Research Paper

CRISPR/Cas9-mediated knockout of p22phox leads to loss of Nox1 and Nox4, but not Nox5 activity

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Abstract

The NADPH oxidases are important transmembrane proteins producing reactive oxygen species (ROS). Within the Nox family, different modes of activation can be discriminated. Nox1-3 are dependent on different cytosolic subunits, Nox4 seems to be constitutively active and Nox5 is directly activated by calcium. With the exception of Nox5, all Nox family members are thought to depend on the small transmembrane protein p22phox. With the discovery of the CRISPR/Cas9-system, a tool to alter genomic DNA sequences has become available. So far, this method has not been widely used in the redox community. On such basis, we decided to study the requirement of p22phox in the Nox complex using CRISPR/Cas9-mediated knockout. Knockout of the gene of p22phox, CYBA/p22phox, led to an ablation of activity of Nox4 and Nox1 but not of Nox5. Production of hydrogen peroxide or superoxide after knockout could be rescued with either human or rat p22phox, but not with the DUOX-maturation factors DUOXA1/A2. Furthermore, different mutations of p22phox were studied regarding the influence on Nox4-dependent H2O2 production. P22phox Q130* and Y121H affected maturation and activity of Nox4. Hence, Nox5-dependent O2*- production is independent of p22phox, but native p22phox is needed for maturation of Nox4 and production of H2O2.

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1. Introduction

The Nox family of NADPH oxidases is an important source of reactive oxygen species (ROS). Through ROS, Nox enzymes play diverse roles in cellular signaling, regulation of gene expression, cell differentiation and posttranslational protein modifications [4]. In mammals, seven members are expressed and grouped according to their mode of activation. Nox1, Nox2 and Nox3 are activated by an assembly with specific cytosolic proteins, which translocate to the plasma membrane. On the other hand, Nox5 and DUOX1 as well as DUOX2 (Dual oxidase 1 and 2) are directly activated by calcium in an EF-hand motif dependent manner. To our current knowledge, Nox4, in contrast, is constitutively active and ROS production of the enzyme is mainly controlled by its expression level [5].

For DUOX1 or DUOX2, maturing factors are needed to achieve proper expression and activity. The small transmembrane protein p22phox/CYBA (Cytochrome B-245 Alpha Chain) was described to be essential for Nox1, Nox2 and Nox3 maturation, stabilization and activation [2,20,21,31]. P22phox has been found to interact with Nox4, indicating importance for Nox4 activity and localization [2,18,19]. The exact site where p22phox interacts and activates Nox4 appears non-canonical with respect to Nox1, Nox2 or Nox3 [17,31]. P22phox consists of two (or four) predicted transmembrane domains and an extended C-terminus with a prolin-rich region, which is required for the assembly with p47phox or NoxO1 to membrane-bound Nox1, Nox2 and Nox3 [5,31]. This feature is dispensable for Nox4 indicating a different role for the p22phox-Nox4 activation [16,17]. Mutations in p22phox are associated with a loss of function leading to severe immunodeficiency called chronic granulomatous disease (CGD), with inability to clear some...
pathogens and subsequent life threatening infections [6,10].

Further analysis of the structural and functional role of p22phox are necessary not just for understanding the Nox2-dependent pathogen defense, but also for the analysis of Nox4, as a protective cardiovascular enzyme.

In the kidney, the endothelium and fibroblasts, Nox4 is the predominantly expressed Nox homolog [1,7,11]. Nox4 is fundamentally different to the other Nox enzymes and exhibits a different dependency on p22phox. Approaches to study p22phox-Nox4 interaction like the p22phox mutant mouse nmf333 could only partially reveal the mechanisms underlying the relevant protein interaction [17,22]. There is, however, no doubt that only partially reveal the mechanisms underlying the relevant protein interaction [17,22]. Approaches to study p22phox-Nox4 interaction like the p22phox mutant mouse nmf333 could only partially reveal the mechanisms underlying the relevant protein interaction [17,22].

