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Identification of NOX2 regions for normal biosynthesis of cytochrome *b*₅₅₈ in phagocytes - Highlighting essential residues for p22^{phox} binding

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ABSTRACT

Cytochrome *b*₅₅₈, the redox core of the NADPH-oxidase complex in phagocytes, is composed of NOX2 and p22^{phox} whose synthesis are intimately connected but not fully understood. We reproduced ten rare Xminus chronic granulomatous disease mutations of highly conserved residues in NOX1–4, in the X⁰CGD PLB-985 cells in order to analyse their impacts on the synthesis process of cytochrome *b*₅₅₈. According to the impact of these mutations on the level of NOX2 expression and activity, mutants were categorized in group A (W18C, E309K, K315del, I325F) characterized by a linear relationship between NOX2 expression and the NADPH oxidase activity and group B (H338Y, P339H, G389A and F656-F570del) showing an absence of NADPH oxidase activity associated with variable levels of NOX2 expression. These last residues belong to the FAD-binding pocket of NOX2, suggesting this functional domain plays a role also in the structural integrity of NOX2. Finally, we observed an abnormal accumulation of p65-kDa, the NOX2 precursor and a p65-kDa/p22^{phox} dissociation in the W18C, E309K, I325F and G389A mutants, pointing out a possible role of the first transmembrane domain (W18) and the region between the membrane and the dehydrogenase domain of NOX2 (E309, I325, G389), in the binding with p22^{phox}.

Keywords: cytochrome *b*₅₅₈; NOX2; precursor p65-kDa; biosynthesis; X^{minus} CGD; p22^{phox}

Summary statement: X⁰CGD mutant analysis provides the first indications on NOX2 regions essential for the normal cytochrome *b*₅₅₈ synthesis in phagocytes; the FAD-binding pocket, the first transmembrane passage and a cytosolic region located between the membrane and the dehydrogenase domain of NOX2.

Abbreviations used : NOX, NADPH oxidase; ROS, reactive oxygen species; CGD, chronic granulomatous disease; PMA, Phorbol 12-myristate 13-acetate; fMLP, formyl-methionylleucyl-phenylalanine; Cyt*b*₅₅₈, cytochrome *b*₅₅₈; DMF, dimethylformamide; DFP, diisopropylfluorophosphate; DPI, diphenylene iodonium; INT, iodonitrotetrazolium, SOD, superoxide dismutase; O₂⁻, superoxide; G6PD, Glucose 6 Phosphate Dehydrogenase.

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INTRODUCTION

Phagocytes, essentially monocytes, macrophages and neutrophils, exert the crucial physiological function of ingesting foreign bodies, including microbes, and cellular debris. Although phagocytes have other microbicidal mechanisms including the secretion of antimicrobial peptides (e.g. defensins) and broadly acting proteases, phagocytosis, with the generation of reactive oxygen species (ROS) by the NADPH oxidase complex, is still regarded as the critical mechanism for killing most invading pathogens. Indeed, the phagocyte NADPH oxidase plays a crucial role in the host's defense against invading microorganisms by catalyzing the formation of superoxide (O₂⁻), which is the precursor of a variety of microbicidal oxidants such as hydrogen peroxide (H₂O₂) (1). The importance of this enzyme in innate immunity and inflammation is illustrated by chronic granulomatous disease

(CGD), a rare inherited syndrome (frequency, about 1/200 000) characterized by the absence of NADPH oxidase activity, severe and recurrent infections in childhood and chronic inflammatory granuloma (2). This enzyme is dormant in resting cells but becomes activated during phagocytosis to produce superoxide. Indeed its activity is subtly controlled by a dynamic assembly process involving several cytosolic proteins (p40^{phox}, p47^{phox}, p67^{phox}, Rac1/2) and a membrane partner which is the catalytic component of this protein complex, the flavocytochrome *b*₅₅₈ (*cytb*₅₅₈). The *cytb*₅₅₈ is composed of a small subunit, p22^{phox}, and a larger one, the glycosylated flavocytochrome gp91^{phox} or NOX2, closely associated in the plasma membrane or in the membranes of specific granules (3). NOX2 is the redox element of the NADPH oxidase complex containing hemes (4) and flavin centers that mediate electron transfer from NADPH to molecular oxygen, ultimately resulting in the production of oxidizing species involved in the destruction of phagocytosed microorganisms. In the last decade, homologues of NOX2 have been identified, including four additional proteins (NOX1, NOX3, NOX4 and NOX5) and two dual oxidases (DUOX1 and DUOX2) (5). These homologues all share a common structure with NOX2. Although NOX2 has, as yet, never been crystallized, some information about its function is available (6). The N-terminus of NOX2 is intracellular (7) and is followed by six membrane-spanning domains that are potential α -helices, which contain two heme groups ligated by four histidine residues in the IIIrd and Vth transmembrane passages (8), three glycosylated asparagines in two external loops C and E (9), and the B and D intracellular loops.

Nevertheless, the regions of NOX2 that are important for its synthesis and stability have not been determined. Indeed, the synthesis of *cytb*₅₅₈ is a complex process because it involves not only the presence of both subunits, NOX2 and p22^{phox}, but also several maturation steps, including heme incorporation, heterodimer formation and the glycosylation of NOX2 (10,11,12). Moreover, in primary cells, the heterodimerization, translocation and enzymatic function of NOX2 but also of NOX1,3 and 4, require the presence of p22^{phox}, NOX5 being an exception (Bedard, 2007 #14). Previous studies showed that NOX2 is cotranslationally glycosylated and first detected as a high mannose 65-kDa (p65) monomer in the endoplasmic reticulum (ER) even in the absence of p22^{phox}. Full maturation of NOX2 requires sequential incorporation of heme into p65, heterodimerization with p22^{phox} occurring in the late ER step and final N-glycosylation in the Golgi, which drives the transport of the complex to the plasma membrane and into specific granules in neutrophils (13). However, heme insertion is decisive for the stable formation of the heterodimer, whereas glycosylation is not. The association of NOXs with p22^{phox} seems to be a prerequisite for the localization of the heterodimer to specific membrane compartments such as the perinuclear vesicles for NOX4 and plasma membranes in the case of NOX1, 2 and 3 [16]. The importance of some sequences of p22^{phox} for its interaction with NOXs has been highly debated (14-17). However, the binding regions of NOX2 that interact with p22^{phox} are not yet known.

One way to decipher the molecular mechanism of *cytb*₅₅₈ synthesis, and to identify decisive regions of NOX2 involved in its maturation, is to study this process in special X-linked chronic granulomatous (CGD) disease variants called Xminus (X⁻). X⁻-CGD mutants are characterized by a partial defect of *cytb*₅₅₈ synthesis associated with no or diminished oxidase activity. These mutants have helped to highlight regions of NOX2 essential for its stable synthesis. The genetic defects in *CYBB* found in these variants are often missense mutations or small deletions located in the coding region (18). The clinical severity of this disease in patients is variable and is probably related to the residual oxidase activity found in the patients' X⁻-CGD neutrophils. Indeed, X⁻-CGD mutations could have a different impact and act at different steps of the *cytb*₅₅₈ synthesis process, and probably affect this synthesis differently. When we started this project, a few X⁻-CGD mutations were listed in the literature (19). The level of NOX2 or *cytb*₅₅₈ expression and the NADPH oxidase activity were reported in half of these mutations (H101Y, A156T, S193F, C244S, C244Y, E309K, K315del, H338Y, P339H, G389A, V534_G538del and F565_F570del) in neutrophils or in Epstein-Barr immortalized B lymphocytes (EBV-LB) from CGD patients (20-29). Others were documented only in reviews which list cases of X-linked CGD (W18C, A53D, C59F, K161R, I187_I189del, I325F and V534_G538del) (19,30-35). The impact of these mutations at the molecular level cannot be studied because of the limitation in the amount of biological material. The molecular mechanism of the defect of *cytb*₅₅₈ synthesis has been highlighted in only two of them (23) (36) (37). The H338Y mutation leads to a defect in FAD incorporation in neutrophils of the X⁻-CGD patient, highlighting the importance of the ³³⁸HPFT³⁴¹ motif of NOX2 in the binding of the isoalloxazine ring of FAD and its

importance in NOX2 stability. In addition, this mutation leads to ER retention related to a partial defect of NOX2 maturation (38). The defect in *cytb*₅₅₈ synthesis caused by the H101Y mutation was also explained by the absence of heme incorporation in NOX2 because this histidine is one of the four that coordinate hemes in NOX2 (24). Thus this mutation probably prevents NOX2 and p22^{phox} interaction, stopping *cytb* synthesis. However, this was not demonstrated in the X⁻CGD patient neutrophils initially published.

Despite recent progress, additional work is needed to improve our understanding of the overall domain structure of NOX2 involved in *cytb*₅₅₈ biosynthesis, most particularly the regions involved in p22^{phox} binding. Therefore, we decided to study the functional and molecular impact of certain X⁻CGD mutations that have never been or only partially characterized using transgenic PLB-985 cells mimicking X⁻CGD neutrophils. A better knowledge of the *cytb*₅₅₈ synthesis process and its relationship with the level of NADPH oxidase activity will help us to better understand the variability of the clinical severity of X⁻CGD variants.

