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# CHARACTERIZATION OF PEROXIDASIN AND PEROXIDASIN-LIKE PROTEINS IN MAMMALIAN CELLS

Ph.D thesis

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**List of Abbreviations**

<sup>32</sup>P: radioactive isotope of phosphorus

7S: N-terminal domain of collagen IV

A431: human epidermoid carcinoma cell line

AH<sub>2</sub>: one-electron donor

AH: free radical

AngII: angiotensin II

Arg: Arginine

ASD: anterior segment dysgenesis

Asp: Aspartic acid

BMPs: Bone Morphogenetic Proteins

Br<sup>-</sup>: bromide ion

C57BL/6: a common inbred strain of laboratory mouse

C736S, C1315S: a peroxidase mutant in which the cysteine 736 is exchanged to serine and cysteine 1315 is also exchanged to serine, this mutant is a monomeric form of peroxidase

CCN: cysteine-rich protein 61, connective tissue growth factor proteins, nephroblastoma overexpressed gene

cDNA: complementary deoxyribonucleic acid

*C. elegans*: *Caenorhabditis elegans*

CGD: chronic granulomatous disease

Cl<sup>-</sup>: chloride ion

CMK: decanoyl-Arg-Val-Lys-Arg-chloromethylketone

COL4A1: collagen type IV alpha 1 chain-antibody

COVID-19: COrona VIRUS Disease 2019

CRISPR-Cas9: Clustered Regularly Interspaced Short Palindromic Repeats-  
CRISPR associated protein 9

C-terminal: carboxyl-terminal

Cys: cysteine

D: aspartic acid

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DPI: diphenyliodonium

Duox: Dual oxidase

DuoxA1: Dual oxidase maturation factor 1

DuoxA2: Dual oxidase maturation factor 2

E: glutamic acid

ECM: extracellular matrix

EF-hand: Ca<sup>2+</sup> binding domain

EGF: Epidermal Growth Factor

EPO: eosinophil peroxidase

ER: endoplasmic reticulum

FAD: flavin adenine dinucleotide

FKBP1B: peptidyl-prolyl cis-trans isomerase FKBP1B

FLAG: common protein tag, an octapeptide with DYKDDDDK amino acid sequence

Gln: Glutamine

Glu: Glutamic acid

GTP: guanosine triphosphate

H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide

HA: epitope tag derived from the hemagglutinin protein

HaCaT: immortalized human epidermal keratinocyte cell line

HA-PXDN: hemagglutinin tag labeled peroxidase

hiPSC-CMs: induced pluripotent stem cell cardiomyocytes

His: Histidine

HOBr: hypobromous acid

HOCl: hypochlorous acid

HOI: hypoiodous acid

HOSCN: hypothiocyanous acid

HOX: hypohalous acid

Hyl: Hydroxylysine

I<sup>-</sup>: iodide ion

IgC2: Immunoglobulin C2 domain

I<sub>Na</sub>: inward sodium current

iPS: induced pluripotent stem cell

I<sub>to</sub>: transient outward potassium current

Kc cell: drosophila cell line

KCND3-WT: wild type Potassium Voltage-Gated Channel Subfamily D Member 3

kD: kilodalton

KDEL: lysine-aspartic acid-glutamic acid-leucine amino acid sequence containing peroxidase mutant, this mutant is unable to be secreted because of the presence of KDEL at the C-terminal

KO PFHR-9: peroxidase knockout mouse embryonal carcinoma cell line

LPO: lactoperoxidase

LRRs: leucine-rich repeats

Lys: lysine

MEF: mouse embryonic fibroblast

Met: Methionine

MG50: Melanoma Gene 50

MPO: myeloperoxidase

mRNA: messenger ribonucleic acid

MW: molecular weight

NADPH: reduced nicotinamide adenine dinucleotide phosphate

NC1: non-collagenous C-terminal domain

NH<sub>2</sub>: amine group

NIS: sodium/iodide symporter

Nox: NADPH oxidase

NOXA1: NADPH Oxidase Activator 1

NOXO1: NADPH Oxidase Organizer 1

N-terminal: amino-terminal

OSCN<sup>-</sup>: hypothiocyanate ion

p22<sup>phox</sup>: component of phagocyte oxidase which molecular weight is 22 kD

p40<sup>phox</sup>: component of phagocyte oxidase which molecular weight is 40 kD

p47<sup>phox</sup>: component of phagocyte oxidase which molecular weight is 47 kD

p53: tumor suppressor protein

p67<sup>phox</sup>: component of phagocyte oxidase which molecular weight is 67 kD

PCR: polymerase chain reaction



PFHR-9: mouse embryonal carcinoma cell line

phox: phagocyte oxidase

PXDN: peroxidasin

PXDNL: peroxidasin-like

PXDNL-WT: wild type peroxidasin-like protein

pxn-2: gene symbol for peroxidasin homolog pxn-2

Q: glutamine

Q823W, D826E: a peroxidasin mutant in which the glutamine 823 is exchanged to tryptophan and aspartic acid 826 is exchanged to glutamic acid, this mutant doesn't have peroxidase activity

Rac: small GTPase

RGR/QKK1333-1335: a peroxidasin mutant in which arginine 1333 is exchanged to glutamine, glycine 1334 is exchanged to lysine, and arginine 1335 is exchanged to lysine as well

RGRR: Arginine-Glycine-Arginine-Arginine sequence motif

RK/AA 1354-1355: a peroxidasin mutant in which the arginine 1354 and lysine 1355 are both exchanged for alanine

ROS: reactive oxygen species

RR/AA 1335-1336: a peroxidasin mutant in which the arginine 1335 and arginine 1336 are both exchanged for alanine