Clustered regulatory interspaced short palindromic repeats (CRISPR) (5% CO2, 3 7°C) was used to mutate p22phox. Primers were designed with the GenJet Plasmid Miniprep Kit (10 U/ml) digestion of the original strand. The mutated plasmid was transformed into XL10-Gold Ultracompetent cells, the generated plasmid was purified with the GenJet Plasmid Miniprep Kit (K0503, Thermo Scientific). Mutations were verified by sequencing. The following primers were used for the introduction of single amino acid mutation: CYBA A91stop 5’-GAG AGC AGA TGA TCC ACC TAG TAA TAA TTC CTG G-3’ and 5’-CCA GGA ATT ACT ATG TTC GGT AGG TCC TGC ATC TCC TGT C-3’; CYBA ESA 5’-CCA CAT GGC CCA CCA CGC CAT TGG CCC ATT CAT-3’ and 5’-ATG GCG CAG CAC TGG TCG GGC ATG TGG-3’; CYBA E12A 5’-CCA GGC CCT GCC CCT TGG CCC AC-3’ and 5’-CTG GCA AAA GCC GCA GGC GCT GG-3’; CYBA G110stop 5’-CTG CTG CCT ACC ATC TTC ACC TGG ACC TGG-3’ and 5’-CCA TGG CGA CCT GCC GGT CTA AAG GAT GGT GGC CAG CAG-3’; CYBA Y121H 5’-CTG CGG GCC TCC ACT ACT CGC CAC GCA C-3’ and 5’-GTT CCG GTC GAG TAC TCG TGG ACC ACC A-3’; CYBA R90Q 5’-GCC CCT TAC CAA TTA TTA TGA ACC CCT GCT CCT-3’ and 5’-ATG CAG GAC GCC TCT AAC ATC ATT GAT GGC C-3’.

Given that protein abundance of p22phox is largely controlled by posttranscriptional mechanisms and that a few studies on the web-based QuikChange Primer Design Program. Brie- lycing-induced tet-on operator (Nox4-pDEST30) were kindly provided by K.H. Krause (University of Geneva, Switzerland) [29]. Induction with 1 μg/ml tetracycline was performed for 24 h in HEK medium. HEK293 cells stably expressing human Nox4 (Nox4-HEK293) were generated as described before using lentiviral transfection and selection [12].

2.2. Cell culture

Human embryonic kidney 293 cells (HEK293) cells were obtained from ATCC (Manassas, VA, USA) and cultured in Modified Eagle’s Medium (MEM, Gibco) supplemented with fetal calf serum (FCS; 8%), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM) and gentamycin (50 μg/ml) in a humidified atmosphere (5% CO2, 37 °C). HEK293 cells expressing human Nox4 with a tetracycline-inducible tet-on operator (Nox4-pDEST30) were kindly provided by K.H. Krause (University of Geneva, Switzerland) [29]. PE-Tag 2.4. Cloning of pHAGE2-NoxO1-NoxA1-Nox1 for transient overexpression

To clone Nox1, NoxO1 and NoxA1 as lentiviral triple overexpression plasmid under control of the human EF1α promoter, in which NoxO1 and NoxA1 are divided by a 2A peptide (2FA) and NoxA1 and Nox1 separated by an internal ribosome entry site (IRES), PCR amplifications of NoxO1, NoxA1 and Nox1 were performed from pCMV6-Nox1 (NM_172167, #RC214973, Origene), pCMV6-NoxA1 (NM_001256067, #RC234211, Origene) and pCMV6-Nox1 (NM_0007052, #RC210426, Origene) Human cDNA ORF clones with the following primers: NoxO1 5’-ATA GGC GCC GCC ATG GAC GCA GCC CCC CGA TAC CCA GGT TCA TGT-3’ and 5’-ATT TGC TTA GCC CCT GCT CCT CCG TCG TGG GGC GGT GC-3’, Nox1 5’-ATT AGC TAG CAT GGC TCT GCC TGG GGA CCT GGT GGC-3’ and 5’-ATT TGC TTA CAT TTC ATG GGA AAC TGG TGT AAC CAC-3’ and 5’-ATT CAT TAG GAT CTA TCG ACT CAA AAA TTT TCT TGT TGG AAG

