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# **Transmembrane Nox4 topology revealed by Topological Determination by Ubiquitin Fusion Assay, a novel method to uncover membrane protein topology**

**Francis Rousset<sup>1</sup>, Leilei Zhang<sup>1</sup>, Bernard Lardy<sup>1, 2</sup>, Françoise Morel<sup>1</sup> and Minh Vu Chuong Nguyen<sup>1</sup> \***

<sup>1</sup> Univ.Grenoble Alpes, CNRS, CHU Grenoble Alpes, Grenoble INP\*, TIMC-IMAG, GREPI, 38000 Grenoble Cedex. \*Institute of Engineering Univ. Grenoble Alpes.

<sup>2</sup> Laboratoire de Biochimie des Enzymes et des Protéines, Pôle de Biologie, Centre Hospitalier Universitaire Grenoble Alpes, 38700 La Tronche, France.

*\*Correspondence should be addressed to Minh Vu Chuong Nguyen. Email: mvchuong@yahoo.fr*

Francis Rousset's present address: Department of Pathology and Immunology, Centre Médical Universitaire, Geneva, Switzerland.

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## **ABSTRACT**

The NADPH oxidase Nox4 is a multi-pass membrane protein responsible for the generation of reactive oxygen species that are implicated in cellular signaling but may also cause pathological situations when dysregulated. Although topological organization of integral membrane protein dictates its function, only limited experimental data describing Nox4's topology are available.

To provide deeper insight on Nox4 structural organization, we developed a novel method to determinate membrane protein topology in their cellular environment, named Topological Determination by Ubiquitin Fusion Assay (ToDUFA). It is based on the proteolytic capacity of the deubiquitinase enzymes to process ubiquitin fusion proteins. This straightforward method, validated on two well-known protein's topologies (IL1RI and Nox2), allowed us to discriminate rapidly the topological orientation of protein's domains facing either the nucleocytosolic or the exterior/luminal compartments. Using this method, we were able for the first time to determine experimentally the topology of Nox4 which consists of 6 transmembrane domains with its N- and C-terminus moieties facing the cytosol. While the first, third and fifth loops of Nox4 protein are extracellular; the second and fourth loops are located in the cytosolic side. This approach can be easily extended to characterize the topology of all others members of the NADPH oxidase family or any multi-pass membrane proteins.

Considering the importance of protein topology knowledge in cell biology research and pharmacological development, we believe that this novel method will represent a widely useful technique to easily uncover complex membrane protein's topology.

## INTRODUCTION

NADPH oxidase (Nox) represent a family of transmembrane protein comprising 7 members which unique function is to produce reactive oxygen species (ROS) [1]. Dysregulation of ROS production of ROS by those enzyme leads to oxidative stress and tissues damage. In particular, increased Nox4 expression and activity has been linked to pathologic states including diabetic nephropathy [2], pulmonary fibrosis [3], or skeletal diseases [4] among others. Targeting Nox enzymes represents a novel and increasingly recognized therapeutic approach for a number of diseases with strong oxidative stress components [5]. However, the lack of data concerning Nox topology limits considerably the possibilities to develop isoform-specific inhibitors. This is particularly true for Nox4 which is constitutively active. Indeed, the development of inhibitors peptide targeting the putative B-loop of Nox4 and the extreme C-terminus of p22phox was unsuccessful, probably in part due to the absence of complete topological data concerning Nox4 [6]. Thus, determining Nox4 membrane topology constitutes a key feature to understand its role in pathophysiological situations and to design specific inhibitors.

Although members of the Nox family display conserved domains with the canonic Nox2, Nox4 overall membrane topology has not yet been experimentally determined. So far, only few studies including previous data from our group described partial topologic organization of Nox4 in particular for the B-loop [7] and the E-loop [8].

To provide deeper insight on Nox4 structural organization, we developed a novel method, named ToDUFA (Topological Determination by Ubiquitin Fusion Assay), aiming to determine the topological organization of transmembrane proteins

in living cells. This novel method utilized the specific subcellular location combined to the enzymatic capability of Deubiquitinases proteins (DUBs) to process ubiquitin (Ub) fusion proteins. This assay provides a fast and easy GFP fluorescence readout and do not require heavy optimization and chemical treatment or modification.

## **EXPERIMENTAL PROCEDURE**

### **Reagents**

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, neomycin (G418, geneticin) were purchased from GIBCO; ECL Western Blotting Detection reagents were purchased from Amersham Biosciences; complete mini EDTA-free protease inhibitor EASYpack, leupeptin, pepstatin, TLCK were purchased from Roche; GFP mAb was purchased from Santa Cruz; Goat anti-Mouse IgG-HRP was purchased from GE healthcare; X-treme GENE 9 Transfection Reagent was purchased from Roche; MassRuler DNA Marker was purchased from Euromedex. Alexa Fluor 488 labelled goat anti-mouse IgG and pCDNA 3.1 plasmid were from Invitrogen.