S1/S2 site: cleavage site between the two subunits of the spike protein

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

SCN<sup>-</sup>: thiocyanate ion

SCN5A-WT: wild type Sodium Voltage-Gated Channel Alpha Subunit 5

SCN9A: Sodium Voltage-Gated Channel Alpha Subunit 9

siRNA: small interfering ribonucleic acid

SNP: single nucleotide polymorphism

S protein: Spike protein

TGF- $\beta$ : Transforming Growth Factor beta

TO-PRO-3: Thiazole Red, fluorescent dye for nuclear counterstaining

TPO: thyroid peroxidase

Trp: Tryptophan

Tyr: Tyrosine

X: halide, pseudohalide

V5: common protein tag, composed of the following amino acid sequence:

GKPIPPELLGLDST

VPO1: vascular peroxidase 1

vWFC: Von Willebrand factor type C domain

W: tryptophan

WT PFHR-9: wild type mouse embryonal carcinoma cell line

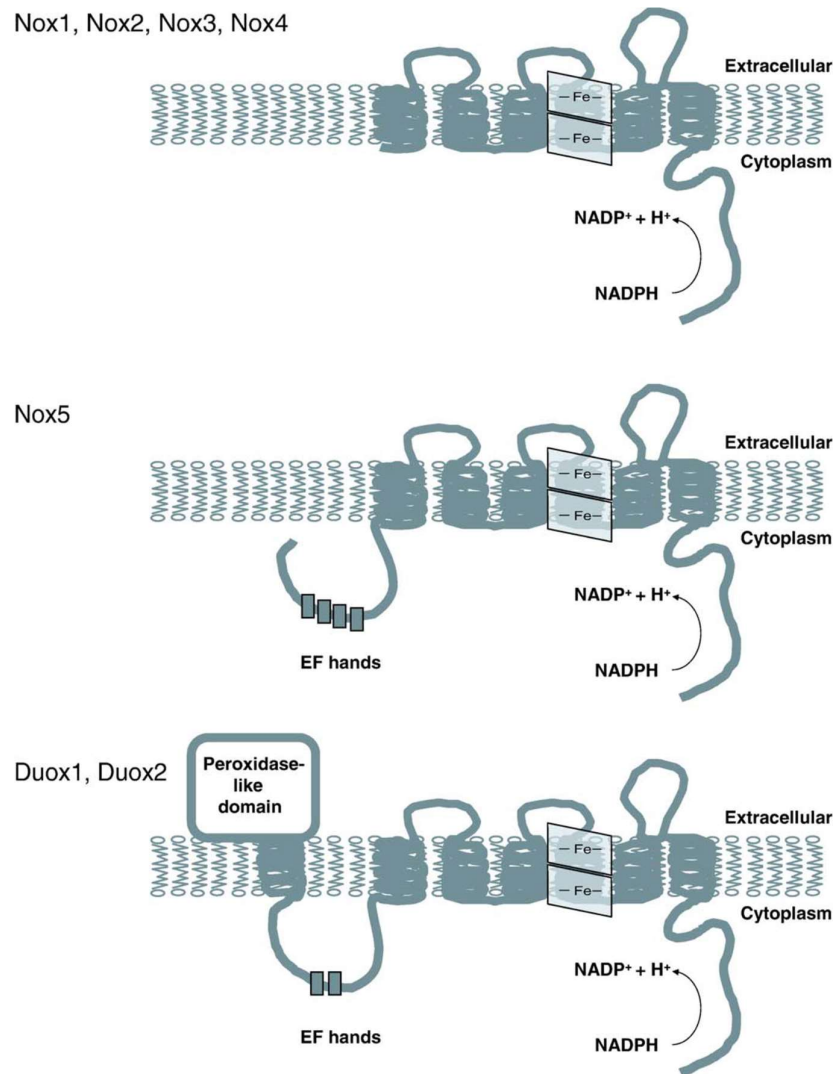
## 1. Introduction

Reactive oxygen species (ROS) are produced continuously in living organisms. Usually, they are considered as harmful chemical agents because of their high reactivity. ROS is either a free radical or a molecule that can generate a free radical. Free radical's high reactivity is due to their unpaired electron in their outer shell. Examples are superoxide, hydroxyl, hydroperoxyl, peroxy, and alkoxy radicals, which are often short-lived and generally they can not diffuse far from the site of synthesis. ROS that don't have an unpaired electron are hydrogen peroxide ( $H_2O_2$ ), and organic peroxides. Usually, they are less reactive, have a longer lifespan, and can more likely leave the cell. ROS can be produced spontaneously or enzymatically in a regulated manner. It's important to note that nowadays, as we get to know more about the biology of ROS, we have to reconsider the original "ROS is bad" way of thinking. It's still true, of course, that uncontrolled ROS production can cause oxidative damage of DNA, proteins, and lipids what is harmful to the organism. Still, ROS have several critical physiological functions in the body, for example, in host defense, hormone synthesis, cell signaling, extracellular matrix (ECM) modification, or even in balance sensation. One of the primary sources of ROS in the cell is the mitochondrion. During the electron transfer, some of the electrons can leak out to reduce oxygen to superoxide. Important regulated superoxide and  $H_2O_2$  sources are the NADPH oxidases (Nox/Duox), the homologs of which we can find in several different tissues. The Nox/Duox family often cooperates with heme peroxidases to further modify target molecules with  $H_2O_2$ . Other ROS generating enzymes in the cell are the cytochrome P450 family, xanthine oxidoreductase, superoxide dismutase, monoamine oxidase, and a major  $H_2O_2$  source is the beta-oxidation of fatty acids in the peroxisome. To limit and prevent the harmful effects of ROS, there are antioxidant mechanisms as well. They can be enzyme-catalyzed ROS elimination ways. For example, superoxide dismutase converts superoxide to less reactive  $H_2O_2$ , catalase breaks down  $H_2O_2$ , glutathione peroxidase also eliminates  $H_2O_2$ , glutaredoxin, and thioredoxin protect thiol-containing proteins, peroxiredoxin reduces peroxides, and glutathione-S-transferase decreases lipid peroxidation. Some non-enzymatic antioxidant mechanisms are executed via molecules like glutathione, N-acetyl cysteine, vitamin C, and vitamin E (1, 2). As I mentioned before, ROS has several beneficial effects in living organisms, and some of these functions are executed through heme-peroxidases. In this Ph.D thesis, our work

mainly focuses on two of the less known members of the peroxidase-cyclooxygenase superfamily: peroxidasin (PXDN) and peroxidasin-like (PXDNL) proteins.