2. Materials and methods

2.1. Mutagenesis of p22phox

The QuikChange II XL Site-Directed Mutagenesis Kit (#200521, Agilent) was used to mutate p22phox. Primers were designed with the web-based QuikChange Primer Design Program. Briefly, the plasmid coding for human p22phox (kindly provided by Leto, NIH) was used for mutant strand synthesis reaction with PhiUltra HF DNA polymerase (2.5 U/μl) according to the kit, followed by a DpnI (10 U/μl) digestion of the original strand. The mutated plasmid was transformed into XL10-Gold Ultracompetent cells, the generated plasmid was purified with the GenJet Plasmid Miniprep Kit (K0503, Thermo Scientific). Mutations were verified by sequencing. The following primers were used for the introduction of single amino acid mutation: CYBA A91stop 5’-GAG AGC AGA TGA TCC ACC TAG TAA TAA TTC CTG G-3’ and 5’-CCA GGA ATT ACT ATG TTC GGT AGG TCC TGC ATC TCC TGT C-3’; CYBA ESA 5’-CCA CAT GGC CCA CCA CGC CAT TGG CCC ATT CAT-3’ and 5’-ATG GCG CAG CAC TGG TCG GGC ATG TGG-3’; CYBA E12A 5’-CCA GGC CCT GCC CCT TGG CCC AC-3’ and 5’-CTG GCA AAA GCC GCA GGC GCT GG-3’; CYBA G110stop 5’-CTG CTG CCT ACC ATC TTC ACC TGG ACC TGG-3’ and 5’-CCA TGG CGA CCT GCC GGT CTA AAG GAT GGT GGC CAG CAG-3’; CYBA Y121H 5’-CTG CGG GCC TCC ACT ACT CGC CAC GCA C-3’ and 5’-GTT CCG GTC GAG TAC TCG TGG ACC ACC A-3’; CYBA R90Q 5’-GCC CCT TAC CAA TTA TTA TGA ACC CCT GCT CCT-3’ and 5’-ATG CAG GAC GCC TCT AAC ATC ATT GAT GGC C-3’.

Given that protein abundance of p22phox is largely controlled by posttranscriptional mechanisms and that a few studies on the web-based QuikChange Primer Design Program. Brie- lycing-induced tet-on operator (Nox4-pDEST30) were kindly provided by K.H. Krause (University of Geneva, Switzerland) [29]. Induction with 1 μg/ml tetracycline was performed for 24 h in HEK medium. HEK293 cells stably expressing human Nox4 (Nox4-HEK293) were generated as described before using lentiviral transfection and selection [12].

2.3. Transfection of Nox constructs

Transient transfection of HEK293 cells was performed using Lipofectamine 2000 (#11668027, ThermoFisher) and 0.75 μg/ml plasmid DNA according to the manufacturer’s instructions. Cells were kept in culture for 48 h. DUOXA1 and DUOXA2 plasmids were kindly provided by H. Grasberger (University of Michigan Medical School, Michigan).

2.4. Cloning of pHAGE2-NoxO1-NoxA1-Nox1 for transient overexpression

To clone NoxO1, NoxA1 and Nox1 as lentiviral triple overexpression plasmid under control of the human EF1α promoter, in which NoxO1 and NoxA1 are divided by a 2A peptide (2FA) and NoxA1 and Nox1 separated by an internal ribosome entry site (IRES), PCR amplifications of NoxO1, NoxA1 and Nox1 were performed from pCMV6-Nox1 (NM_172167, #RC214973, Origene), pCMV6-NoxA1 (NM_001256067, #RC234211, Origene) and pCMV6-Nox1 (NM_0007052, #RC210426, Origene) Human cDNA ORF clones with the following primers: NoxO1 5’-ATA GGC GCC GCC ATG GAC GCA GCC CCC CGA TAC CCA GGT TCA TGT-3’ and 5’-ATT TGC TTA GCC CCT GCT CCT CCG TCG TGG GGC GGT GC-3’, Nox1 5’-ATT AGC TAG CAT GGC TCT GCC TGG GGA CCT GGT GGC-3’ and 5’-ATT TGC TTA CAT TTC ATG GGA AAC TGG TGT AAC CAC-3’ and 5’-ATT CAT TAG GAT CTA TCG ACT CAA AAA TTT TCT TGT TGG AAG
TAG-3. In a parallel, a F2A region for placing between NoxO1 and NoxA1 was amplified with 5′-ATT TGC TAA CCA CGG GTG CGG CGG CAC TAA AGA AGA C-3′ and 5′-ATT TGC TAG CTT GAC CGT GAT TTG ACT CTA CAT CAC-3′ from pHAGE2, which was a gift from G. Mostoslavsky (Boston University, MA, USA) [30]. PCR products were cut in the following way to achieve a specific order of integration into pHAGE2: NoxO1 (NotI/BspI (#ER0591, #ER0091, ThermoFisher)), F2A (BspI/Nhel (#ER0909, #ER0972, ThermoFisher)), NoxA1 (Nhel/BamHI (#ER0972, #ER0055, ThermoFisher) and Nox1 (NdEl/BsaBI (#ER0581, #ER1711, ThermoFisher)). In a first ligation reaction, pHAGE2 cut by BamHI/NotI (#ER0055, #ER0591, ThermoFisher) was used to be ligated with restricted NoxO1, F2A and NoxA1. This intermediate product was purified and further cut by NdEl/BsaBI (#ER0581, #ER1711, ThermoFisher) to be ligated in a second reaction with restricted Nox1. The final plasmid (pHAGE2-NoxO1-NoxA1-Nox1) was purified and sequenced.