### **Plasmid constructions**

GFP sequence was obtained by PCR and inserted into the pCDNA 3.1 empty plasmid, using *XhoI* and *ApaI* restriction sites, to generate the pCDNA GFP plasmid. The forward GFP primer sequence was designed to add a *Clal* restriction site between the *XhoI* site and GFP sequence. pCDNA Ub-GFP was constructed by inserting the ubiquitin sequence obtained by PCR between the *XhoI* and *Clal* restriction site in frame with the GFP sequence into the pCDNA GFP plasmid. Finally, Nox2N131, IL1RI and Nox4 full length or truncated sequences were obtained by

PCR and inserted into either pCDNA GFP or pCDNA Ub-GFP plasmids using *KpnI* and *XhoI* restriction sites in frame with GFP or Ub-GFP sequences. The primers used in this study are indicated in the supplementary table 1.

### **Cell culture and transfection**

HEK293 cells were cultured in DMEM containing 4.5 g/L glucose and 0.11 g/L sodium pyruvate, supplemented with 10% (v/v) fetal bovine serum and 100 units/ml penicillin and 100 mg/ml streptomycin and 2 mM glutamine at 37°C in atmosphere containing 5% CO<sub>2</sub>. For transfections, 5×10<sup>5</sup> HEK293 cells were seeded in 6-well plates and allowed to grow for 24h to reach a 60% confluence in 2 ml of culture medium. Cells were transfected with 1 µg of vectors according to the manufacturing protocol (X-treme GENE 9, Roche). After 24h, transfected cells were stably selected with 500 µg/ml geneticin for 3 weeks before analysis.

### **Flow cytometry**

Cells were suspended in PBS (10<sup>7</sup>/ml), incubated with 0.01% saponin in PBS or PBS alone for 3 min on ice. After a centrifugation step, saponin treated cell supernatant was collected and stored for GFP Western Blot. Meanwhile, cells were washed twice and suspended in PBS buffer. GFP fluorescence intensity of the cells (GMean) was measured on a FACScalibur (Becton Dickinson) cytometer.

### **SDS/PAGE and Western Blotting**

Cells were suspended in PBS buffer containing 2 µM leupeptin, 2 µM pepstatin, 10 µM TLCK and complete mini EDTA-free protease inhibitor. The cells were fractionated by sonication. The homogenate was centrifuged at 1,000 g for 15 min at

4°C to remove unbroken cells and nuclei. The post-nuclear supernatant was centrifuged at 200,000 g for 1 h at 4°C. This high-speed centrifugation supernatant was referred to the cytosol, and the pellet consisting of crude membranes was suspended in the same buffer. Immunodetection was performed using primary monoclonal antibodies against GFP (dilution 1:1,000) (Santa Cruz). The immune complexes were detected with a secondary antibody combined with peroxidase and detected using ECL reagents.

## RESULTS

### General principle of the Topological Determination by Ubiquitin Fusion Assay (ToDUFA)

This method is based on the properties of deubiquitinase enzymes to process ubiquitin fusion proteins. Deubiquitinases are exclusively expressed in the nucleocytoplasm and are involved in the synthesis and recycling of monomeric ubiquitin proteins by processing the polyubiquitin chain [9]. To determine the topology of transmembrane proteins, the idea is to attach to the C-terminus moiety of the reference protein a chimera protein, composed of an ubiquitin molecule fused to a fluorescent protein, GFP (UbGFP). They are then to possible scenarios: 1) if the C-terminus moiety is located in the extracellular or luminal side, the ubiquitin is not accessible to DUBs and therefore protected from their cleavage (**Fig. 1a**); 2) conversely, if the C-terminus moiety is exposed to the nucleocytoplasmic side, GFP protein will be released from the chimera UbGFP by the DUBs enzymes proteolytic activity (**Fig. 1b**). Consequently, the release of “free” GFP molecule will serve as an

indicator for the orientation of the C-terminus moiety of the reference transmembrane protein.