### 1.1 Nox/Duox family

The Nox/Duox family members are the primary source of regulated ROS production in several organisms. The enzyme family has seven members in humans: Nox1,2,3,4,5 and Duox 1,2. Surprisingly mice and rats don't have the Nox5 isoform. Various cells and tissues express NADPH oxidases (3). It's part of the homeostasis to maintain the appropriate level of ROS, but we also know that a high level of these compounds can easily cause oxidative damages to molecules. Still, we also learned that inappropriately low levels of them could lead to severe immune deficiency like chronic granulomatous disease (CGD, phagocytic superoxide production by Nox2 complex is impaired)(4), or to hormone synthesis deficiency like congenital hypothyroidism caused by impaired function of Duox2 (5). All the family members have transmembrane domains and transport electrons across biological membranes (**Figure 1.**). The electron donor is an NADPH molecule, and it reduces oxygen to superoxide. Nox/Duox enzymes have several conserved structures in common: an NADPH binding site at the C-terminal part of the protein, a FAD-binding region, six conserved transmembrane domains, and four conserved heme-binding histidines (3). Nox1, Nox2, Nox3 complexes are composed of the Nox subunit and p22<sup>phox</sup>, and they have associated cytosolic partners. Nox5, Duox1, Duox2 don't need cytosolic factors, but they have EF-hands that sense intracellular Ca<sup>2+</sup>. Duox1 and Duox2 have an additional transmembrane domain, and an extracellular peroxidase-like domain, and these enzymes mainly release H<sub>2</sub>O<sub>2</sub> instead of superoxide. Duox enzyme's activation requires DuoxA1 and DuoxA2. Nox4 is associated with p22<sup>phox</sup> and can further reduce superoxide to H<sub>2</sub>O<sub>2</sub> (6, 7). In recent years our knowledge of the Nox/Duox family increased a lot thanks to the development of knockout animal models (8).



**Figure 1. Domain structure of Nox/Duox family.** Nox1,2,3,4 have six transmembrane domains, Nox5 has additional Ca<sup>2+</sup> binding EF-hands, Duox1 and 2 have EF-hands and an extracellular peroxidase-like domain as well (9).

Nox1 was identified as the first homolog of Nox2 (10). It has a high expression level in the epithelial cells of the colon. To produce superoxide, it requires two other proteins, NOXO1 and NOXA1. In the first Nox1-deficient mice, it wasn't identified any obvious phenotype. Still, probably Nox1 has a role in host defense based on its homology with Nox2 and because many bacteria inhabit the colon that generally don't invade through the epithelial layer (8). Nox1 was also described in vascular smooth muscle cells. The data on the possible blood pressure modulating role of the enzyme is controversial (11, 12). Nox1 mRNA was also detected in dorsal root ganglion neurons and microglia (8).

Nox2 (gp91phox) was the first discovered Nox enzyme. It was shown to be expressed mainly in neutrophilic granulocytes and macrophages that is why it is often called the phagocyte NADPH oxidase. Nox2 is constitutively coupled with p22<sup>phox</sup>, and for its activation, the Nox2 complex requires the “organizer subunit” p47<sup>phox</sup>, the “activator subunit” p67<sup>phox</sup>, a small subunit p40<sup>phox</sup>, and the GTPase Rac protein. Nox2 expression is mainly phagocyte-specific, but it was found also in neurons, skeletal muscle cells, cardiomyocytes, endothelial cells, hematopoietic stem cells, and hepatocytes. In phagocytes, the enzyme localizes to both plasma and intracellular membranes. Upon the stimulation of resting phagocytes, Nox2 translocates to the surface and starts to generate ROS-superoxide. This phenomenon will cause a sudden increase in cellular oxygen consumption known as oxidative burst. The superoxide will be further converted into H<sub>2</sub>O<sub>2</sub> then, finally, hypochlorous acid (HOCl) is produced, a potent antimicrobial molecule (3). In addition, Nox2 was described in B lymphocytes, where it is necessary for bacterial killing during the phagocytic activity of B cells (13).

Nox3 is only expressed in the inner ear. It is necessary for the otoconia crystals formation in the vestibular system. Loss of function mutation in the gene causes balance and spatial orientation problems in mice (8).

Nox4 was first shown to be expressed in kidney epithelial cells (14). Since its discovery, it was identified in vascular smooth muscle cells, endothelial cells, cardiomyocytes, fibroblasts, osteoclasts, neurons, microglia, skeletal muscle, and adipocytes (3). Nox4 is mainly present in the proximal tubules of mice kidneys. They investigated the kidney phenotype in the absence of the enzyme and didn't find apparent alteration, not even in different kidney disease models (8). Zhang *et al.* studied the function of Nox4 in the adaptation of the heart to pressure overload with the help of various genetic mice models. They found that Nox4 has a protective effect in chronic load-induced stress, probably by enhancing angiogenesis (15). This finding is remarkable because it was the first one that stated the absence of Nox4 might have severe pathophysiological consequences. Endothelial cells express a high level of Nox4, where it probably plays a protective role for example against atherosclerosis (8). In the lungs, they also found Nox4 as a protective enzyme in the bleomycin-induced fibrosis model. In an ischemic stroke model, it was found that the lack of the enzyme was advantageous, probably because of the reduced

oxidative stress. The bone density is higher in Nox4 knockout animals, likely caused by impaired osteoclast function (8).