2.5. Cloning of pHAGE2-Nox5 for stable overexpression

To clone pHAGE2-Nox5 for lentiviral overexpression under the control of the human Efa1 promoter, Nox5 was amplified from pCMV6-Nox5 (NM_001184779) Human cDNA ORF Clone (KC230471, Origene) with the following primers: 5′-ATT GCC GCC GCC ATG ACA ACA TCT GGA GAC CCA GCC CAG CAC-3′ and 5′-ATT TGA TTA GGA TCT ATC GAC CTA GAA ATT CTC TTG GAA AAA TCT G-3′. pHAGE2 was a gift from Gustavo Mostoslavsky [30]. Nox5 PCR product and pHAGE2 were cut with NotI/BsaBI (#ER0581, #ER1711, ThermoFisher) followed by ligation. Plasmid was purified and sequenced.

2.6. Lentiviral transfection of HEK293 cells with pHAGE2-Nox5

Vesicular stomatitis virus (VSV) pseudotyped lentivirus was produced by transfection of HEK293T cells with pHAGE2-Nox5 in combination with HCPM2, Tat1B, Rev1B and VSV-G, which were a gift from Bianling Liu. Viral supernatants were collected, concentrated and snap-frozen after four days. Transfection of 70% subconfluent HEK293 with Nox5 viral particles was performed for 12 days, followed by clonal expansion.

2.7. CRISPR/Cas9

The web interface of CRISPR design (http://crispr.mit.edu/) was used to develop gRNAs. Off-target activity was evaluated additionally with Blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The pSpCas9(BB)-2A-Puro (PX459) v2.0 was a gift from Feng Zhang (Addgene plasmid # 60248) and used as cloning backbone. CRISPR/Cas9 was carried out similar as in [14,25]. Briefly, phosphorylation and annealing was performed with the following oligonucleotides harboring a BbsI overhang: For (A) CYBA_1_Ext1 with 5′-CAC CGG CGG GGT TGT TCG CTC CAT G-3′ and 5′-AAA CCA TGG CCA GCA CCC GCC C-3′; For (B) CYBA_2_Ext1 with 5′-CAC CGG GAC GGC AGC TGT TCG T-3′ and 5′-AAA CAC GAA CAG CGG CTG CGG TCC C-3′; For (C) CYBA_3_Ext1 with 5′-CAC CGG GCC ATG TGG GCC AAC GAA C-3′ and 5′-AAA CTT GTG TCG GCC CAG ATG GCC C-3′; For (D) CYBA_4_Intron1 with 5′-CAC CGG TGG ACG TCA CGG ACC GTG G-3′ and 5′-AAA ACC ACC GTC CCT GAC GTG CAC C-3′; For (E) CYBA_2_Intron1 with 5′-CAC CGG ACC TGG CCG TGC TTA C-3′ and 5′-AAA CTT ACT CAG TAG CCC ACC GTC CCT GAC GTG CAC C-3′; For (F) CYBA_3_Intron1 with 5′-CAC CGG ACC TGG CCG TGC TTA C-3′ and 5′-AAA CTT ACT CAG TAG CCC ACC GTC CCT GAC GTG CAC C-3′; For (G) CYBA_2_5UTR with 5′-CAC CGG GGT TGT GGG CAG CCA GCC GCA GCC C-3′ and 5′-AAA ACC ACC GTC CCT GAC ACC CCG C-3′. Afterwards, BbsI (#FD1014, ThermoFisher) mediated digestion and T7 DNA ligase (#M0318L, NEB) directed ligation was performed followed by PlasmidSafe exonuclease treatment (#E3101K, Epicenter/Biozym) according to the manufacturers protocol. Plasmids were purified and sequenced. After transformation of the indicated combinations of pSpCas9(BB)-2A-Puro-gRNAs (Fig. 1B), positive cells were selected using puromycin (2 µg mL−1) for five days prior to clonal expansion. Empty pSpCas9(BB)-2A-Puro was used as negative control.

