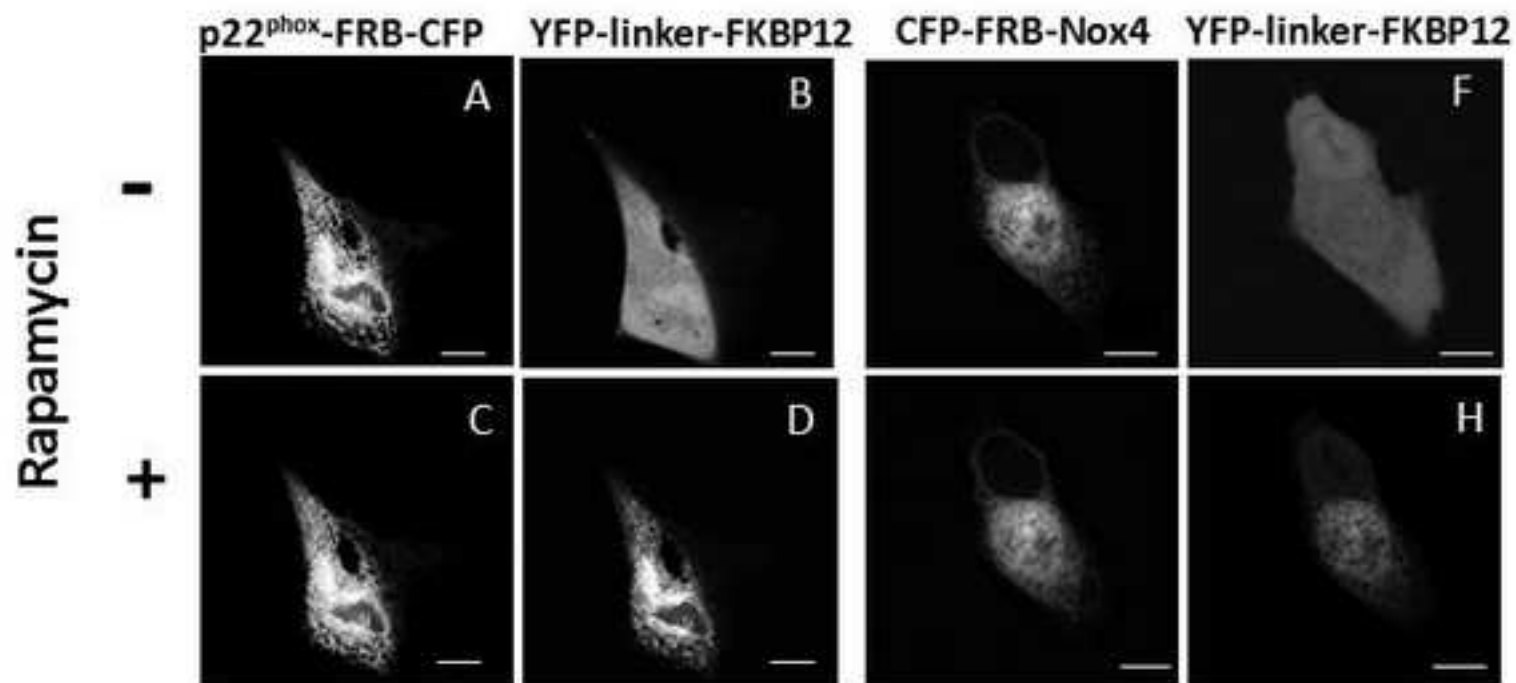


Figure 6.



Supplementary figure 1.