MATERIALS AND METHODS

Materials

The materials used were: Phorbol 12-myristate 13-acetate (PMA), dimethylformamide (DMF), diisopropyl-fluorophosphate (DFP), actinomycin D, pepstatin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and Hoechst 33258 (Sigma Chemical Co); Foetal bovine serum, RPMI 1640, Alexa Fluor 488 goat-F(ab')₂ fragment anti-mouse IgG1 (H+L) (ref A21121) and Cyanin5 goat-F(ab')₂ fragment anti-rabbit IgG (H+L) (ref A10523) (Life Technologies); 7D5 (ref D162-3, Clinisciences); Anti-Lamin B1 (ref Ab 16048), Anti-GM130 (ref Ab 52649), Anti-Calnexin antibodies (ref Ab 22595, Abcam); anti-NOX2 54.1 (ref sc-130543), goat anti-mouse IgG-HRP, monoclonal antibody CS9 (anti p22^{phox}) (sc-130551, Santa Cruz Biotechnology); monoclonal antibodies 48 (anti-NOX2, MW1842) and 449 (anti-p22^{phox}, MW1843) (Sanquin Research at CLB Amsterdam); Geneticin (GE Healthcare); Endofree plasmid Maxi Kit, QIAprep Spin Miniprep Kit (Qiagen); The Sephaglas kit and molecular weight markers (Page Ruler®) (Amersham) and (Fermentas); Nitrocellulose sheets for Western blotting (Bio-Rad).

Site-directed mutagenesis and expression of recombinant NOX2 in the X⁻CGD PLB-985 cell line

Site-directed mutagenesis was performed with the Quick ChangeTM Mutagenesis Kit (Stratagene) according to the manufacturer's instructions, using two sets of complementary oligonucleotides harbouring the desired mutation. DNA sequences were verified by DNA sequencing on both strands using the Big Dye Kit (Eurofins). The wild type or mutated gp91^{phox} cDNA were then sub-cloned into the mammalian expression vector pEF-PGKneo (39). The X⁰-CGD PLB-985 cell line (lacking NOX2 expression and NADPH oxidase activity) (40) was transfected with the pEF-PGKneo constructs by nucleofection (Amaxa) and clones were selected by limiting dilution.

Cell culture and granulocyte differentiation

Transfected PLB-985 cell lines were grown in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-Glutamine at 37°C in a humidified 5% CO₂ atmosphere. Geneticin (0.5 mg/mL) was added to maintain the selection pressure of the plasmid in the transfected cells. For granulocytic differentiation, PLB-985 cells (2×10⁵ cells/mL) were exposed to 0.5% (v/v) dimethylformamide for 6 days (41). Geneticin (0.5 mg/mL) was added to maintain the selection pressure in the transfected cells.

Analysis of recombinant NOX2 expression in PLB-985 cell lines

NOX2 expression was analysed using flow cytometry and immunoblot in transfected PLB-985 cell lines as described previously (42). 5.0×10⁵ PLB-985 cells labelled with 5 µg/ml of monoclonal 7D5 or irrelevant IgG1 and then incubated with phycoerythrin-labelled goat-F(ab')₂ fragment anti-mouse IgG (Beckman Coulter) were analysed by Flow cytometry with a FACSCanto II (BD Biosciences) [42]. We detected 15–20 different clones for each mutant, and to minimize clone-to-clone variation in NOX2 expression, two to three independent clones of each X⁻CGD mutant were used for subsequent analysis (data not shown). The purity of the clonal population was higher than 97%. Expression of

NOX2, its precursor p65-kDa, p22^{phox} and p47^{phox} were examined by Western blot analysis (43,44) using monoclonal antibodies 48, 449 and anti-p47^{phox} (ref 610354 BD Biosciences) respectively (45,46). Polyclonal goat anti-mouse IgG-HRP was used as a second antibody and the immune complexes was detected by chemiluminescence using an ECL kit Fentomax (Rockland Immunochemicals).

Measurement of ROS production by chemiluminescence

ROS production by intact differentiated PLB-985 cells (5×10^5 cells) in PBS containing 0.9 mM CaCl₂, 0.5 mM MgCl₂, 20 mM glucose, 20 μ M luminol, and 10 U/mL HRPO was measured after PMA stimulation (77 ng/mL). Relative light units (RLUs) were recorded at 37°C over a time course of 60 min in a Mithras LB 940 (Berthold Technologies) (42).

Analysis of NOX2/p65-kDa location in subcellular compartments of X⁻CGD PLB-985 cells by confocal microscopy

The differentiated PLB-985 cells (5×10^5) were deposited on coverslips at 37°C for 15 min then fixed in 4% formaldehyde for 10 min and permeabilized with 0.25% Triton X 100. After extensive washing, cells were incubated for 1 h at 37°C with diluted 54.1 anti-NOX2 monoclonal antibody, and the rabbit polyclonal antibodies directed against calnexin in the endoplasmic reticulum, the rabbit monoclonal directed against GM-130 in the Golgi or the rabbit polyclonal directed against lamin B1 in the nuclear membrane. All primary antibodies were diluted at 1/100 and secondary antibodies at 1/200. After washing, cells were incubated in 5% BSA/PBS buffer containing 2 μ g/mL Alexa Fluor 488 F(ab')₂ fragments of goat anti-mouse IgG (H+L) and cyanin5 F(ab')₂ fragments of goat anti-rabbit IgG (H+L) used as second antibodies. Cellular nuclei were visualized using Hoechst 33258 staining. Cells were examined with a confocal laser scanning microscope and analysed using Leica confocal software (42).

Preparation of the Triton X100 soluble extract from X⁻CGD PLB-985 cell lines

Differentiated PLB-985 cells were harvested by centrifugation (1000 *g* for 10 min at room temperature) and treated with 3 mM diisopropyl-fluorophosphate (DFP) for 15 min on ice. After centrifugation, the cells were resuspended at 1×10^8 cells/mL in the lysis buffer A (1% Triton X100 (v/v), HEPES 10 mM, MgCl₂ 3.5 mM, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 μ M leupeptin and 4 μ M pepstatin) for 30 min on ice (47). After centrifugation, the soluble extract was recovered in the supernatant and the protein content was estimated using the BCA Protein Assay (Interchim).

Co-immunoprecipitation of p65-kDa and p22^{phox} from X⁻CGD PLB-985 membrane fractions

Soluble extract (SE) (100 μ g) was incubated with mAbs CS9 directed against p22^{phox} (2 μ g) in 500 μ L of buffer A for 30 min on ice. We added 100 μ L of μ MACS protein G MicroBeads (Miltenyi Biotec, Paris, France) and incubated the solution for 30 min on ice. The sample was loaded onto a MACS Column placed in the magnetic field of the μ MACS Separator. The magnetically labelled antibody–protein complex and associated molecules were retained on the column and the flow-through (–) was recovered. After washing, 50 μ L pre-heated (95°C) gel loading buffer (50 mM Tris HCl (pH 6.8), 50 mM DTT, 1% SDS, 0.005% bromophenol blue, 10% glycerol) were loaded onto the column matrix for elution of the (co-)immunoprecipitated proteins as the positive fraction (+), which was recovered. The protein content of flow-through (–), positive and initial soluble extract fractions (100 μ g of proteins in each sample), was assessed by immunoblot analysis as described above. IgG1 antibodies were used as irrelevant antibodies.

Protein determination

Protein content was estimated by the Pierce method using a Bio-Rad protein-assay kit II and bovine serum albumin as standard (48).

cDNA generation by reverse transcription amplified by PCR (RT-PCR)

Total RNA was isolated from cells using a modified single-step method (49),(50). cDNA was synthesized from 5 μ g of total RNA by the reverse transcriptase reaction according to the manufacturer's instructions (QBiogen, Montréal, Canada). NOX2 or p22^{phox} cDNA were immediately amplified by the polymerase chain reaction (PCR) using specific primers (27). RT-PCR

was performed with geneticin primers as a control for RNA quality and equal loading. Aliquots of 5 μ L PCR products in bromophenol blue solution were run together with a DNA ladder (markers XIV, Roche Diagnostics, Indianapolis, IN, USA) on 1% (wt/vol) agarose. The bands were photographed under UV light (Gel Doc XR⁺, Bio-Rad).

Quantitative PCR

Real-time ready assays were conducted with the LightCycler® 2.0 Instrument according to the manufacturer's instructions (Roche). Total RNA was extracted from differentiated X⁻CGD PLB-985 cells treated or not with actinomycin D 5 μ g/mL for 4 h as previously described (51). qPCR reactions were performed using Faststart Taqman probe master 1x (Roche, ref 4707494001) in 20 μ L reaction volume with 10 ng cDNA/RNA, specific primers forward and reverse (500 nM each) of NOX2 and the reference gene (*G6PD*). Specific probes (250 nM each) of NOX2 and G6PD were labeled with FAM and Yellow 555, respectively. Reaction conditions were: +95°C for 10 min, 40 cycles of +95°C for 10 s, +59°C for 30 s (with single acquisition) followed by +40°C for 30 s final cooling. Color compensation was applied between specific emission of FAM (530 nm) and Yellow 555 (560 nm). Relative gene expression was calculated according to the Δ DCt method¹² using the LightCycler® 2.0 Software with normalization to the G6PD RNA level as the endogenous reference. Normalized values represent average \pm SD of at least four independent experiments.

RESULTS

Position and conservation of X⁻CGD mutated amino acids in the NOXs

Approximately 30 mutations leading to X⁻CGD cases, characterized by a lower level of *cytb*₅₅₈, have been described in the literature to date (see Roos et al. [35] for a review). As can be seen in Figure 1A according to their location in the NOX2 protein, four distinct regions are hot-spots; the second intracellular loop D, a cytosolic region close to the last transmembrane passage VI, the FAD-binding site and the extreme N terminal region of NOX2. We concluded that these regions were probably highly involved in the stability or the synthesis/maturation process of *cytb*₅₅₈. However until now, the majority of these mutations have been classified as X⁻CGD mutations on the grounds of the NADPH oxidase activity and *cytb*₅₅₈ expression in the patients' neutrophils, or their inclusion as X⁻CGD in reviews without any other information. Therefore, we reproduced some X⁻CGD mutations within these regions (I187_I189del, E309K, K315del, I325F, H338Y, P339H, F565_F570del) and in isolated regions (W18C and G389A) in the X⁰CGD PLB-985 cell line, in order to decipher the role played by these residues in the synthesis process of *cytb*₅₅₈. The H101Y mutation, which is one of the binding His of the hemes, was also reproduced because it serves as a control of the heme incorporation defect leading to the absence of NADPH oxidase activity and probably binding of its precursor p65kDa with p22^{phox}, as was demonstrated previously by treatment with a heme synthesis inhibitor [28]. The orientations of the mutations were also chosen because of the high conservation of these mutated residues in NOX1 to NOX4 (Figure 1B), suggesting a common and important role, possibly in the interaction with p22^{phox}.