2.8. Determination of ROS production

ROS production was measured as described before [24,26]. As probe, luminol (100 µM, #123072, Sigma) catalyzed by horseradish peroxidase (HRP, 1 U/mL, #P6782, Sigma) or L012 (250 µM, #120-04891, Wako) was used and measured in a TriStar2 Multimode Reader (LB942, Berthold, Wildbad, Germany).

2.9. Western blot analysis

Triton-lyzed samples were substituted with sample buffer (8.5% glycercin, 2% SDS, 6.25% TRIS/HCl pH 6.8, 20 mM DTT, 0.01% bromphenol blue) but heat-denatured. As a reducing agent TCEP (tris[2-carboxyethyl]phosphine, Thermo Scientific) was used as described before [28]. Protein amounts were determined by Bradford protein assay. Proteins were separated by SDS–PAGE, substituted to Western analysis and detected by primary antibodies. Infrared-fluorescent-labeled secondary antibodies were used and visualized in the Odyssey system (Loric, Bad Homburg, Germany). Nox4 was detected by an anti-Nox4 antibody (1:2000) reported by Anilkumar et al. [3]. Primary antibodies against Nox1 (Nox1, #sc-5821, 1:500), Nox5 (#sc-67006, 1:500) and p22phox (8.5% glycerin, 2% SDS, 6.25% TRIS/HCl pH 6.8, 20 mM DTT, 0.01% bromphenol blue) but heat-denatured. As a reducing agent TCEP (tris[2-carboxyethyl]phosphine, Thermo Scientific) was used as described before [28]. Protein amounts were determined by Bradford protein assay. Proteins were separated by SDS–PAGE, substituted to Western analysis and detected by primary antibodies. Infrared-fluorescent-labeled secondary antibodies were used and visualized in the Odyssey system (Loric, Bad Homburg, Germany). Nox4 was detected by an anti-Nox4 antibody (1:2000) reported by Anilkumar et al. [3]. Primary antibodies against Nox1 (Nox1, #sc-5821, 1:500), Nox5 (#sc-67006, 1:500) and p22phox (#sc-20781, 1:500) were purchased from Santa Cruz, β-Actin (#A1978, 1:2000) was obtained from Sigma.

2.10. RNA isolation and RT-qPCR

RNA was isolated with the RNA Mini-Kit (#BS67311, Bio&Sell) according to the manufacturer’s instructions. Random hexamer primers (#C1181, Promega) and SuperScript III Reverse Transcriptase (RT, #18080044 ThermoFisher) were used for reverse transcription. A non-RT control was used. CDNA was used for semi-quantitative real-time PCR using iTQ™ SYBR® Green Supermix (#1708880, BioRad) in a Mx3000P qPCR cycler (Agilent Technologies). Relative mRNA expression was normalized to RNA Polymerase Ilia (POLR2A) and analyzed by the delta-delta-Ct method. Results are shown as relative to the corresponding control. Primer sequences are summarized in Table 1.