### **Establishment and proof-of-principle of the ToDUFA method**

To establish the proof-of-principle of the ToDUFA method, we used two proteins of known membrane topology as models (**Fig. 1c**) : 1) Interleukin 1 Receptor Type I (IL1RI) which is a cytokine receptor presenting a single-pass transmembrane protein with its C terminus facing the cytoplasm [10] and 2) Nox2, the prototype of the NADPH oxidase family, which topology have been intensively studied. A truncated form of Nox2 corresponding to its second extracellular loop, Nox2N131, have been chosen because its C-terminus faces the extracellular or luminal side [11,12]. Cells overexpressing IL1RI or Nox2N131 tagged with UbGFP were treated or not with 0.01% saponin, a determined concentration allowing the allowing the diffusion of “free” GFP out of the cell (**Supplementary Fig. S1**), and analyzed by FACs for GFP fluorescence (**Fig. 2a**). As shown in **Fig. 2b and 2c (left panels)**, fluorescence of IL1RI-UbGFP expressing cells was lost upon saponin treatment compared to that of the untreated cells suggesting that the GFP attached to the IL1RI-Ub has been cleaved and released out of the cells (**Fig. 2b, left panel cartoon**). Indeed, the Western blot analysis showed the presence of “free” GFP in the supernatant collected after cell permeabilization (**Fig. 2d**). Furthermore, the GFP fluorescence remained unchanged upon saponin treatment for cells expressing IL1RI-GFP (**Supplementary Fig. S2a**) and no “free” GFP was found in the supernatant collected after permeabilization (**Fig. 2d**).

To further validate the method, we next examined the fate of the GFP fluorescence of cell expressing Nox2N131-UbGFP which C-terminus moiety faces



the extracellular or luminal side (**Fig. 2b, right panel cartoon**). The addition of saponin did not decrease the GFP fluorescence of the Nox2N131-UbGFP cells (**Fig. 2b and 2c, right panels**) similarly to that of cells expressing Nox2N131-GFP (**Supplementary Fig. S2a**). Consistently, no “free” GFP was found in the supernatant collected after cell permeabilization (**Fig. 2d**).

We next corroborated these results by using a standard Western blot protocol on membrane and cytosol fractions (**Fig. 3a**). In theory, if the UbGFP tag is not processed by DUBs, GFP fused protein location should remain in the membrane fraction; and conversely, if the UbGFP tag is targeted by the DUBs enzymes, GFP should be released into the cytosol (**Fig. 3b cartoons**). In agreement with the data described above, “free” GFP molecules were found only in the cytosolic fraction of cells expressing IL1RI-UbGFP (**Fig. 3b upper panel**) and GFP labeling was found solely in the membrane fraction extracted from Nox2N131-UbGFP expressing cells (**Fig. 3b lower panel**).

#### **Application of ToDUFA method to reveal the membrane topology of NADPH oxidase 4 proteins.**

UbGFP or GFP tags were fused to the C-terminus side of full length or truncated Nox4 proteins according to the location of the predicted transmembrane domains (**Supplementary Fig. S3**). All constructs were carefully designed to keep the amino acid charges surrounding the transmembrane domain in order to not interfere with its orientation [13]. As seen in **Fig. 4a** in the FACS experiments, the fluorescence of Nox4FL-UbGFP, Nox4N341-UbGFP, Nox4N184-UbGFP and Nox4N98-UbGFP expressing cells strongly decreased upon saponin treatment.

Meanwhile, as control, fluorescence of Nox4FL-GFP, Nox4N341-GFP, Nox4N184-GFP and Nox4N98-GFP, did not change (**Supplementary Fig. S2b**). By contrast, fluorescence intensity associated with Nox4N46-UbGFP, Nox4N144-UbGFP and Nox4N239-UbGFP expressing cells remained unchanged after the saponin treatment (**Fig. 4a**), similarly to that of their control without ubiquitine Nox4N46-GFP, Nox4N144-GFP and Nox4N239-GFP expressing cells (**Supplementary Fig. S2b**).

This topological organization was confirmed by examining the GFP expression in the cytosol and crude membrane fractions by Western blot. As in **Fig. 4b**, “free” GFP was detected only in the cytosol fraction of Nox4FL-UbGFP, Nox4N341-UbGFP, Nox4N184-UbGFP and Nox4N98-UbGFP expressing cells at its monomeric molecular weight. By contrast, the expression of Nox4N46-UbGFP, Nox4N144-UbGFP and Nox4N239-UbGFP protein was detected at their predicted fusion size with UbGFP tag in the crude membrane fraction.

Altogether, those data summarized in **Fig.4c and 4d** permitted us to experimentally reveal Nox4 topology which consists of 6 transmembrane domains with its N- and C-terminus moieties facing the cytosol. Furthermore, we were able to identify 5 intermembrane loops a, b, c, d, and e. Loops a, c and e are located in the extracellular or luminal side whereas loops b and d face the cytosol (**Fig. 4d**). Thus, ToDUFA appears to be an original, easy, robust and suitable method to determine the topology of complex multi-spanning membrane proteins.

## DISCUSSION

Based on the range of predictions by the different methods more than 30% of the human proteome is dedicated to be membrane proteins [14] which are considered to play a key role in cell's life by transducing and propagating signals from the surrounding to the cell. The proper function of these integral membrane proteins is dictated by their topological distribution throughout the cellular membrane.