Characterization of the NOX2 expression in the X⁻CGD PLB-985 mutant cells

The first challenge was to exactly reproduce the phenotype of X⁻CGD human neutrophils in the transfected X⁰CGD PLB-985 cell line. To be sure that the defect of NOX2 expression in X⁻CGD mutant cells was not due to a defective transfection, mutated NOX2 mRNA was quantified by RT-PCR by comparing it with the amplification of the geneticin resistance gene located in the plasmid used to clone the mutated NOX2 cDNAs (Figure 2A). A nearly equivalent level of NOX2 mRNA was transcribed in mutant PLB-985 cells. This confirmed the correct transfection and transcription of the mutated cDNAs NOX2 and p22^{phox} mRNA. To confirm these previous data and to test NOX2 mRNA stability, we used real-time PCR to measure the amount of NOX2 mRNA of the mutant PLB-985 cells before and after treatment with actinomycin D 5 μ g/mL for 4 h (Figure 2B). We also observed that all the mutants except H101Y, I325F and P339H have 50% or less steady state mRNA levels compared with WT-NOX2. While this likely cannot explain the greater decreases in cell surface protein, it could contribute to it. However we found that the values for the transfected PLB-985 cells (WT-NOX2 or

mutated NOX2 cDNA) were always higher than the values obtained for the human neutrophils (PMN) and the WT PLB-985 cells. In addition, no difference between group A and group B mutants (as classified in Table 1) was observed. The actinomycin treatment slightly affected the level of mRNAs, suggesting good stability of the mutated NOX2 mRNA. These results suggest that X⁻CGD mutations do not affect the transcription step drastically but they could probably influence later stages during protein synthesis.

NOX2 expression at the plasma membrane of undifferentiated and differentiated PLB-985 cells was checked by flow cytometry using the 7D5 monoclonal antibody. A striking point was that the membrane NOX2 expression diminished in differentiated mutant PLB-cells compared to undifferentiated mutant PLB-985 cells (Figure 3A). The entire human CGD X⁻-mutations were reproduced in the PLB-985 cell model except the I187_I189del where no NOX2 expression was observed as an X⁰-CGD phenotype. In order to analyse the total NOX2 expression of the mutant cells (at the plasma membrane plus in intracellular compartments), Western blot analysis was performed from each mutant cell line (Figure 3B). This method makes it possible to analyse not only the total NOX2 expressed in the cells, but also its precursor of p65-kDa and p22^{phox} to be visualized. However the levels of proteins' expression seen in the western blot of undifferentiated and differentiated cells, cannot be compared because both results from unequal times of exposure. P47^{phox} was revealed in order to normalize the quantification of protein expression. The level of NOX2 expression was variable in all the mutants and corresponds to what we found with flow cytometry except for the H338Y and P339H mutants (Table 1, Figure 3B). Indeed the total amount of NOX2 assessed from Western blot (46.4% and 38% of the control cells, respectively) was considerably higher than those measured by flow cytometry (10% and 5% of the control cells, respectively). This suggests an intracellular location of NOX2. In addition, the level of p22^{phox} expression seems to mimic the level of NOX2 when it is expressed. In some mutants (W18C, E309K, I325F, E389) there is an accumulation of the precursor p65-kDa (Table 1, Figure 3B) and the ratio p65/NOX2 is higher than in WT NOX2 cells (5, 6, 18 and 27, respectively) (Table 1).

NADPH oxidase activity in X⁻CGD PLB-985 mutant cells

The NADPH oxidase activity was measured in all X⁻-CGD mutant PLB-985 cells using chemiluminescence in the presence of luminol and HRPO as described in the Material and Methods section. As seen in Table 1, in group A mutants there was a faint residual NADPH oxidase activity (W18C, E309K, K315del, I325F), whereas in group B mutants the NADPH oxidase activity was totally abolished (H338Y, P339H, G389A and F565_F570del). In addition, in group A mutants, the level of NOX2 expressed at the plasma membrane measured by flow cytometry was directly proportional to the level of NADPH oxidase activity (Table 1). This suggests that these X⁻-CGD mutations lead to a defect in the efficiency of the *cytb*₅₅₈ synthesis process only and do not affect the intrinsic NADPH oxidase activity. However, in the group B mutants, the NADPH oxidase activity was null, whereas NOX2 was expressed at the plasma membrane at levels between 2 and 12% of that seen in controls. In this case, the X⁻-CGD mutations affecting not only *cytb*₅₅₈ synthesis, but also the NADPH oxidase activity point to functional regions of NOX2 that are essential for the stable conformation of the protein.

Cellular location of NOX2 and p65-kDa in X⁻CGD PLB-985 mutant cells

In mutant cells characterized by an accumulation of the precursor p65-kDa (W18C, E309K, I325F and G389A) or of NOX2 in intracellular compartments (H338Y, P339H), we analysed the location of both proteins by conducting a co-staining experiment and confocal microscopy using antibodies directed against calnexin, an endoplasmic reticulum marker, GM130, a Golgi apparatus marker and lamin B1, a nuclear membrane marker. We used the 54.1 monoclonal antibody, which can recognize both p65-kDa and NOX2.

In the Mock-X⁰CGD PLB-985 cells, no NOX2 was expressed, but calnexin delimited the endoplasmic reticulum (Figure 4A). In WT-NOX2 PLB-985 cells, NOX2 was mainly localized at the plasma membrane (Figure 4A) and there was no colocalization of NOX2 and calnexin, as suggested by the absence of overlapping between the clouds of green (NOX2 or p65-kDa) and red (calnexin fluorescence (open angle in both clouds) assessed by the Leica Instruments colocalization software (Figure 4B). In the H101Y mutant considered as an X⁰-CGD phenotype (no NOX2 expression and no

oxidase activity), the 54.1 antibody revealed the presence of the precursor p65-kDa that accumulates in the ER or in compartments close to it because of a yellow fluorescence of the merge (Figure 4A). Colocalization was evidenced in some cells, as seen in Figure 4B. In the group A mutants with an abnormal p65/NOX2 ratio (W18C, E309K and I325F), a yellow merge was seen inside the cells, as in the H101Y mutant (Figure 4A). Using the colocalization program, we showed that part of the cells coexpressed p65-kDa and calnexin in the ER (yellow cells in the quarter). In addition, both green (p65-kDa) and red (calnexin) clouds moved closer (more closed angle in both clouds) compared to the WT-NOX2 cells. As we demonstrated above, the H338Y and P339H mutants are characterized by a normal p65/NOX2 ratio (i.e., no accumulation of the p65-kDa precursor in subcellular compartments); the co-location of NOX2/p65-kDa with the ER was less evident than for the previous mutants (Figure 4B). In addition, as in the WT-NOX2 PLB-985 cells, a green fluorescence can be clearly seen over the yellow merge, suggesting NOX2 expression at the plasma membrane (Figure 4A). Finally, for the G189A mutant, p65-kDa is mainly in intracellular compartments, and the analysis of the colocalization is difficult because of the very low expression of NOX2 and p65-kDa. Neither NOX2 nor p65-kDa colocalizes with the Golgi apparatus or the nuclear membrane in the mutants analyzed (Supplementary Figures S1 and S2).

Study of the interaction between the precursor p65-kDa and p22^{phox} in X⁻CGD PLB-985 mutants with an abnormal p65/NOX2 ratio

In order to determine if the defective synthesis of *cytb*₅₅₈ in the mutants with an abnormal accumulation of the p65-kDa precursor in intracellular compartments was caused by faulty p22^{phox} and p65-kDa assembly, we performed co-immunoprecipitation experiments from X⁻CGD mutant cells. The same amount of proteins (100 µg) in each lane was analysed using SDS-PAGE and Western blot. Following immunoprecipitation of membrane proteins with CS9 (an antibody against p22^{phox}) in the MACS column, the p65/p22^{phox} complex was eluted by the SDS-PAGE sample buffer (positive fraction) in the case of the WT NOX2 PLB-985 cells (Figure 5). However, the surplus of the p65/p22^{phox} complex was recovered in the flow-through (negative fraction). Indeed, we adapted the quantity of CS9 antibody against p22^{phox} to trap the amount of p65-kDa of the mutants but this quantity may not be sufficient to trap the p65-kDa/p22^{phox} complex of the WT-NOX2 PLB-985 cells. IgG1 antibodies as irrelevant antibodies demonstrated the specificity of the co-immunoprecipitation in WT NOX2 PLB-985 cells. We also analyzed the mutant H101Y PLB-985 cell lines as a control as follows. The H101Y mutation is assumed to disturb *cytb*₅₅₈ synthesis because it impairs heme incorporation in NOX2 leading to a lack of p65-kDa and p22^{phox} binding, as demonstrated previously (13). Indeed, we found a total dissociation of p65-kDa, eluted in the unbound fraction, whereas p22^{phox} was eluted with the SDS-PAGE sample buffer (Figure 5). In the group A X⁻CGD mutants and in the G389A mutant, the totality of p65-kDa was eluted in the flow-through fraction, whereas most of the p22^{phox} was eluted with the sample buffer for SDS-PAGE. We conclude that the W18C, H101Y, E309K, I325F, and G389A mutations prevent p65/p22^{phox} binding. For group B mutants (H338Y, P339H), whereas some p65-kDa is eluted directly in the flow-through, probably because of a nonspecific effect of detergent treatment (1% Triton X100). In addition, we cannot exclude that part of p65-kDa may be unbound in the cells. However, part of the precursor p65-kDa is eluted with p22^{phox} in the positive fraction (SDS-PAGE sample buffer). Thus the defect of *cytb*₅₅₈ synthesis in the H338Y and P339H mutants probably stems from another cause than a defect of p65-kDa/p22^{phox} binding. This point will be discussed further.