2.11. Statistical analysis

Values are mean ± SEM (standard error of mean) if not otherwise indicated. Statistical analysis was carried out using Prism 5.02. For multiple testing ANOVA followed by post hoc testing (Bonferroni’s Multiple Comparison Test) was used, unless otherwise indicated; n indicates the number of individual experiments. Densitometry was performed using the Odyssey-software (Image studio lite 5.0) and normalized to corresponding reference protein β-actin. A p-value of less than 0.05 was considered statistically significant (*p < 0.05; **p < 0.01; ***p < 0.001).

3. Results

3.1. CRISPR/Cas9-mediated knockout of CYBA/p22phox

To study the role of endogenous p22phox on Nox-dependent ROS formation, different guide RNAs (gRNAs) against the p22phox
gene CYBA were designed (Fig. 1A, B). Seven different gRNAs were transfected into stable Nox4- (Fig. 1C; Nox4-HEK293) or tetracycline-inducible Nox4-HEK293 cells (Fig. 1D; tetNox4-HEK293) and CRISPR/Cas9-mediated knockout was validated by Western Blot. Whereas single gRNA usage resulted in p22phox protein reduction, most of the gRNA combinations displayed loss of p22phox in both HEK293 cell types (Fig. 1B, C, D). Complete loss of p22phox was achieved with a combination of three gRNAs (Fig. 1B and C; combination 17) or all seven gRNAs (Fig. 1B and C; combination 18). For the subsequent experiments, the most effective combinations were used with cells targeted with two or three gRNAs with the combinations 1, 4, 7, 8, 10 and 17.

3.2. Knockout of endogenous p22phox reduces Nox4-dependent H2O2 production, but not Nox4 expression

To test the influence of CRISPR/Cas9-mediated p22phox knockout on Nox4-dependent ROS production, Nox4 constitutively overexpressing (Fig. 2 left) and induced tetNox4-overexpressing HEK293 cells (Fig. 2 right, tetracycline 1 μg/mL, 24 h) were studied in the luminol/HRP assay. P22phox and Nox4 mRNA as well as protein expression were studied in the cells subjected to ROS measurements (Fig. 2A, B, E, F, G, H). Deletion of p22phox resulted in a strong reduction of ROS production, and the extent of the effect correlated with the p22phox mRNA and protein expression but not with Nox4 mRNA or protein expression. Interestingly, the inhibitory effect on ROS formation appeared more prominent in the constitutively overexpressing than in the tet-inducible cells (Fig. 2C and D). Although deletion of p22phox lowered ROS formation, it certainly did not abolish it. This effect could potentially be a consequence of insufficient transfection efficiency.

3.3. Knockout of endogenous p22phox completely abolishes Nox4-dependent H2O2 production

To generate defined p22phox-deficient cells, Nox4- and tetNox4-HEK293 cells, harboring the most effectively reduced p22phox expression (gRNA combination 1 and combination 10), were clonally expanded. Multiple clones were tested on p22phox expression and H2O2 production and three of those were chosen for the subsequent studies. The three tested clones of either constitutive expressing Nox4-HEK293 cells (Fig. 3A) or tetracycline-induced tetNox4-HEK293 cells (Fig. 3B) displayed a complete loss of H2O2 production measured by luminol/HRP assay, in comparison to the empty vector control. Despite some unspecific bands in the Western blot, p22phox could not be detected in all three clonally expanded CRISPR/Cas9-knockout cell lines (C1 or C10). As the starting population of the clonal expansion was variable regarding Nox4 expression, Nox4 expression level also varied in the different p22phox-knockout cell lines. Hence, the H2O2 production is not dependent on Nox4 expression as seen in the Western blot, but on p22phox expression only. The clones C1-1 and C10-1 were used for all following experiments.
3.4. ROS production after p22phox knockout can be restored with p22phox, but not with DUOXA1 or DUOXA2

The clonally expanded p22phox knockout cells are an ideal tool for reconstitution experiments. Transfection of the cells with plasmids coding for human p22phox (hp22) as well as rat p22phox (rp22) restored the ROS production in Nox4-HEK293 cells as well as tetNox4-HEK293 cells (Fig. 4A and B). To test the hypothesis that DUOX1/2 maturation factors may substitute p22phox, DUOX1 (DA1) and/or DUOX2 (DA2) were also transfected. Importantly, the DUOX maturation factors did not affect ROS formation (Fig. 4A and B) and therefore cannot substitute p22phox.