Although an increasing number of membrane protein structures emerged recently, the vast majority of those structures are of membrane proteins from prokaryotic sources and high resolution structural data of eukaryotic membrane proteins remains largely limited [15]. Techniques available to estimate the membrane protein topology of protein require substantial optimization and are often time consuming such as cystein substitution [16], N-glycosylation scanning [17], antibody mapping [12], protease protection [18], specific protease cleavage sequence [19] or protease accessibility [20]. Our method based on the activity of native DUBs offers the advantage to study the membrane topology of proteins *in situ* without the need of performing biochemical modification of the reference protein or time-consuming optimization steps prior the analysis. Based on the analysis of "free" GFP status, ToDUFA method may be applied in a variety routinely techniques utilized in laboratory such as Western blot, FACS or fluorescent microscopy (**Supplementary Fig. S4**)

Although GFP tag allows an easy readout that do not require additional tools such as specific monoclonal antibodies [12], many other reporter system such as luciferase, Cre recombinase, hemagglutinin or myc tags for example could be used instead reducing substantially the steric interference with the native topological distribution. While GFP tag most of the time does not affect the properties of the

protein and retain native conformation of the untagged protein [21], rare case reported otherwise. For some specific case, additional investigation such as enzyme activity or proper cellular addressing of the tagged protein may be necessary to verify its correct conformation.

ToDUFA method is based on the protease activity of DUBs enzymes and is therefore applicable to cells that expressed endogenously DUBs proteins. DUBs which function is to generate active monomeric ubiquitin molecules from polyubiquitin chain are ubiquitously expressed, similarly to ubiquitin proteins, in all eukaryotic cells from *Saccharomyces cerevisiae* to *Homo sapiens* [22]. Ubiquitous expression of DUBs opens a wide range of applicability of ToDUFA method to numerous cellular systems but prokaryotic system though. Although we did not experimentally test this eventuality, it is conceivable that exogenous expression of DUBs enzymes in prokaryotic cells may overcome this limitation.

Unlike NADPH oxidase Nox2 which has been studied for decades and represents the prototype of the NADPH oxidase family, very little is known about Nox4 structure organization. Our group has previously generated specific monoclonal antibodies directed against Nox4. The identification of their epitopes on Nox4 protein allowed us to partially reveal the topology of Nox4 by showing that its C terminus domain is located in the cytosol and that the amino acid  $^{221}\text{H-E}^{241}$  (located in the E-loop represented in the **Fig.4d**) is exposed to the extracellular side [8]. Furthermore another group has shown that the dehydrogenase domain (located in the C terminus domain) binds to the amino acid sequence corresponding to the B-loop (**Fig.4d**) [7] suggesting a cytosolic localization of this loop. Moreover, we provided additional data

that demonstrate the complete topology of Nox4 protein which organization appears to be similar to that of Nox2 [11-18]. However, further investigation using other complementary methods [13,19,23-26] may strengthen and fully validate our present data. Although we did not study the topology of other Nox such as Nox1, Nox3 and Nox5 with our method, our topological data of Nox4 tend to confirm that indeed according to their sequence homology the NADPH oxidase family seems to share a common topological organization *i.e.* 6 transmembrane domains separated by 5 loops with the N- and C-terminus moieties facing the cytosol.

Finally, ToDUFA represents a useful technique for topology determination of transmembrane proteins, which is performed on the protein in its cellular environment using native endogenous cellular enzymes such as DUBs. Its applicability and robustness have been successfully demonstrated in validating the topology of two known protein (IL1RI and Nox2) but also in defining the undetermined yet topology of a multi-pass membrane protein NADPH oxidase Nox4. Regarding the relative importance of membrane proteins in cellular process and drug design and the technical difficulties to obtain membrane protein crystallization structure, the easy and straightforward ToDUFA method appears to be an attractive alternative approach that can be widely used to reveal topology of integral membrane proteins.

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« programme Emergence 2003–2006 »; the « CGD research Trust 2006–2007 », UK; the « Groupement des Entreprises Françaises de la Lutte contre le Cancer », délégation de Grenoble; the « Direction Régionale de la Recherche Clinique », Center Hospitalier Universitaire, Grenoble ; The « Etablissement Français du Sang EFS », Grenoble, the « Fondation Arthritis Courtin » ; the « TRALI fellowship », EFS, Paris and the « Fonds National de la Recherche Luxembourg ». We thank Dr M.H. Paclet for English language corrections and editing of the manuscript and Prof. J.L. Lenormand for helpful discussions.

## **AUTHOR CONTRIBUTIONS**

M.V.C.N., F.R. and L.Z. designed and performed experiments, and analyzed data. B.L. contributed to experiments. M.V.C.N. and F.R. wrote the manuscript. F.M. contributed to discussions and to the editing of the manuscript. M.V.C.N. developed the concept and managed the project.