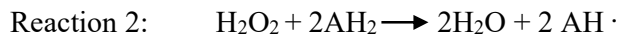
The strongest Nox5 expression was identified in the testis, spleen, and fetal tissues. In the testis mostly it is present in the spermatocytes. Recently it was also detected in blood vessels. Unfortunately, the enzyme gene is not present in the mouse genome, making it harder to investigate its function. In rabbits, its expression was detected in the lymph node, testis, and aorta. With the generation of a Nox5 knockout rabbit, Petheő and Kerekes *et al.* were able to study the function of the protein, and they found that after feeding the rabbits with atherogenic chow diet, in the Nox5 knockout rabbit, the aortic plaque formation was increased, suggesting a protective role for the enzyme in atherosclerosis (16).

Duox1 and Duox2 were first cloned from the human and porcine thyroid glands. These enzymes require the Duox activator proteins DuoxA1 and DuoxA2. The complete loss of function of Duox2 causes congenital hypothyroidism (8). Duox enzymes are also present on the mucosal surfaces of the gastrointestinal and respiratory tract. Geiszt *et al.* introduced a model where Duox is the source of H<sub>2</sub>O<sub>2</sub>, which oxidizes thiocyanate (SCN<sup>-</sup>) into hypothiocyanate (OSCN<sup>-</sup>) in cooperation with lactoperoxidase to form an effective antimicrobial compound (17). In *Drosophila*, Zebrafish, and *Caenorhabditis elegans* (*C. elegans*), an increasing amount of data suggest that Duox has an essential role in controlling the gastrointestinal tract microorganism environment (8). Duox 1 expression was detected in mouse urothelium, where in vivo cystometry experiments showed that Duox1 produced H<sub>2</sub>O<sub>2</sub> decreases the urinary bladder contractions (18). Duox1 was also shown to play a role in intracellular redox signaling. Sirokmány *et al.* showed that in A431 and HaCaT cells, there is a Ca<sup>2+</sup> induced Duox1 dependent H<sub>2</sub>O<sub>2</sub> production upon EGF stimulus, and this H<sub>2</sub>O<sub>2</sub> can cause the oxidation of thioredoxin-1 and cytosolic peroxiredoxins (19).

## **1.2 Peroxidase enzymes and their functional cooperation with NADPH oxidases**

Peroxidase enzymes oxidize a great variety of substrates with the help of H<sub>2</sub>O<sub>2</sub>. There are four heme peroxidase superfamilies, the members of which contain heme as a prosthetic group in the catalytic site. They are expressed in different kingdoms of life. One of the superfamilies is the peroxidase-cyclooxygenase superfamily, the unique feature of which

is the post-translationally modified heme group. This heme group is covalently bound to the protein, usually with two covalent linkages or, exceptionally, in the case of myeloperoxidase (MPO), with three covalent bonds. These enzymes can enter the peroxidase or the halogenation cycle during their catalytic activity. First, the native ferric peroxidase heme reduces the  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  via the formation of Compound I (oxoiron(IV) with porphyrin  $\pi$ -cation radical). Compound I can return to the resting enzyme form through reaction with two-electron donors (halides or pseudohalides,  $\text{X}^-$ ) in the halogenation cycle. Meanwhile, the electron donors become oxidized into hypohalous acids (HOX, Reaction 1). Compound I can also return to the native form via a two-step mechanism reacting with one-electron donors ( $\text{AH}_2$ ) and oxidizing them into free radicals ( $\text{AH}\cdot$ ; Reaction 2) via the formation of Compound II (20-23). I will further go into detail about the mammalian peroxidases: MPO, eosinophil peroxidase (EPO), lactoperoxidase (LPO), thyroid peroxidase (TPO), PXDN, and PXDNL. I will also describe the examples of cooperation between NADPH oxidases and peroxidase enzymes.



MPO mainly produces HOCl – a highly reactive oxidant- which can effectively damage invading pathogens at its production site. The azurophilic granules of neutrophilic granulocytes store the MPO. During neutrophilic granulocyte stimulation, granules are either fusing with the phagosome or release their content to the extracellular space. At the same time, the Nox2 complex is assembled to its active form and produces superoxide, which will be converted to  $\text{H}_2\text{O}_2$  by superoxide dismutase. Thus MPO will go through the halogenation cycle to oxidize chloride ( $\text{Cl}^-$ ) with  $\text{H}_2\text{O}_2$  into HOCl. Impairment of this system can cause immune deficiency disorder. In Nox2 complex deficiency, the disease CGD will develop with frequent bacterial and fungal infections. In MPO deficiency, usually, the patients are more susceptible to fungal infections (22). Besides its beneficial role in host defense, MPO is also implicated in various diseases like Alzheimer's, rheumatoid arthritis, kidney, and cardiovascular disorders (21, 24).

EPO is present in the granules of eosinophil granulocytes. Similar to MPO, it can oxidize halides with  $\text{H}_2\text{O}_2$  (Nox2 complex is the source of superoxide, which will be converted to  $\text{H}_2\text{O}_2$ ) (22), but mainly it converts bromide ( $\text{Br}^-$ ) and  $\text{SCN}^-$  into hypobromous acid



(HOBr) or hypothiocyanous acid (HOSCN), respectively. These compounds hurt pathogens, especially EPO-mediated eosinophil attack is vital in the fight against parasites. EPO is also linked with the pathogenesis of different diseases like asthma (24).