3.5. P22phox is required for Nox1-, but not Nox5-dependent O$_2^{**}$ production

To test whether other Nox isoforms are also influenced by the knockout of endogenous p22phox, p22phox deficient cells were transiently transfected with either a NoxO1-NoxA1-Nox1 expressing plasmid or with a Nox5 plasmid. Despite knockout of p22phox, Nox5 transfected cells exhibited PMA (phorbol 12-myristate 13-acetate; 100 nM)-stimulated ROS production as detected by L012 chemiluminescence (Fig. 5A, CYBA KO1&2). A similar effect was observed in stably transduced Nox5-HEK293 cells subjected to CRISPR/Cas9 p22phox knockout with different
combinations of gRNAs (1, 4, 7, 8, 10, 17) (Fig. 5B). In contrast, p22phox deficiency completely abolished Nox1-dependent O2− production (Fig. 5C, CYBA KO1&2), whereas Nox1 expression was not changed (Fig. 5C). Thus, Nox5 but not Nox1-mediated ROS is independent of p22phox in contrast to Nox1 or Nox4.

**3.6. Analysis of p22phox mutants and their effect on Nox4-dependent ROS production**

To gain insights into the function of p22phox in Nox4-dependent ROS production, mutations were studied. First, C-terminal truncations were analyzed (Fig. 6A and B). Truncation beyond Q130 resulted in a complete loss of enzyme activity, whereas the protein truncated at Q130 still showed some ROS activity. Interestingly, this effect was more pronounced in the tetNox4-HEK293 cells as compared to the Nox4 stably expressing cells, potentially suggesting that the dynamic of Nox4 protein formation is relevant for this mutation. Importantly, these observations are in stark contrast to a previous publication, which reported that a p22phox-Q130 truncation was fully active [17]. Truncations at aa90, aa91 or aa110 were indispensable for Nox4 activity (Fig. 6A and B).

As several CGD patients carry the p22phox missense mutation R90, we also tested a R90Q mutant [27] on its effect on Nox4 (Fig. 6C and D). The mutation not only leads to a loss of Nox4-dependent H2O2 formation, it also exhibited reduced p22phox protein expression. Mutations of negatively charged amino acids at the beginning of the N-terminus (E5A, E12A) or aliphatic amino acid close to the predicted Nox1/2-p22phox interaction site (I120C) had no influence on Nox4-dependent ROS production (Fig. 6C and D). Interestingly, the Y121H mutation, previously described by Banfi et al. in Cyba-deficient mice [22] only resulted in ROS formation in the tetracycline-induced Nox4-HEK293 cells, whereas in the constitutive Nox4-HEK293 cells only a minimal effect on ROS production was achieved. None of the mutants had a dominant negative effect. I.e. transfection of the p22phox mutants into HEK cells overexpressing Nox4 but expressing endogenous p22phox had only a very minor effect on ROS formation (Sup. Fig. 1).

These results highlight the importance of R90, the transmembrane region aa90-130 and also indicate importance for Y121 and aa130-195 for Nox4 activity.

**4. Discussion**

In this study using CRISPR/Cas9-mediated knockout cell lines we demonstrate that p22phox deficit completely abolishes Nox1 and Nox4 but not Nox5-dependent ROS production. In contrast,
expression levels of these Nox enzymes were independent of 
p22phox. The effect of the knockouts could be rescued with either 
human or rat p22phox, but not with the DUOX-maturation factors 
DUOXA1/A2. Truncation of p22phox till the amino acid 130 was 
without a functional consequence, while further truncation leads 
to a reduced function of the protein. Exchange of conserved ne-
gatively charged amino acids at the very N-terminus did not im-
pact on Nox4-dependent ROS formation, whereas the R90Q mu-
 tant was not able to produce significant amounts of ROS. Sur-
prisingly, the C-Terminus of p22phox was just dispensable in tet-
racycline-induced HEK293 cells. In constitutive Nox4-expressing 
cells, truncation of p22phox with Q130stop could not rescue the 
p22phox knockout. The same was observed for Y121H p22phox, 
which just restored the ROS producing capacity of tetracycline-
induced Nox4. This might suggest that these parts of the protein 
are important during the de novo formation of the Nox-p22phox 
complex.