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## FIGURE LEGENDS

**Figure 1 | General principle of Topological Determination by Ubiquitin Fusion Assay (ToDUFA) method.** C-terminus amino acid residue of transmembrane protein of unknown topology is tagged with Ubiquitin fused to GFP (UbGFP). **(a)** Cartoon representing a cell expressing membrane proteins tagged with an UbGFP that faces the extracellular or luminal compartment: ubiquitin is protected from DUBs processing and GFP proteins remain attached to the reference protein. **(b)** Cartoon representing a cell expressing membrane proteins tagged with an UbGFP that faces the nucleocytoplasmic compartment: ubiquitin is cleaved by surrounding DUBs releasing the “free” GFP in the nucleocytoplasm. **(c)** Schematic representation of Interleukin-1 Receptor Type I protein (IL1RI) and truncated Nox2 containing the first 131 amino acids (Nox2N131) displaying a C-terminus amino-acid residue exposed to the cytosol and to extracellular side.

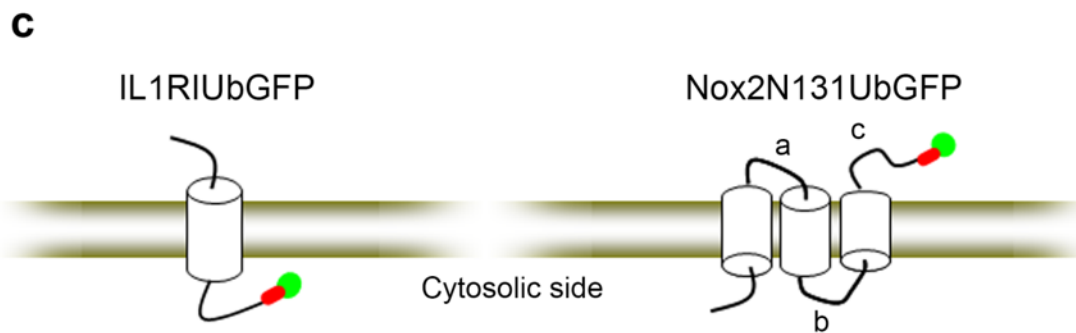
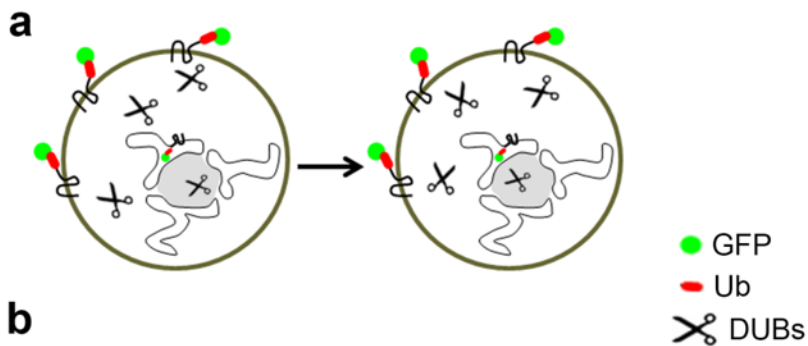
**Figure 2 | ToDUFA proof-of-principle by FACS analysis.** **(a)** Scheme describing the FACS protocol. HEK293 cells expressing Ubiquitin fusion proteins were scraped, treated or not with saponin and centrifuged. Supernatants were collected for further western blot analysis and cells were washed and suspended in PBS buffer for FACS analysis **(b)** FACS measurement of GFP fluorescence intensity in IL1RI-UbGFP and Nox2N131-UbGFP expressing cells treated or untreated with saponin. In graphs,

grey area indicates wild type HEK293 cells control fluorescence intensity. **(c)** Quantification of GFP fluorescence in IL1RI-UbGFP expressing cells or Nox2N131ubGFP cells treated or not with saponin. Data are expressed relatively to untreated cells. **(d)** GFP western blot from IL1RI-UbGFP, IL1RI-GFP, Nox2N131-UbGFP and Nox2N131-GFP cells supernatants after saponin treatment. Ctl, recombinant GFP positive control.