DISCUSSION

We successfully reproduced the majority of the X⁻CGD cases in the PLB-985 cell model. Surprisingly, we observed that in most X⁻CGD PLB-985 cells, NOX2 expression at the plasma membrane, as measured by flow cytometry, decreases after differentiation. We suggest that the increase of p22^{phox} during the differentiation step (data not shown) could lead to controlled expression and trafficking of the recombinant mutated NOX2 protein that is continuously produced in transfected PLB-985 cells. We should point out that all the results described here were obtained using

differentiated mutated PLB-985 cells. The first point was that all the X⁻-CGD mutations did not alter the transcription step, as suggested by the equivalent level of NOX2 mRNA analysed by RT-PCR. Among the ten X⁻-CGD mutations reproduced in the PLB-985 cell line, one mutation **I187_I189del** was considered as having an X⁰CGD profile because no expression of NOX2 and no NADPH oxidase activity could be measured. However, this mutation was only listed as an X⁻-CGD case in a review without any results supporting this phenotype (33). The **I187_I189del** is in the IVth transmembrane region of NOX2 closed to the D-loop. The D-loop of NOX2 was demonstrated to be involved in the control of NADPH oxidase activity (42) (52). In addition, a new role for the D-loop of NOX4 in folding and interaction with p22^{phox} has been proposed (17). Thus I187–I189 residues are in an important functional and structural region of NOX2. The cell model of the **H101Y** mutation also leads to an X⁰CGD profile, like the I187_I189del mutation, whereas Tsuda et al. described 8% NOX2 (24). However, in the figure of the Western blot in his article we did not see any NOX2 band. This highly conserved histidine in NOX1–4, is one of the four involved in the coordination of hemes in *cytb*₅₅₈, described as an essential step for the assembly of NOX2 with p22^{phox} (13). Thus, the defect of heme incorporation in H101Y mutant cells affects *cytb*₅₅₈ synthesis by blocking the association between the precursor p65-kDa and p22^{phox} in ER compartments. Indeed in our work, this mutant served as a useful control of the dissociation of the two proteins in co-immunoprecipitation experiments and to visualize the accumulation of p65-kDa in the ER or in nearby compartments using confocal microscopy.

Then according to the NOX2 expression and the NADPH oxidase activity, we classified the X⁻-CGD mutants into two groups. Group B mutants **H338Y**, **P339H**, **G389A**, and **F565_F570del**, lead to the same phenotype in PLB-985 cells; a discrepancy between NOX2 expression at the plasma membrane as measured by flow cytometry (4–12%) and the complete absence of NADPH oxidase activity. This phenotype was previously reported in human neutrophils (36) and confirms the robustness of our approach. These X⁻-CGD mutations affect not only the structure of NOX2, but also its activity. Both H338 and P339 residues are located in the binding site for the FAD isoalloxazine ring (Figure 6A) [43]. FAD depletion in the neutrophils from a patient exhibiting the H338Y mutation has been previously reported (36). The absence of FAD could explain the absence of NADPH oxidase activity in these mutants as previously demonstrated [43]. In addition, both mutations do not affect p65/p22^{phox} binding. Thus both H338Y and P339H mutations contribute to disturbing the catalytic site of NOX2 (Figure 6A). Surprisingly, in the H338Y and P339H mutants, NOX2 was found in both the plasma membrane and the intracellular compartments. This was previously shown for the H338Y mutation only [37,38]. It is possible that both mutations lead to partial structural deformation of the *cytb*₅₅₈ such that it cannot be optimally incorporated into the plasma membrane and accumulates inside the cells in undefined compartments different from ER.

The **F565_F570del** mutation originally reported in the X⁻-CGD patient's neutrophils (22) belongs to the group B mutants like the previous ones. In competition experiments using peptides, the carboxy terminus of NOX2 has been shown to be directly or indirectly involved in p47^{phox} subunit binding (53,54). Then using directed mutagenesis of the last 11 residues of NOX2, Zhen et al. demonstrated that this region plays a structural role rather than a functional one (55). In addition, the F570 is highly conserved in the FAD binding in the proteins of the ferredoxin reductase family and is involved in the stabilization of the isoalloxazine ring of the FAD [43]. Indeed, our present work demonstrates that the last six residues of NOX2, highly conserved in the NOX1–4 proteins, play both structural and functional roles. In addition, the *cytb*₅₅₈ synthesis defect in this mutant is due to an intrinsic instability of the mature NOX2 protein rather than a defect in its maturation process itself. The absence of p65-kDa accumulation in intracellular compartments for this mutant consolidates this conclusion.

The effect of the **G389A** mutation in the *cytb*₅₅₈ synthesis process is intriguing. This glycine is highly conserved in NOX1–4 and is located in a highly conserved region ³⁸⁸DGPFG³⁹². Through sequence analogy with the ferredoxin reductase family, we found a match with sequences conserved in the FAD binding pocket (<http://www.ebi.ac.uk/interpro/sequencesearch/iprscan5-R20140418-130743-0988-23575230-pg>). Like the previous group B mutants, the NADPH oxidase is null, but unlike the others, a substantial accumulation of the precursor p65-kDa demonstrated a blockage in the maturation process of *cytb*₅₅₈ due to a defect in p65-kDa and p22^{phox} binding. Other cases of X⁰-CGD originate from Gly 389 mutations – G389R, G389V and G389E – were reported that underlie the importance of this residue (19). From its location in the dehydrogenase domain (Figure 6B), at the interface with the transmembrane part of the protein, Gly 389 is not in a position where it can be a direct contributor to

the interaction with p22^{phox}. However, the presence of a glycine, without any bulky side chain, seems to be essential to ensure the structural integrity required for p22^{phox} binding. In addition, this Gly is essential to maintain the electron transfer inside NOX2, possibly by preserving the integrity of the coupling between the FAD of the dehydrogenase domain and the heme moieties within the membranous domain.

Finally, in the following group A mutants – **W18C**, **E309K**, **K315del** and **I325F** – NOX2 expression is directly proportional to the NADPH oxidase activity; therefore, these mutations affect NOX2 synthesis and stability only. We observed a high p65/NOX2 ratio caused by the abnormal accumulation of the p65-kDa precursor in structures close to the ER or in the ER itself in all but K315del. This deletion affects the stability of NOX2 only. As for the G389A mutant, the accumulation of the p65-kDa precursor is explained by a defect in binding with p22^{phox}, as demonstrated by the co-immunoprecipitation experiment. It remains uncertain as to whether these residues are directly or indirectly involved in p22^{phox} binding. **W18C** is one of two missense mutations of XCGD found in the first potential transmembrane passage of NOX2, whose role has not yet been determined (19). W18 is conserved in NOX1, NOX3 and NOX4 (but not in NOX5) for which a connection with p22^{phox} is necessary for their activity. Therefore, the first transmembrane passage of NOX2 and particularly W18 could potentially be directly involved in the binding of p22^{phox}. **I325** and **G389** residues are located in the same region of the FAD-binding domain, at the interface between the dehydrogenase and the transmembrane domain (Figure 6B). Mutations in these positions introducing some bulkier residues may induce a packing problem at this interface between both domains of the NOX2 protein. The fact that it abrogates p65/p22^{phox} binding suggests that the p22^{phox} binding site is spread over both regions, the transmembrane region and the dehydrogenase domain, that need to be well oriented to provide an extended and functional interaction interface for p22^{phox} (red circle in Figure 6B). Nonetheless, these residues are also postulated as being close from the surface and considering the size of some substitution in X⁻CGD mutants (like in I325F), a direct perturbation of a surface interaction site could also be postulated (Figure 6B). Thus, these mutations could impact either directly or indirectly on p22^{phox} binding interface. The role played by the **E309** residue could be quite different. Although the E309K mutation prevented p65/p22^{phox} binding (as I325F and G389A mutations), E309 is likely to be exposed on the dehydrogenase domain surface (Figure 6B). Indeed this mutation of a charged amino acid side chain oriented toward the solvent is not expected to disorganize domain orientations. Its deleterious effect and the fact that it is strictly conserved from NOX1–4 support the notion that it could play a direct role in the interaction with p22^{phox}.

In conclusion we increased the understanding of the impact of these mutations on the process of *cytb*₅₅₈ synthesis, previously restricted by insufficient biological material. In the absence of a NOX2/p22^{phox} structure, the analysis of the X⁻CGD mutants reported herein provides the first indications on the structural requirement and potential NOX2 regions required for p22^{phox} interaction, spread over the entire NOX2 structure, like the first transmembrane passage (W18), the intermediate region between the membrane and the cytosolic dehydrogenase domains of NOX2 (E309, I325, G389) and a surface docking site on the dehydrogenase domain located with the E309K mutation. In addition, this study has demonstrated that FAD-binding pocket integrity is closely related to NOX2 stability [H338, P339, G389 and 570_570del].