LPO was detected in various body fluids (such as milk, saliva, tears, and airway fluids) secreted by exocrine glands. In the Duox/LPO system, Duox is the source of H<sub>2</sub>O<sub>2</sub>, and LPO catalyzes the formation of HOSCN. Duox2 is expressed in major salivary ducts and rectal epithelial cells, Duox1 is described in the epithelial cells of the airway (17, 24). LPO can oxidize both I<sup>-</sup> and SCN<sup>-</sup>. These ions are transported at the site of action by sodium/iodide transporter (NIS). The main product is the HOSCN which has potent antimicrobial effects on the mucosal surfaces (22).

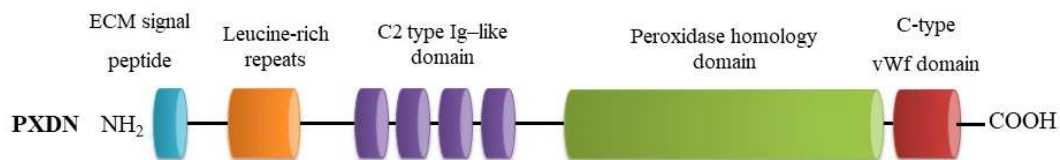
TPO is expressed by the thyroid gland's follicular cells. Thanks to the high local concentration of iodide (I<sup>-</sup>), it can produce hypiodous acid (HOI), which is necessary for synthesizing triiodothyronine and thyroxine (25). In the Duox2/TPO system, Duox2 produces H<sub>2</sub>O<sub>2</sub>, which TPO uses. This peroxidase is in the apical membrane of the thyrocytes. Its active site faces the colloid and catalyzes the thyroglobulin iodination. Loss of function mutations of Duox2, DuoxA2, or TPO causes hypothyreosis, underlining the essential role of these proteins in thyroid hormone synthesis (22).

Another example of the cooperation between NADPH oxidases and peroxidases is the urchin Duox1/ovoperoxidase system, Duox1 is the H<sub>2</sub>O<sub>2</sub> source, and ovoperoxidase catalyzes the crosslinking of protein tyrosyl residues in the fertilization envelope. The fertilization envelope is a hardened membrane that can prevent polyspermy (22).

### 1.3 PXDN

PXDN is a member of the peroxidase-cyclooxygenase superfamily. It was first discovered in *Drosophila melanogaster* (26). Nelson *et al.* purified the protein from the conditioned media of Kc cells, and through gel electrophoresis experiments, they found that PXDN molecular weight is 170kD. After determining the amino acid sequence of an N-terminal peptide fragment of PXDN, the authors generated <sup>32</sup>P-labeled oligonucleotides with all the possible coding sequences corresponding to this peptide sequence and used these probes to screen cDNA libraries. By identifying several overlapping cDNA clones, they managed to clone the entire open reading frame of the *Drosophila* peroxidase, which consists of 1535 amino acids. They also proved that it forms homotrimers through

disulfide bridges. The purified protein had peroxidase activity in many different assays, and the mRNA of PXDN was detected mainly in the hemocytes of *Drosophila* during embryogenesis. PXDN has a unique domain structure, beyond its peroxidase domain, it also carries several modules typical for ECM proteins. The peroxidase domain has high homology with the other members of the enzyme superfamily (23, 26). At the N-terminal of PXDN, there is a 23 amino acids long secretory signal peptide, followed by six leucine-rich repeats (LRRs), then four immunoglobulin (Ig) domains. After this, there is the peroxidase domain, then an amphipathic alpha-helix, and at the C-terminal, there is a C-type von Willebrand factor domain (vWFC) (**Figure 2.**)



**Figure 2. Domain structure of PXDN.** The protein has domains characteristic for ECM proteins (Leucine-rich repeats, C2 type Ig-like domain, C-type vWf domain) and a peroxidase homology domain. The figure is modified from Figure 1.A of (27).

### 1.3.1 Human PXDN, structural elements of the protein

The human PXDN gene was first cloned as a p53 responsive gene from a colon cancer cell line (28). The gene was also expressed in melanoma samples and was called melanoma-associated gene 50 (MG50) (29). Another group named the mammalian protein vascular peroxidase 1 (VPO1) because the expression was high in vascular tissues (30). This name of PXDN is not in use anymore since it has a more widespread expression than it was thought initially. The mammalian PXDN's structure is very similar to the one in *Drosophila* (**Figure 2.**). The human protein is 1479 amino acids long. Its calculated molecular weight is 165kD. PXDN has N-terminally an ECM signal peptide followed by LRRs. These repeats are not a unique feature of PXDN, they appear in several different proteins, and primarily it is involved in the mediation of protein-protein interactions. This ability can facilitate ECM assembly, cell adhesion, signal transduction, neuronal development, platelet aggregation, and immune response (31). This module has a