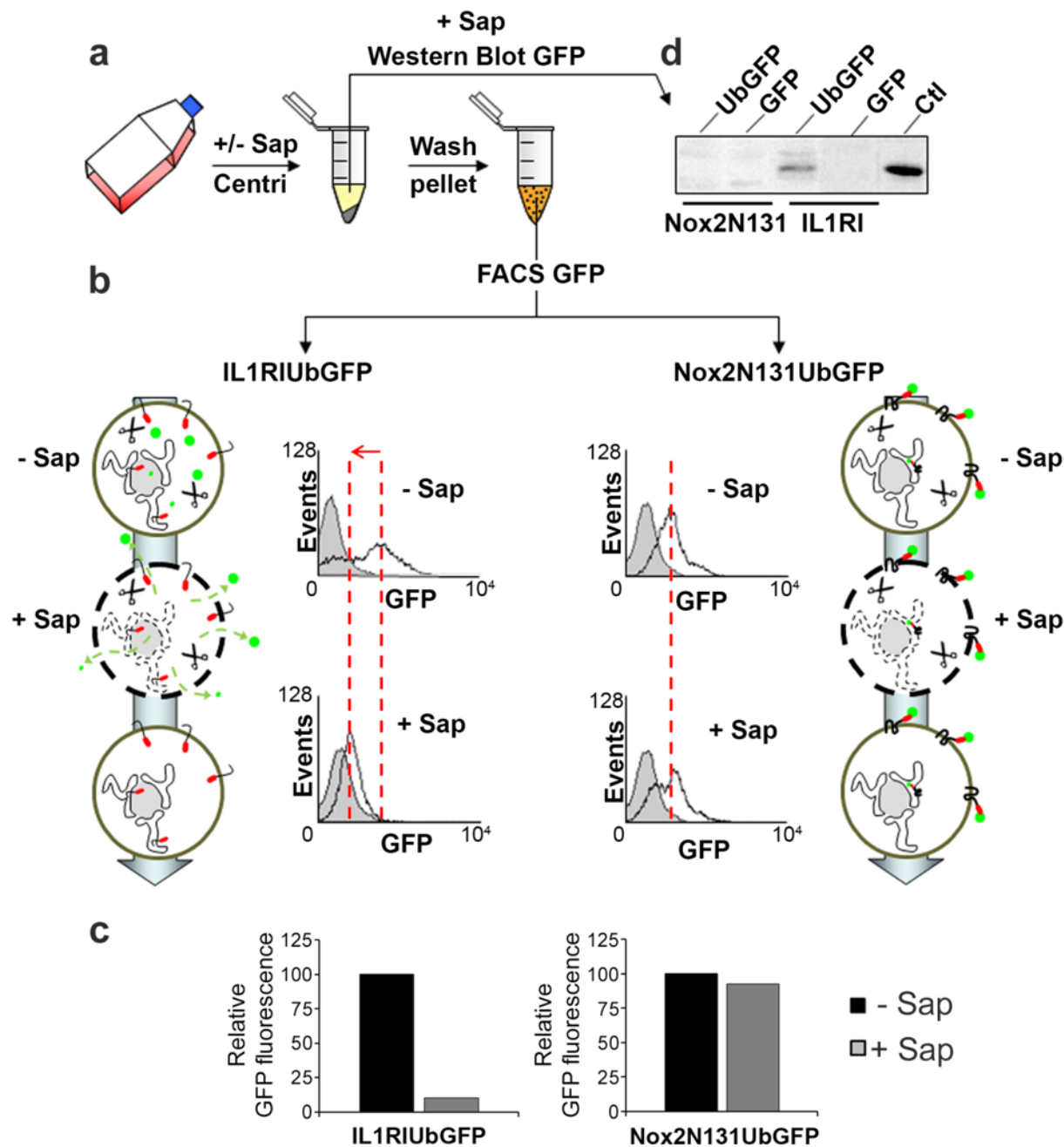
**Figure 3 | Establishment of the ToDUFA method by western blot.** **(a)** Scheme describing the protocol used for Western blotting analysis of the ToDUFA method. Cells expressing UbGFP fusion proteins were scrapped, lysed by sonication. Cytosol and membrane fractions were separated by ultracentrifugation and 100 µg of proteins from each fraction were then loaded on SDS-PAGE for GFP immunoblotting. **(b)** Upper panel: cartoon of a cell expressing ubiquitin fusion protein facing the nucleocytoplasmic side. Western blot from the cytosolic and membrane fraction of IL1RI-UbGFP transfected cells showing the presence of “free” GFP (27kDa) released in the cytosol fraction. Lower panel: cartoon of a cell expressing ubiquitin fusion protein facing the exterior/luminal side. Western blot of the cytosolic and membrane fraction extracted from Nox2N131-UbGFP transfected cells showing the non-cleaved fusion protein (41kDa) in the membrane fraction. Ctl: recombinant GFP positive control.