AUTHOR CONTRIBUTION

Sylvain Beaumel undertook the experiments in Figures 2-5; Didier Grunwald performed the confocal microscopy analysis and took photos from the slides prepared by Sylvain Beaumel. Marie José Stasia designed the experiments, analysed the experimental data and wrote the paper. Franck Fieschi analysed the data, wrote part of the discussion and prepared the Figure 6.

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Table 1 Expression of NOX2, p65-kDa and NADPH oxidase activity in the X⁻-CGD transfected PLB-985 cells

The relative level of recombinant NOX2 (and p65-kDa) was measured after PLB-985 cell differentiation with 0.5% DMF for 6 days. NOX2 expression measured by flow cytometry was calculated using G mean fluorescence of each PLB 985 cell line. NOX2 and p65-KDa expression were also estimated by Western blot based on scanning densitometry of immunoblots performed with the 48 monoclonal antibodies (GelDoc® BioRad system). ROS production was measured by luminol-amplified chemiluminescence in 5.10⁵ differentiated and transfected X⁻-CGD PLB-985 cells after PMA stimulation. Results are represented as total activity by the sum of relative luminescence units measured in 60 min at 37°C. Percentages were calculated from the values of the control WT NOX2 PLB-985 cells. Data are the mean ± SD of (n) range experiments. In group A mutants, the level of NOX2 expressed at the plasma membrane measured by flow cytometry is directly proportional to the level of NADPH oxidase activity. In group B mutants, the NOX2 expression at the plasma membrane was variable, whereas the NADPH oxidase activity was totally abolished. Values of NOX2 expression measured by flow cytometry and NADPH oxidase activity for mutants A and B are in bold.

	FLOW CYTOMETRY		WESTERN BLOT (n = 3)				CHEMILUMINESCENCE	
	NOX2		NOX2		p65-kDa		H ₂ O ₂ PRODUCTION	
	G mean	% of control	% of control	% of control	ratio p65/NOX2	Sum RLU x 10 ³	% of control	
WT NOX2 (control)	123.0 ± 28 (n=10)	100	100	100	1	10,122 ± 1,215 (n=10)	100	
Mock X-CGD	2.9 ± 0.7 (n=10)	0	0	0	/	14 ± 9 (n=10)	0	
Group A								
W18C	24.0 ± 5.0 (n=10)	17	12.1 ± 2.8	59.1 ± 19.6	5	2,034 ± 446 (n=10)	20	
P56L	6.3 ± 3.9 (n=10)	3	1.0 ± 0.8	50.3 ± 19.3	50	597 ± 233 (n=10)	6	
E309K	8.1 ± 3.6 (n=10)	4	8.2 ± 2.1	48.3 ± 17.7	6	415 ± 243 (n=10)	4	
K315del	13.0 ± 4.8 (n=10)	8	11.0 ± 1.8	11.5 ± 1.3	1	891 ± 314 (n=10)	9	
I325F	4.6 ± 2.4 (n=10)	2	1.5 ± 0.6	26.8 ± 6.6	18	423 ± 139 (n=10)	4	
Group B								
H338Y	14.4 ± 2.8 (n=5)	10	46.4 ± 16.3	56.6 ± 16.4	1	11 ± 6 (n=5)	0	
P339H	8.7 ± 2.4 (n=5)	5	38.0 ± 17.1	36.7 ± 15.3	1	9 ± 5 (n=5)	0	
G389A	4.4 ± 2.5 (n=5)	2	0.8 ± 0.8	21.5 ± 4.5	27	7 ± 4 (n=5)	0	
F565_F570del	18.5 ± 7.0 (n=5)	12	16.3 ± 8.1	16.0 ± 5.0	1	18 ± 7 (n=5)	0	
X⁰ CGD								
H101Y	2.6 ± 0.6 (n=5)	0	0	71.7 ± 10.0	/	10 ± 2 (n=5)	0	
A156T	2.5 ± 0.4 (n=5)	0	0	36.0 ± 9.2	/	32 ± 11 (n=5)	0	
I187_I189del	2.6 ± 0.8 (n=5)	0	0	19.3 ± 5.9	/	30 ± 12 (n=5)	0	
C244S	2.6 ± 0.5 (n=5)	0	0	3.0 ± 2.0	/	30 ± 11 (n=5)	0	
C244Y	2.5 ± 0.7 (n=5)	0	0	38.7 ± 6.0	/	16 ± 6 (n=5)	0	
V534_G538del	2.6 ± 0.9 (n=5)	0	0	6.3 ± 2.1	/	10 ± 4 (n=5)	0	

FIGURE LEGENDS**Figure 1 Location in NOX2 and conservation among NOX family members of amino acids involved in X⁻CGD mutations.**

(A) Glycosylated asparagines are located in the external loops of NOX2 and numbered by their residue numbers. The four heme-binding histidines located in the third and fifth transmembrane domains are shown by single-letter code as H. The potential FAD- and NADPH-binding domains are illustrated by dotted boxes. The X⁻CGD mutations studied in the article are in red and other mutations causing X⁻CGD are in blue (Roos et al. 2010). These mutations are present in the whole sequence, whereas four clusters can be delimited by the high frequency of the location of X⁻CGD mutations as red circles; the intracytosolic D-loop, the potential FAD-binding sites, the C-terminal region and a region between the sixth transmembrane passage and the FAD-binding site. (B) The X⁻CGD mutations studied in this work (in red) are totally preserved among NOX1–4. The potential binding sites of NADPH and FAD are in pink and orange, respectively, the histidines that coordinate hemes are in green and the glycosylated asparagines are in blue. The alignments were done using the website <http://www.ebi.ac.uk/Tools/msa/clustalw2/>.

Figure 2: RT-PCR and quantitative PCR analysis of the mRNA of the transfected X⁻CGD PLB-985 cells. (A) RT-PCR was performed on total RNA extracts with primers specific for p22^{phox} or NOX2 cDNA. As a control for RNA quality and equal loading, RT-PCR was performed with primers for geneticin. The results are representative of three independent experiments. (B) Real-time PCR was performed as described in Materials and Methods. Results are expressed as means of the ratio from four different qPCR performed from two different RT-PCR from one total RNA extraction for each type of differentiated cell. Data are the mean ± SD of four independent experiments.

Figure 3 Analysis of mutated NOX2 expression in transfected X⁻CGD PLB-985 cells by flow cytometry and Western blot

(A) Flow cytometry analysis of NOX2 expression in undifferentiated (UD) and differentiated (D) transfected X⁻CGD PLB-985 cells. ($5 \cdot 10^5$) cells were incubated with the NOX2 monoclonal antibody 7D5 and then with a phycoerythrin-labelled goat-F(ab')₂ fragment anti-mouse IgG as described in Material and Methods. The mouse IgG1 isotype was used as an irrelevant monoclonal antibody. In Mock X⁰-CGD PLB-985 cells there is no NOX2 expression (dotted line) after WT NOX2 cDNA transfection; NOX2 is stably expressed in the WT NOX2 PLB985 cells (white histogram). A diminished NOX2 expression is visible in the X⁻CGD PLB-985 cells (grey histogram). The same observations were obtained in at least four independent experiments. (B) Immunoblot analysis of NOX2, p22^{phox} and the precursor of NOX2 p65-kDa was performed on 1% Triton X-100 soluble extracts (100 µg) from X⁻CGD PLB-985 cells, subjected to SDS-PAGE, blotted onto a nitrocellulose sheet and revealed with monoclonal antibodies 48 and 449, respectively. P47^{phox} monoclonal antibody served as a loading control. Western blots of undifferentiated and differentiated PLB-985 cells have unequal times of exposure.

Figure 4 NOX2 and/or p65-kDa location in subcellular compartments of X⁻CGD mutants

(A) NOX2 and p65-kDa were localized using confocal microscopy analysis in control and mutated PLB-985 cells as described in Material and Methods. NOX2 and/or p65-kDa were visualized in green and calnexin as an endoplasmic reticulum marker (ER) in red. Cellular nuclei were stained with Hoechst 33258 (blue). (B) Colocalization analysis of NOX2/p65-kDa with calnexin was performed using the Leica confocal software. Cells presenting a colocalization of NOX2/p65-kDa with calnexin are in yellow (upper right quarter). The same observations were obtained in at least two independent experiments.

Figure 5 Co-immunoprecipitation of the precursor p65-kDa and p22^{phox} from X⁻CGD PLB-985 cells

After co-immunoprecipitation of the precursor p65-kDa and p22^{phox} as described in the Material and Methods section, the same amount of proteins of the initial soluble extract fractions (SE), the flow-through of the column with the CS9 antibody (–) and the positive fraction collected after washing with

the sample buffer for SDS PAGE (+) were assessed by immunoblot analysis using monoclonal antibodies 48 and 449. HC IgG1 and HL IgG1 indicate positions of the heavy and light chains, respectively, of immunoglobulin. Black boxes indicate the presence or absence of the precursor p65-KDa and p22^{phox} in the positive fraction (A) Control cell lines were the WT NOX2 PLB-985 cells in which p65-KDa and p22^{phox} are associated in the positive fraction, the H101Y mutant PLB-985 cells where the mutation affects the p65-kDa and p22^{phox} association. (B) Group A X⁻-CGD mutants with an abnormal accumulation of the precursor p65-kDa. (C) Group B X⁻-CGD mutants with mutations in the FAD binding site. The same observations were obtained in at least three independent experiments.