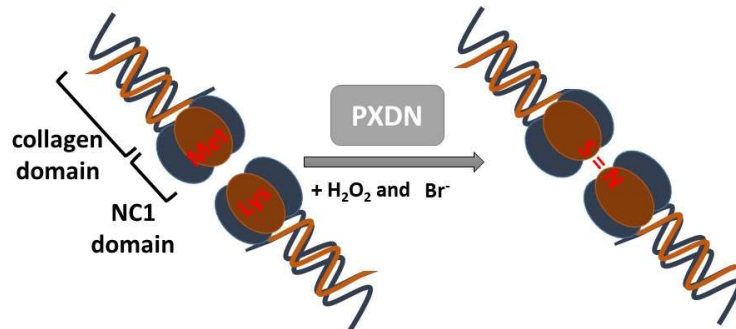
solenoid structure. Each repeat corresponds to a turn that usually has a length of 20-30 amino acids. The conserved leucines and other primarily hydrophobic amino acids form the inner tightly packed core of the solenoid and are responsible for the stability of the domain. N- and C-terminally cysteine-rich flanking regions protect the core from opening in PXDN (32). After the LRRs, the following domains are the immunoglobulin domains in PXDN. There are different categories like C-domains, C-like domains, V-domains, and V-like domains (33). There are four C-like Ig domains in PXDN. The biological function of the Ig-fold motif is typically cell adhesion and pattern recognition (34). The only known enzymatic motif in PXDN is the heme-containing peroxidase domain (this domain will be described in detail in the next part). At the C-terminal of PXDN, there is a vWFC domain. A consensus sequence of ten cysteines defines this motif, and usually, it is 60-80 amino acids long (35). The name of this domain comes from a multimeric blood glycoprotein, the von Willebrand factor, which facilitates platelet adhesion and stabilizes clotting factor VIII in the blood (36). The vWFC domain is present in a significant number of ECM proteins, including procollagen, glycosylated mucins, thrombospondin, neuralins, and CCN (cysteine-rich protein 61, connective tissue growth factor proteins, nephroblastoma overexpressed gene). The number of domains in different proteins can be different. Even amongst peroxidases from different species can be variable. In the human PXDN, there is one domain at the C-terminal of the protein. One of the most common functions of the vWFC module is the binding and regulation of transforming growth factor beta (TGF- $\beta$ ) and bone morphogenetic proteins (BMPs) (37). Another role of the motif is the mediation of protein oligomerization (36). The oligomerization was described first in *Drosophila* PXDN (26). Later on, our research group and an other research team independently showed that human PXDN also exists in the form of homotrimers linked by disulfide bridges (38, 39). The trimers are formed both in heterogeneously and endogenously PXDN-expressing cells. Our group proved with site-directed mutagenesis experiments that Cys736 and Cys1315 are required for the disulfide bond formation in the process of trimerization (39). The other group concluded that a PXDN truncated at amino acid position 1288 couldn't form trimers (38). PXDN's only known physiological role is the crosslinking of collagen IV (this function will be explained in detail later) molecules (40). This crosslinking activity seems to be decreased in heterogeneous expression systems where cells are expressing a mutant form of PXDN,

which is incapable of trimerization because of the absence of these critical cysteines (39). PXDN is highly glycosylated, seven glycosylation sites were identified in the peroxidase domain, and additional three sites were described: one in the second Ig domain, one between the peroxidase motif, and the vWFC domain, and the last one in the vWFC domain (38).

### 1.3.2 The enzymatic activity of PXDN

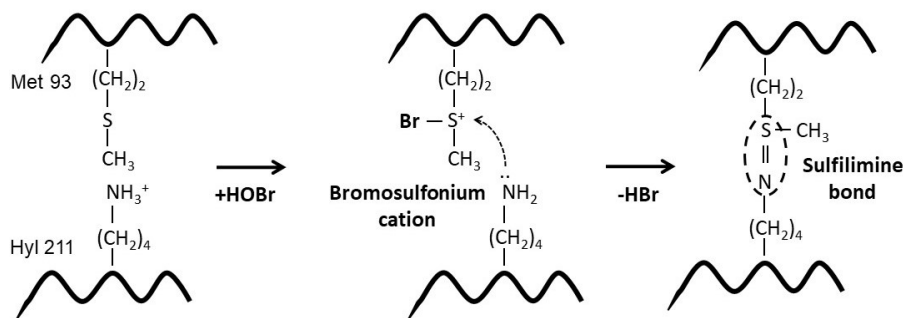
The peroxidase domain of PXDN is homologous to other animal peroxidases (MPO, EPO, LPO, TPO), and it is closest in similarity to LPO (23). The catalytic motif has a covalently bound heme group (38). Many critical residues play a role either in the cleavage of  $H_2O_2$  (in the distal heme cavity His827 and Arg977) or in the binding of halides (Gln823) (23). As an active peroxidase enzyme, it can catalyze reactions following halogenation and the peroxidase cycles (41, 42). As was mentioned before, PXDN is responsible for the crosslinking of collagen IV molecules which is the major component of basement membranes. The crosslink is made between Met93 and hydroxylysine (Hyl)/lysine (Lys) 211 of two neighboring NC1 domains of collagen IV by forming a sulfilimine covalent bond (43) (**Figure 3**). The crosslinking activity is a specialty of PXDN, although EPO can also form HOBr, but its crosslinking activity is not comparable with PXDN (44). The sulfilimine chemical bond is unique in living organisms, it has been shown only between the collagen IV molecules. The crosslinking occurs through the halogenation cycle and another unique feature is that it uses HOBr. Till now this is the only known physiological role of the trace element bromine in the body.  $H_2O_2$  oxidizes the resting enzyme during the catalysis to form compound I, then compound I can directly react with two-electron donors like  $Br^-$  or pseudohalide ( $SCN^-$ ) to make HOBr or HOSCN. The substrate specificity and pre-steady-state kinetics of PXDN were investigated with an enzymatically active truncated variant (composed of the four Ig and the peroxidase domains). This construct proved that compound I can only react with bromide, iodide, and thiocyanate but not with chloride (41). Amongst these possible substrates, for the crosslinking reaction, PXDN prefers to use  $Br^-$  as it was detected *in vitro* in PFHR-9 cell culture (40). This observation was further confirmed *in vivo* in *Drosophila* (45). First, HOBr is formed during the crosslinking reaction, which reacts with Met93 to make a bromosulfonium cation. This intermediate can react with water to form methionine sulfoxide (a “dead end” in collagen IV crosslinking) or can

interact with the  $\epsilon$ -NH<sub>2</sub> of Hyl/Lys 211 to make the sulfilimine bond (this pathway has a low energetic barrier) (**Figure 4**).



**Figure 3. Schematic figure of sulfilimine bond formation.** The crosslink is made between the Met and Lys residues of two adjacent NC1 domains of collagen IV molecules. The reaction is catalyzed by PXDN with the help of Br<sup>-</sup> and H<sub>2</sub>O<sub>2</sub>. The figure is modified from Figure 1.B of (27).