**Figure 4 | ToDUFA reveals the topology of the membrane protein NADPH oxidase Nox4.** UbGFP tag (green dots) was added to the C-terminus moiety of the full-length (FL) or truncated forms of Nox4 corresponding to each putative loop (residues 46, 98, 144, 184, 239, 341). For each panel: **(a)** the upper graph corresponds to untreated cells and the lower to saponin treated cells. Grey area

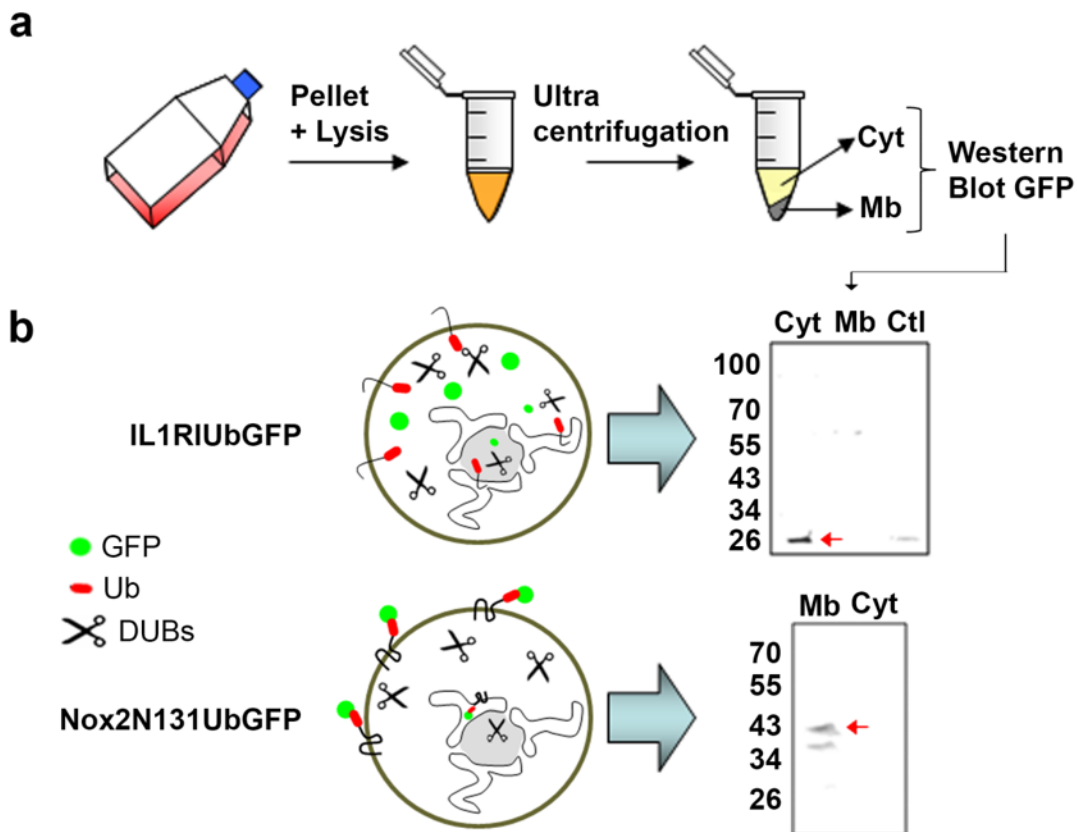
indicates wild type HEK293 cells. **(b)** GFP Western blot of cytosol and membrane fractions from cells expressing ubiquitin fusion proteins. **(c)** Histogram summarizing fluorescence data obtained by FACS experiments of different forms of Nox4 fused to UbGFP. Fluorescence measured in the saponin treated cells is expressed relatively to the fluorescence of untreated cells (100%). Red bars show a significant decrease in GFP fluorescence intensity indicating an cytosolic orientation of the corresponding C-terminus amino acid residue. **(d)** Schematic representation of Nox4 topology, as determined by ToDUFA. Epitopes of monoclonal antibodies (6B11, 5F9 and 8E9) confirm the correct topological determination by ToDUFA of the corresponding Nox4 sequence [21].