Figure 6 Ribbon representation of the 3D model of the NOX2 dehydrogenase domain

(A) X⁻-CGD mutated residues H338 and P339 are located in the binding site for the FAD isoalloxazine ring. H338Y and P339H mutations contribute to disturbing the catalytic site of NOX2 and the localization of NOX2/p22^{phox} to the plasma membrane. (B) X⁻-CGD mutated residues E309, I325 and E389 (affecting the p65/p22^{phox} binding) are located in the intermediate region between the membrane and the dehydrogenase domain of NOX2. Residues are represented as yellow sticks. NADPH and FAD are also represented as sticks in the CPK colour code. The red circle corresponds to a potential p22^{phox} binding region. The figure was drawn using Pymol software.

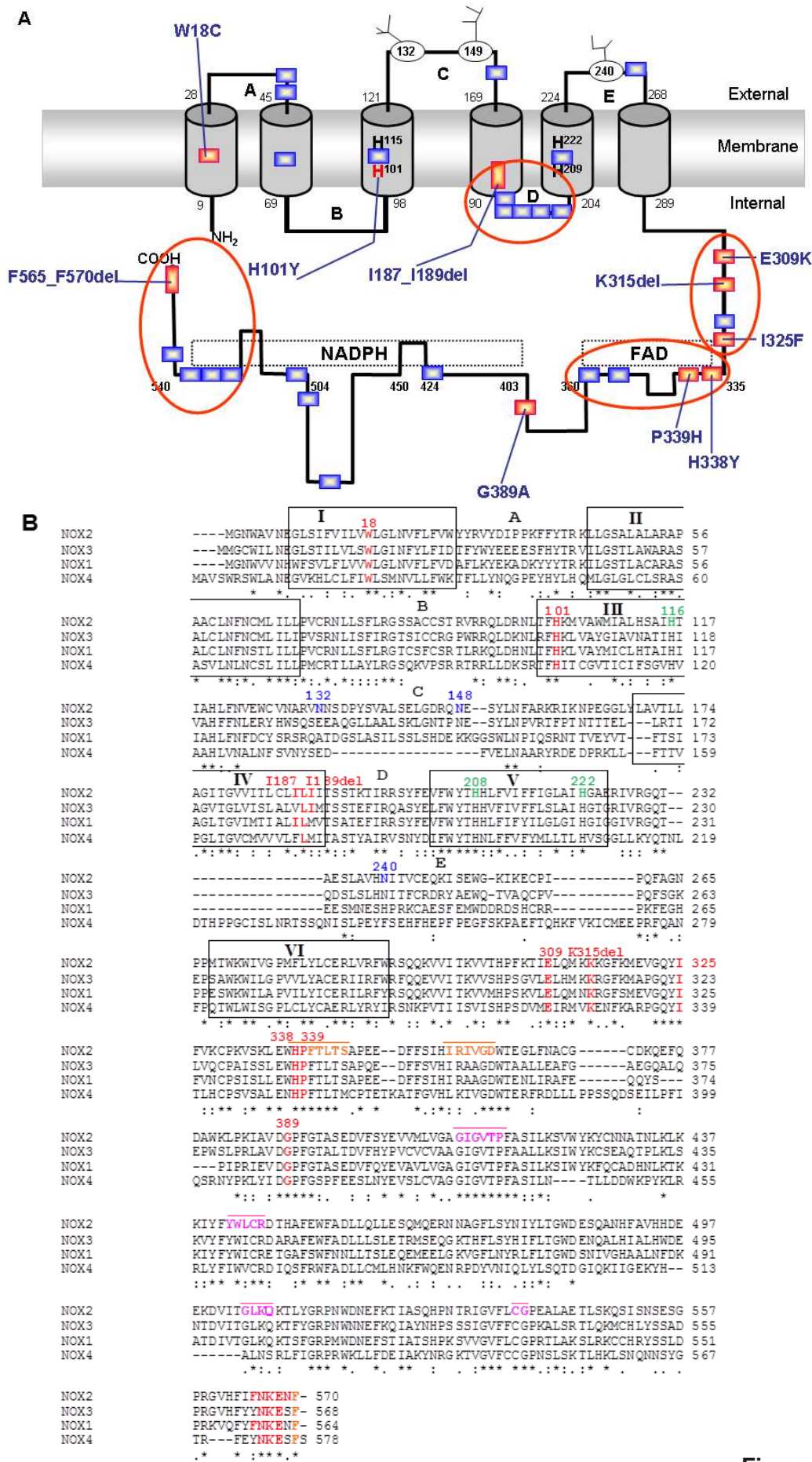


Figure 1

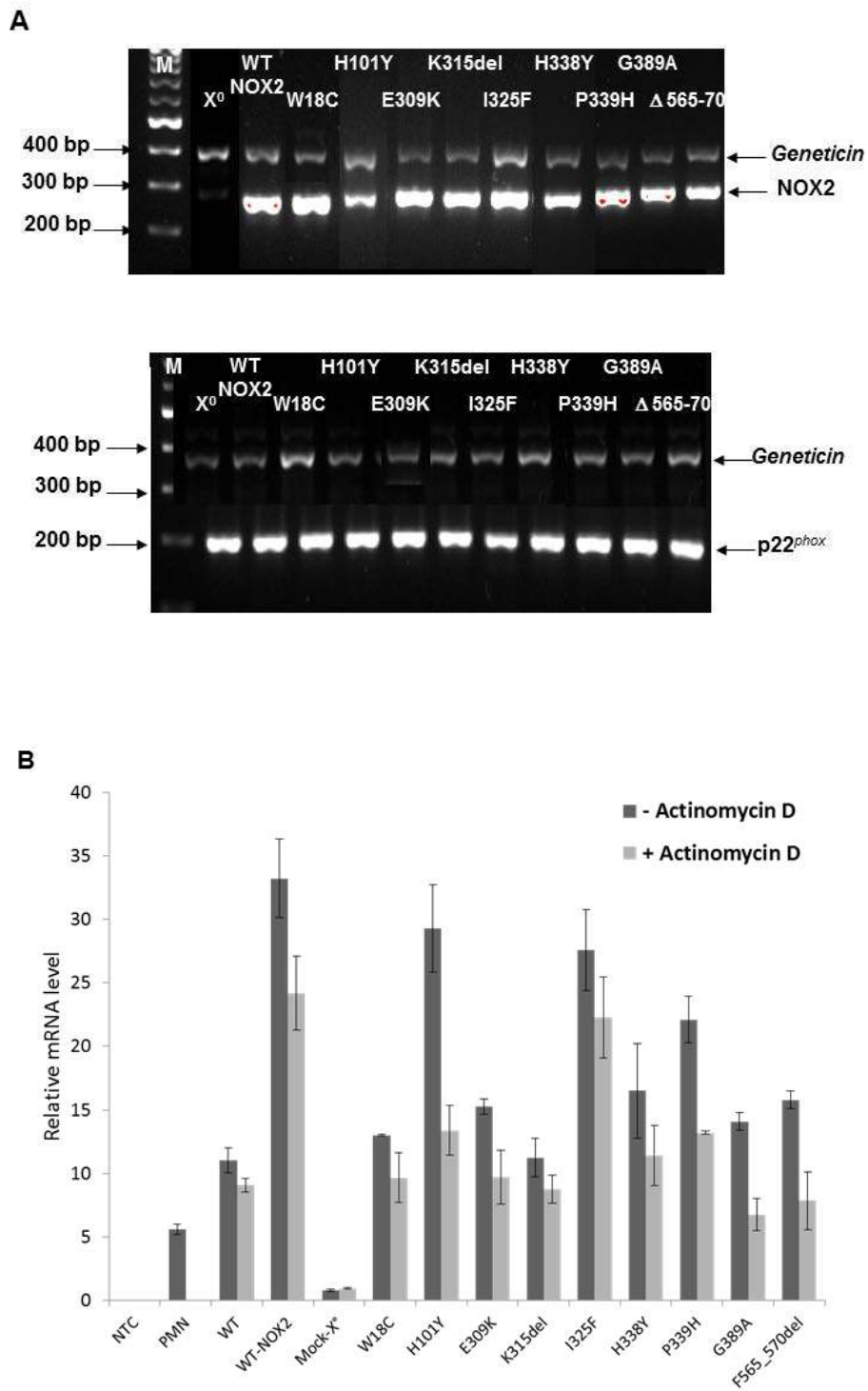


Figure 2

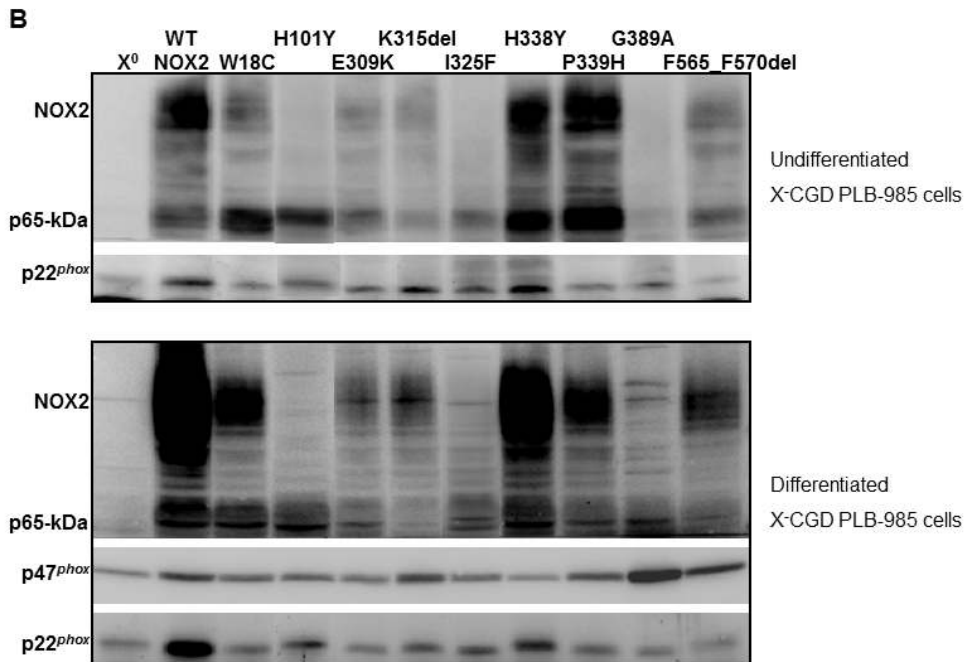
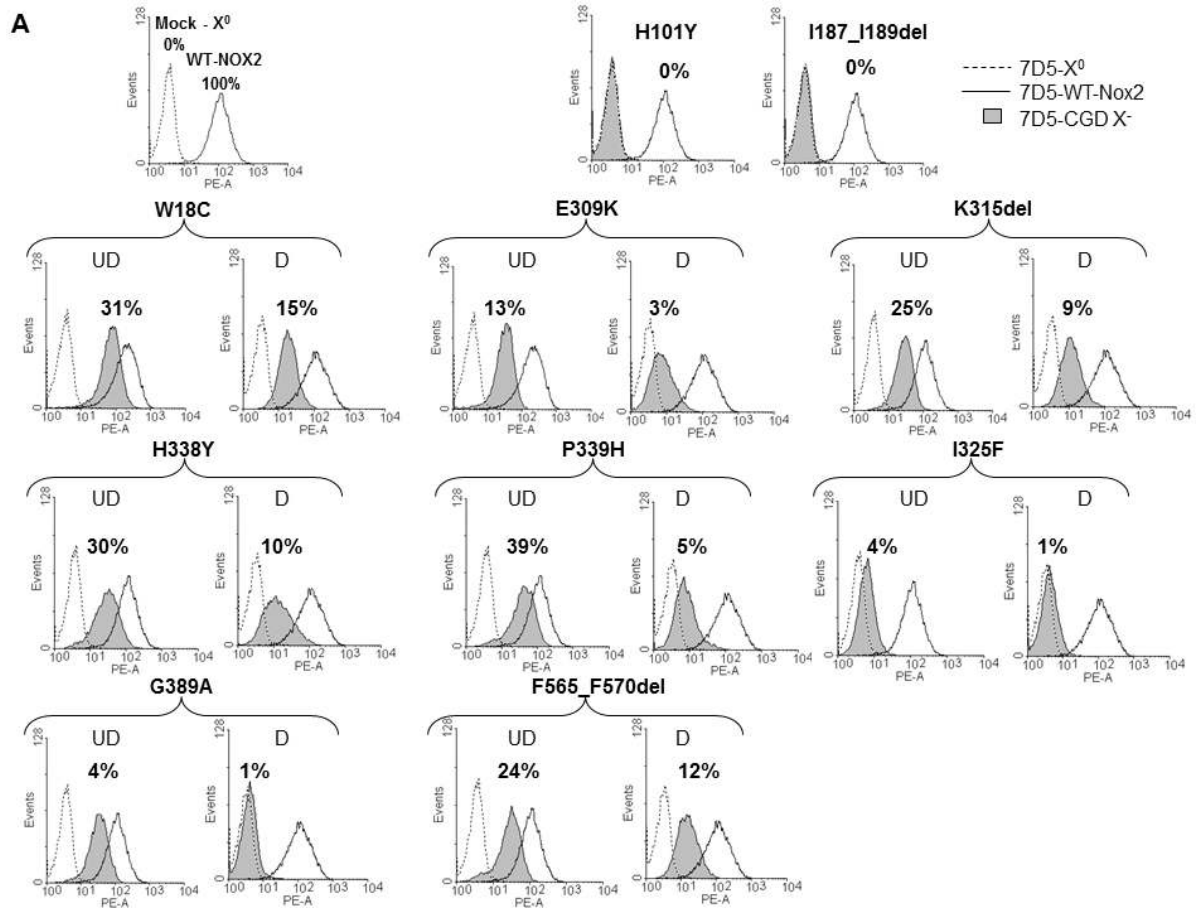