Fluoride is inert to this reaction. I<sup>-</sup> and SCN<sup>-</sup> can inhibit the sulfilimine bond formation *in vitro*. A possible explanation for the inhibition is that these ions can compete with Br<sup>-</sup>, on the other hand, their corresponding hypohalous acids do not form halosulfonium ions in a significant amount (40, 45, 46). It is essential to highlight that in the human plasma Br<sup>-</sup> concentration is 10-100  $\mu$ M, which is enough for the crosslinking reaction based on the observations with *Drosophila*. The *in vivo* plasma I<sup>-</sup> level is 0.2-0.4  $\mu$ M. It can not interfere with the crosslinking, but SCN<sup>-</sup> plasma level is higher than Br<sup>-</sup> concentration. Especially in smokers, it can be even 70-300  $\mu$ M (47). This elevated SCN<sup>-</sup> level may interfere with the sulfilimine bond formation in basement membranes. Those domains of the enzyme that are important in forming intermolecular connections can help localize the formation of HOBr close to the NC1 domains preventing the possible oxidative damage on the surrounding other molecules (48, 49). PXDN can bind to laminin (another important component of basement membranes), positioning PXDN next to the collagen IV molecules (50).



**Figure 4. Molecular mechanism of sulfilimine bond formation.** The covalent bond is formed via the bromosulfonium cation intermediate formation between the Met93 of the NC1 domain and the Hyl/Lys 211 of an adjacent NC1 domain. The figure is modified from Figure 1.C of (27).

The halogenation cycle is undoubtedly crucial in crosslink formation. Still, PXDN can also go through the peroxidase cycle by reacting with H<sub>2</sub>O<sub>2</sub> and two one-electron donors. This alternative pathway can decrease the capacity of PXDN to participate in the halogenation cycle. *In vitro* cell culture experiments with PFHR-9 cells showed decreased crosslinking activity in the presence of urate (48). The source of H<sub>2</sub>O<sub>2</sub> for PXDN is still an unresolved question. Our laboratory studied the role of Nox/Duox family members as a potential source of H<sub>2</sub>O<sub>2</sub>. That work will be discussed later in the results and discussion chapter, as the current Ph.D thesis is partly based on that publication. Another still open question is whether PXDN catalyzes the bromination of proteins *in vivo*. Some data support this idea. For example, kidney Bowman's capsule-, glomerular- and tubule basement membranes are shown to accumulate bromine in a PXDN dependent manner both in mice and humans (51). ECM extracted from PFHR-9 cells also contains 3-Br-Tyr residues produced by PXDN (52).

### 1.3.3 Proprotein convertase processing of PXDN

Colon *et al.* investigated the proteolytic processing of PXDN. With the help of a proprotein convertase site prediction algorithm, they found a potential cleavage site in the PXDN sequence. This motif is the RGRR close to the C-terminal of the protein at Arg-1336. Cell culture experiments could prove that the cleavage can indeed happen at this site and that the cleaved protein has an increased crosslinking activity compared to the

uncleaved form (53). This Ph.D work analyzed further the details of the proprotein convertase processing.

#### **1.3.4 The biological function of PXDN**

The gene of PXDN in *C.elegans* (pxn-2) was studied through pxn-2 mutant animals. Seven mutations were identified in the peroxidase gene that turned to be lethal. The phenotype of the mutant animals showed epidermal elongation defect and disturbed epidermal-muscle interactions, which led to larval arrest and muscle detachment. The same study also found that PXN-2 might hurt axon regrowth after injury (54). Peroxidase was first discovered in *Drosophila* (26). In a later study, the effect of peroxidase mutation was examined on the phenotype. The homozygous genotype for the mutant allele was lethal at the larval stage, and it had a severely damaged basement membrane in the midgut visceral muscles (40). In zebrafish (*Danio rerio*), inhibition of PXDN translation results in decreased eye size, trunk patterning effects, and cardiac edema (55). PXDN mutation in mice causes an ocular developmental defect called anterior segment dysgenesis (ASD). Another phenotype of the PXDN knockout mouse is a pigmentation problem: black mice (C57BL/6) have white spots on their belly (56, 57). PXDN was also studied in differentiating mammalian fibroblasts, where it was proved that it participates in the building of ECM (58).

#### **1.3.5 PXDN role in diseases**

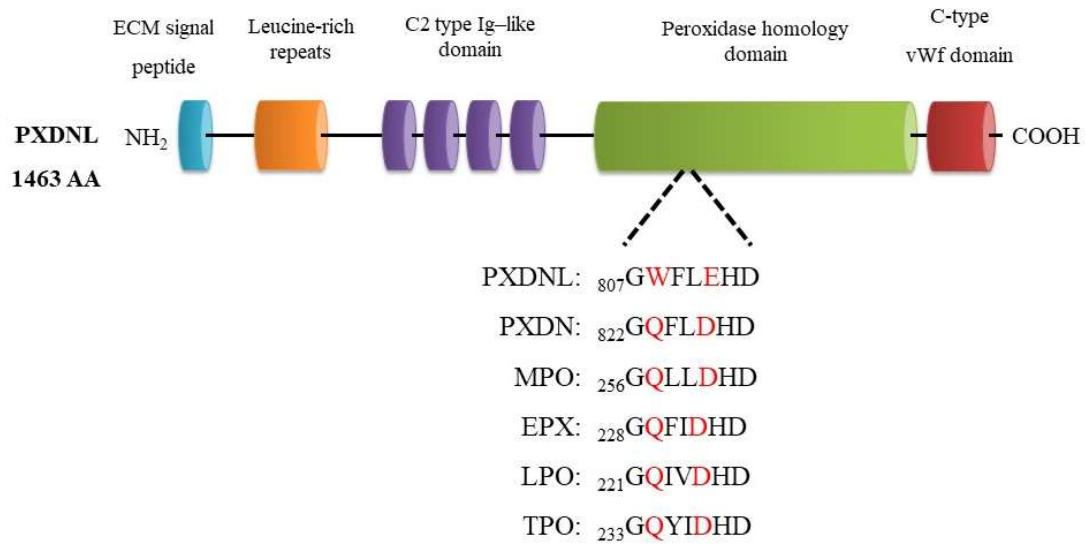
PXDN's function is highly investigated in the kidney. Bhave *et al.* reported - based on experiments with wild type (WT) and PXDN knockout mice - that the presence of sulfilimine crosslinks contributes to renal tubular basement membrane stiffness (59). This finding can help understand more kidney diseases that involve the basement membrane, like Alport syndrome. A murine unilateral ureteral obstruction model found that pharmacological inhibition of PXDN or its genetic absence can decrease renal fibrosis (60). In the serum of patients with Goodpasture syndrome, McCall *et al.* could identify anti-PXDN autoantibodies. These immunoglobulins displayed an inhibitory effect *in vitro* on PXDN-mediated HOBp production. The same work could also detect anti-PXDN antibodies in anti-MPO vasculitis, associated with a more active clinical disease (61). PXDN was also identified as a target of autoantibodies in lupus nephritis (62). As was mentioned before, the most apparent phenotype related to the dysfunction of PXDN is ASD. ASD is a group of developmental eye disorders involving the anterior chamber