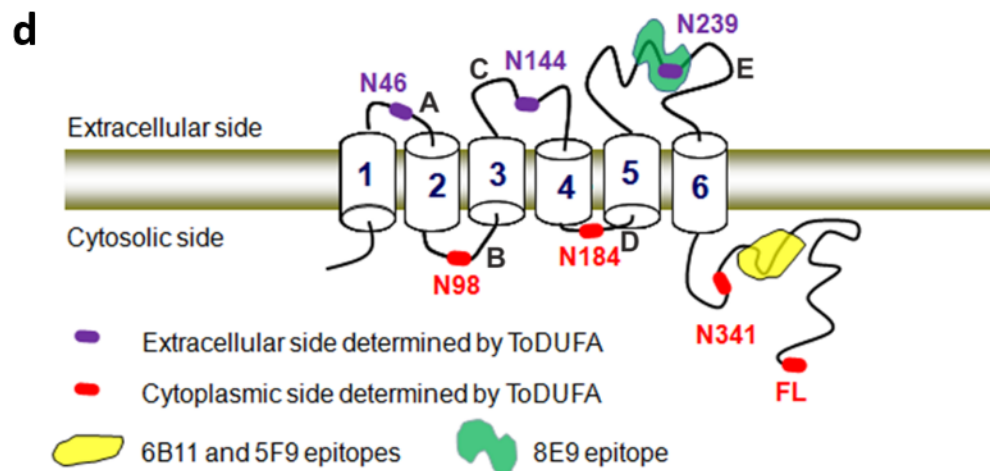
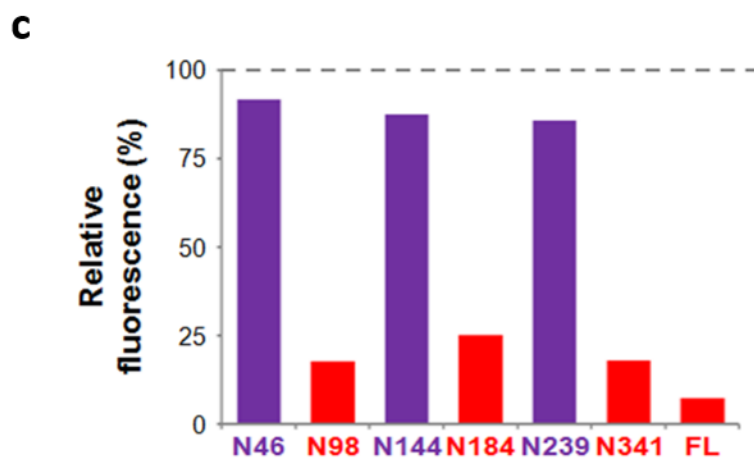
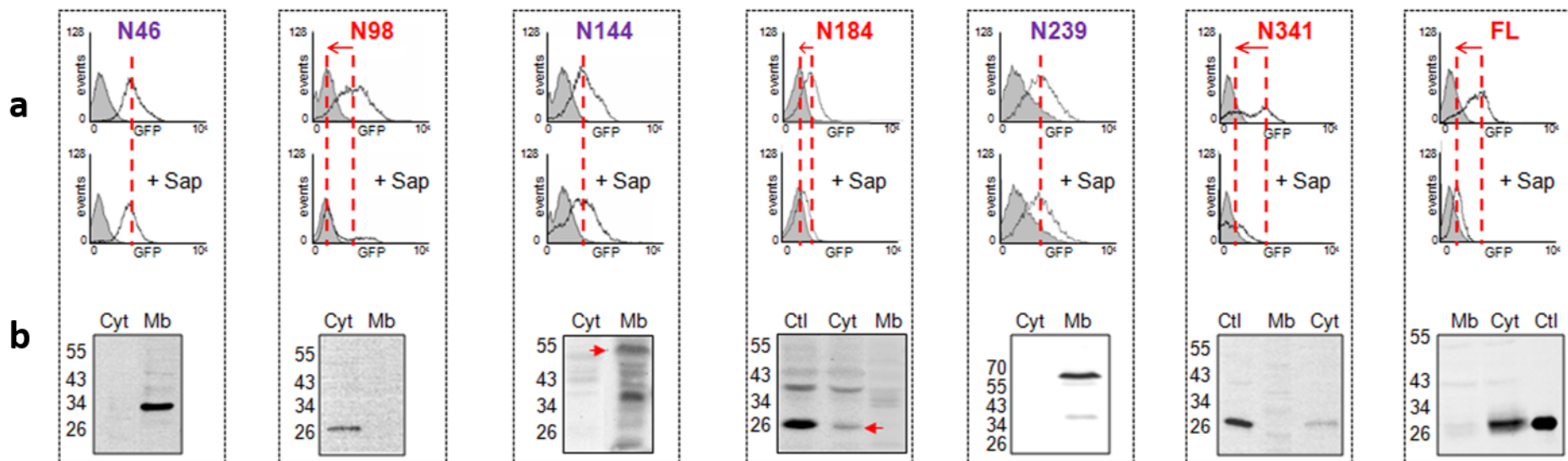
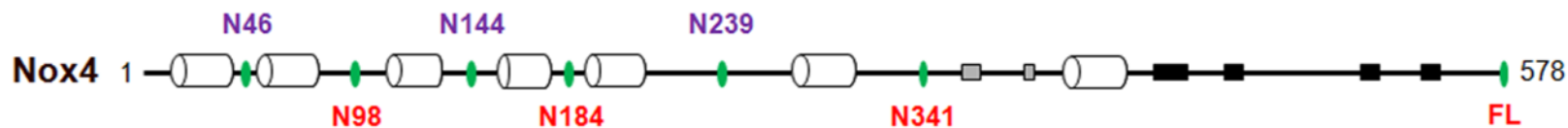
**Figure 1**



**Figure 2**



**Figure 3**



**Figure 4**