Figure 3

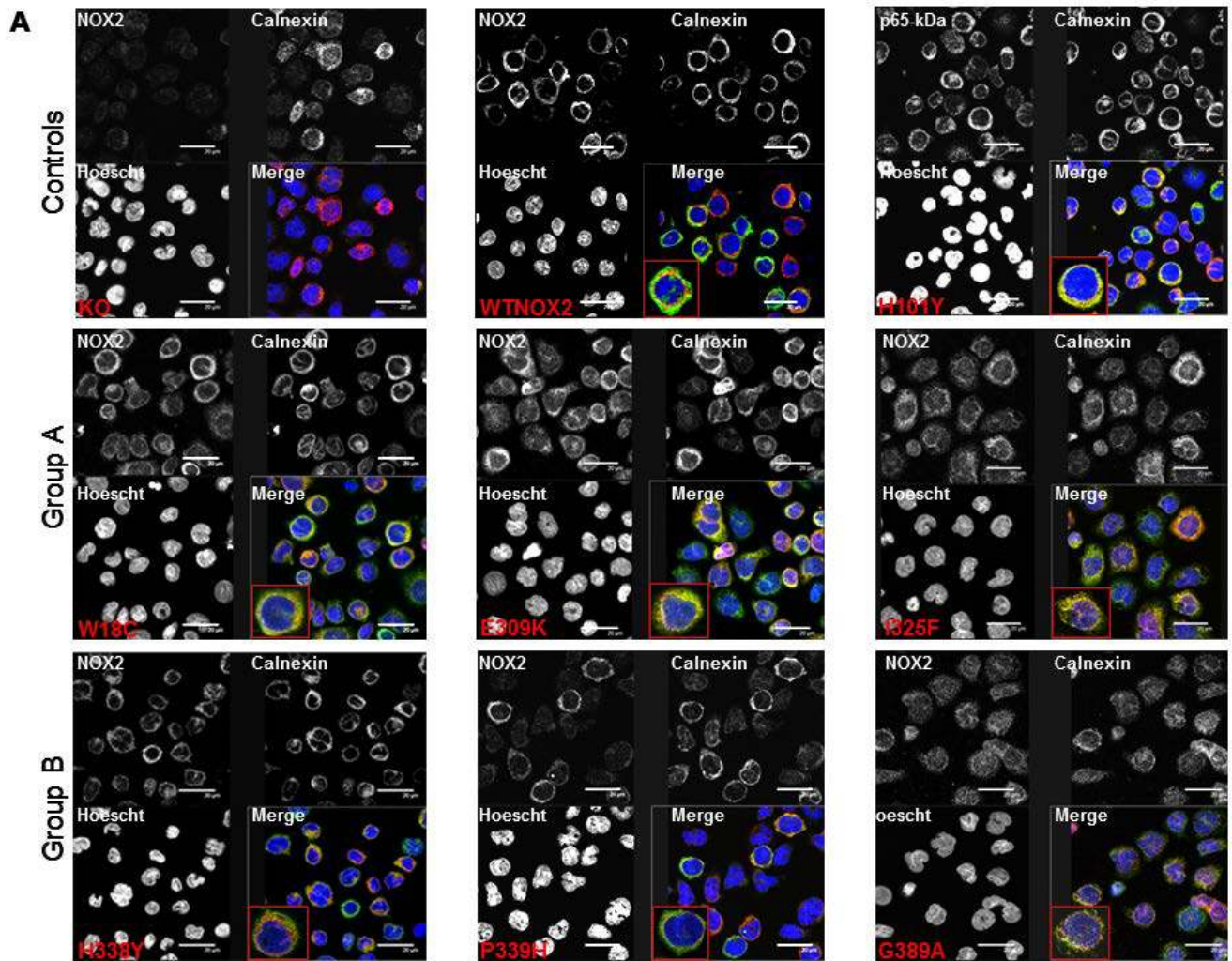


Figure 4A

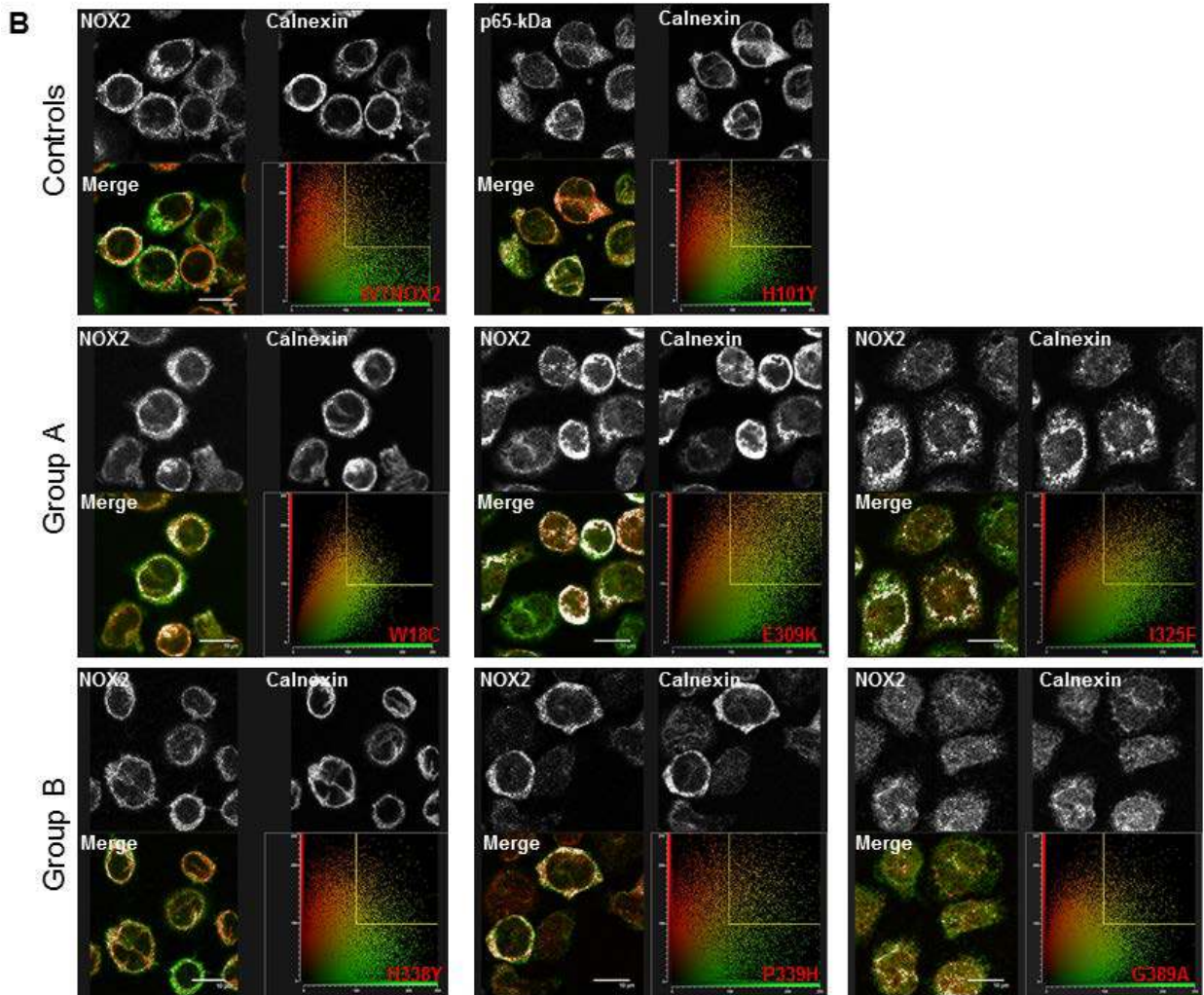