The functional interaction of the catalytically active Nox enzyme 
with p22phox on ROS formation is in line with previous work 
employing p22phox shRNA, siRNA or overexpression of Nox and 
p22phox [16,17,19]. The rescue with either truncated Q130stop or 
Y121H p22phox was described for co-overexpression of p22phox 
and Nox4 [17]. This could be confirmed with the tetracycline-
ducible Nox4 expression 24 h after the transfection with p22phox. 
Therefore, Nox4 is transcribed, translated and matured after the 
p22phox Q130stop or Y121H p22phox overexpression. In the con-
stitutively expressing Nox4-HEK293 cells, Nox4 is already expressed 
in the absence of p22phox. Our data therefore suggest an involve-
ment of the C-terminal part of p22phox in the maturation of the 
p22phox-Nox4 interaction. Since Nox4 is not dependent on cytosolic 
subunits to form a functional Nox complex the role of p22phox is 
rather stabilization then activation[9,23,29]. Nox1, Nox2 and Nox3 
mattruion and glycosylation have also been described to be 
p22phox-dependent, but these isoforms are furthermore dependent 
ion the direct activation by p22phox by the prolin-rich region in the 
C-terminus of p22phox [20,21]. This region is dispensable for Nox4 
activation as shown in the tetNox4-HEKs and co-expression studies 
by others [16,17].

Fig. 5. Nox1- or Nox5-dependent O2•− production in p22phox-CRISPR/Cas9 knockout HEK293 cells. A, p22phox-knockout HEK293 cells (KO) or control cells (Vec) were transiently transfected with control (Ctl) or human Nox5 overexpression (OE) plasmid and treated with PMA (100 nM, 30 min). Relative L012 assay of the transiently 
transfected cells was performed. Also shown are representative Western blots for p22phox, Nox5 and β-Actin. B, Stably transduced Nox5-HEK293 cells were treated with empty vector (Vec) or different gRNAs combinations targeting p22phox (combi. 1, 4, 7, 8, 10, 17) and treated with PMA (100 nM, 30 min). Relative L012 assay was performed. Normalized, relative Western Blot for Nox5 and mRNA expression of p22phox (CYBA) are shown. C, p22phox-deficient HEK293 cells (KO) or control cells (Vec) were transiently transfected with human NoxO1-NoxA1-Nox1 triple overexpression plasmid (OE) and treated with PMA (100 nM, 30 min). Relative L012 assay was performed. n ≥ 3, mean ± SEM, ***p < 0.001.
Zhu et al. described the dependence of Nox2 maturation on amino acids 6-142 [31]. Therefore we propose that also Nox4 maturation is dependent on the amino acids 6-130. In line with this, deletion of the first five amino acids, but not 11 amino acids, is tolerated by Nox4 as shown in a previous study [17]. Controversially, a peptide targeting the amino acids 6-11 (WAMWAN) is tolerated by Nox4 as shown in a previous study [17]. Furthermore, it was also reported that the D-Loop of Nox4 is responsible for the non-p22phox. We demonstrated that rapid deletion of p22phox is possible and that the activity of Nox1 and Nox4 but not Nox5 exclusively depends on p22phox.

**Conflict of interest**

The authors declare that they have no relevant financial, personal or professional relationships to disclose which could be perceived as a conflict of interest or as potentially influencing or biasing the authors’ work.

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**Appendix A. Supplementary information**

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2016.08.013.

**References**


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**Fig. 6.** Nox4-dependent H2O2 production with p22phox mutants in p22phox-CRISPR/Cas9 knockout HEK293 cells. Either subclonally expanded p22phox knockout (KO) Nox4-HEK293 (C1-1 and C10-1; A and C) or induced tetNox4-HEK293 cells (C1-1 and C10-1; B and D) were transiently transfected (overexpression: OE) with full-length human (hp22): truncated (; stop (A and B)) or point mutated versions of p22phox (C and D). Relative luminol/HRP assay in Nox4-HEK293 (A and C) or induced tetNox4-HEK293 cells (B and D) was performed. n = 6, mean ± SEM. **p < 0.01; ***p < 0.001, relative to the corresponding p22phox (hp22) transfected cells.