Figure 4B

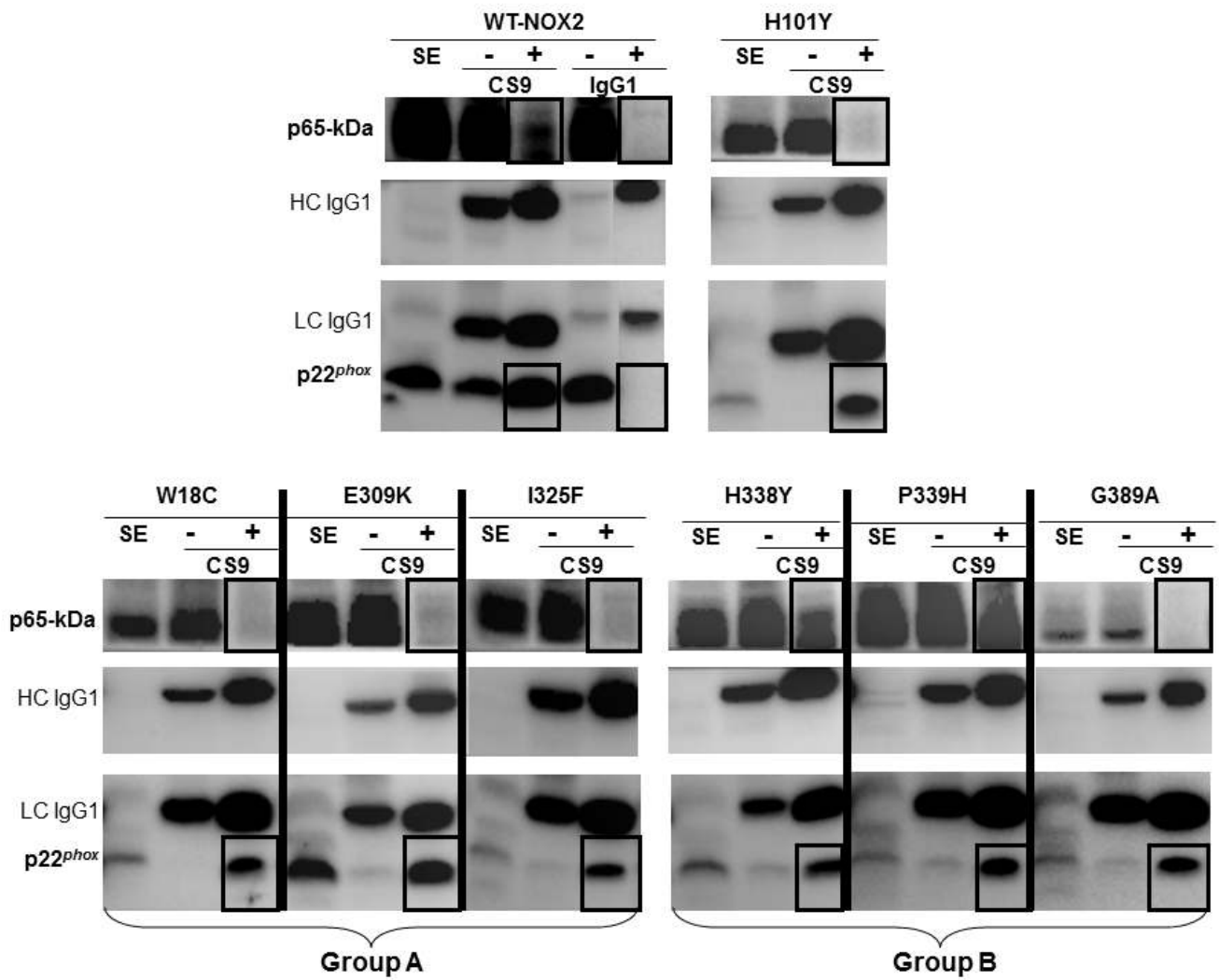


Figure 5

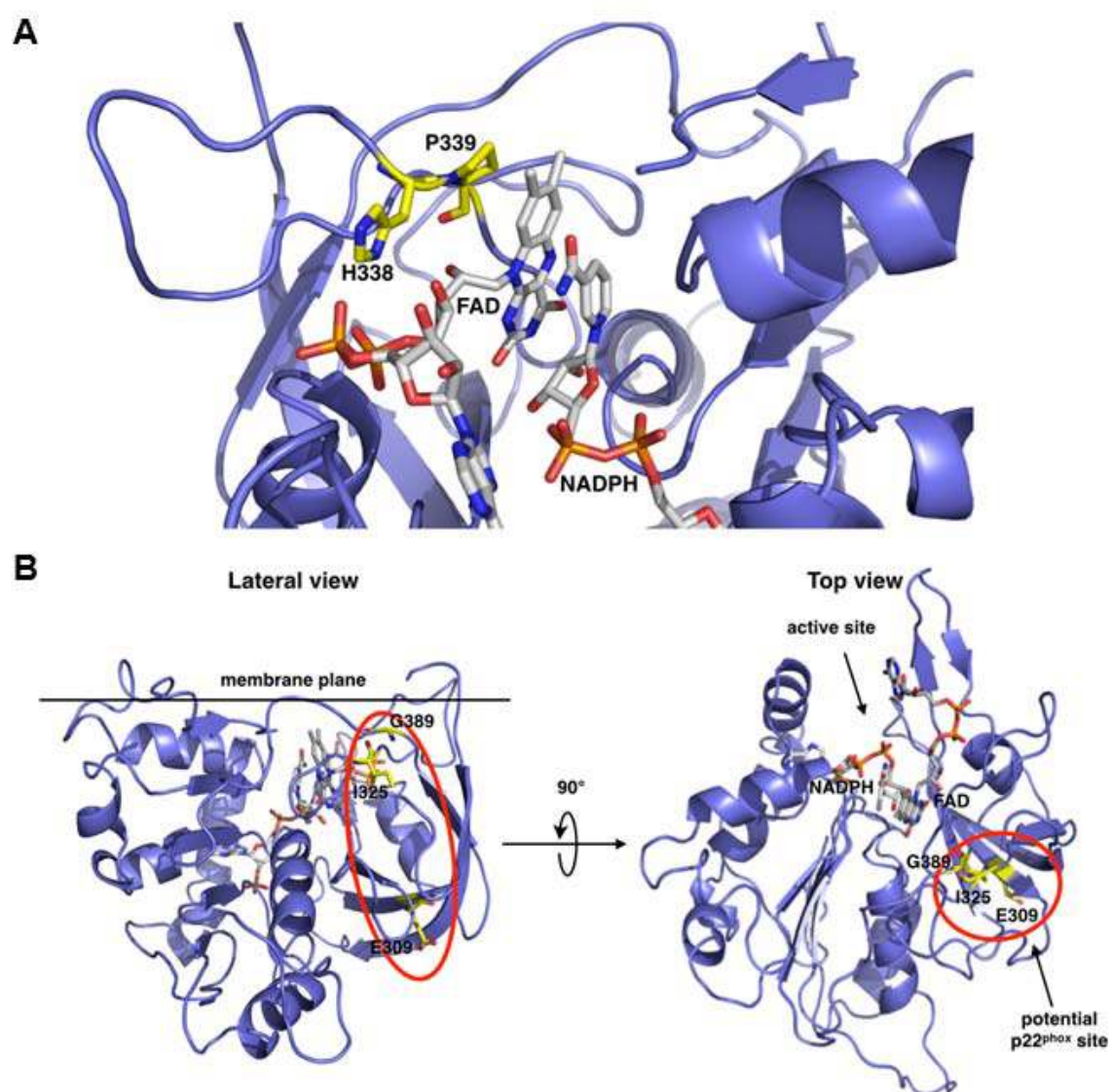


Figure 6