structures. It can cause glaucoma, corneal opacity, or microcornea with cataracts and aniridia. Homozygous mutations in PXDN cause ASD in humans as well (63). Several other mutations in PXDN causing developmental eye problems in patients have been described recently (64, 65). Later on, experiments on mice proved that PXDN is essential for eye development (56, 57). A publication found upregulated PXDN levels in mice during atherogenesis and suggested that PXDN may contribute to arterial stiffening during atherosclerosis (66). PXDN was linked to distinctly different cancer pathology as well. It was reported as a glioma endothelial marker gene that is upregulated in primary and metastatic brain tumors (67). It was also found that PXDN might have an essential role in promoting melanoma cells invasion (68, 69), which can be related to the observed white spots on the belly of PXDN knockout mice, suggesting a role for PXDN in melanocyte migration or differentiation. In ovarian cancer, it was also found that PXDN might have a promoter role in the proliferation and migration of cancer cells (70). PXDN was suggested in prostate cancer as a potential therapeutic target because of its tumor-promoting effect (71). PXDN was also studied in oral squamous cell carcinoma as a tumor promoter gene (72, 73).

#### **1.4 PXDNL**

PXDNL is the less known member of the mammalian peroxidase family. The gene is absent from mice and rats, making its research more complicated, but it is expressed in humans and many other mammalian species. Our group described and characterized the human PXDNL first (74). We found that it has 1463 amino acids (calculated molecular weight is 164 kD), and it is highly homologous to PXDN (58% identity and 72% similarity). The domain composition of PXDNL is practically the same as of PXDN. Still, notably in its peroxidase domain, highly conserved amino acids are replaced with other residues (808Q/W, 811D/E), which can explain the lack of measurable peroxidase activity. **(Figure 5).**





**Figure 5. Domain structure of PXDNL.** The protein has domains characteristic for ECM proteins (Leucine-rich repeats, C2 type Ig-like domain, C-type vWf domain) and an inactive peroxidase homology domain. Highly conserved amino acids (indicated with red color) are different in the peroxidase domain of PXDNL compared to other mammalian peroxidases. The figure is modified from Figure 1.A of (27).

In the same publication, we show that PXDNL mRNA is only detectable in the heart from the examined human tissues. In a heterologous expression system, it doesn't have peroxidase activity, localizes to the endoplasmic reticulum (ER), and can be detected extracellularly as well. After the co-expression of PXDNL with PXDN, we could show that they form a complex, and in the presence of PXDNL, PXDN has less peroxidase activity. In human cardiomyocytes of iPS origin, it was located at the plasma membrane beside the intracellular localization. Upon AngII stimulus of iPS cell-derived cardiomyocytes, PXDNL mRNA level was elevated. The protein was present on the lateral surface of the cells and at the intercalated discs on human heart sections. With *in situ* hybridization and quantitative PCR, we detected an increased level of PXDNL in the diseased heart sample compared to the healthy one (74). Besides our observation in the PXDN-PXDNL co-expression system, another group also suggested an antagonistic role between the two proteins in *C.elegans* (54).

#### 1.4.1 PXDNL roles in diseases

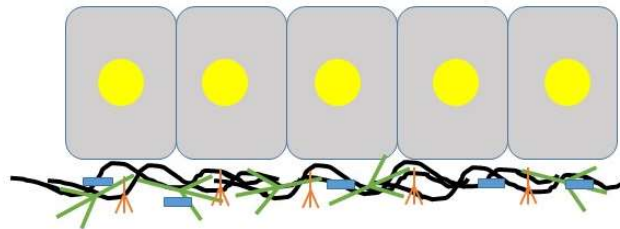
There are many findings related to PXDNL and different pathologies that can be partly understood based on the data we know about this protein.

One of the risk factors of metabolic syndrome and cardiovascular diseases is hyperuricemia (75, 76). A study investigated the genetic background of the correlation between obesity and elevated uric acid level. They performed a genome-wide association study and found a weak association between PXDNL SNPs and plasma uric acid levels (77). Pongor *et al.* identified PXDNL as a top tumor suppressor gene in breast cancer (78). Another group further investigated this gene in breast cancer and reported that PXDNL expression is a potential and independent prognostic biomarker in the manner that the high expression of the gene is related to the poor overall survival of the tumor (79). PXDNL was also found as an outstanding susceptibility gene for schizophrenia, bipolar disorder, and major depressive disorder (80). There is a publication that reported a patient who has a homozygous splicing mutation in the *pxdnl* gene. This patient has an intellectual disability, partial agenesis of the corpus callosum, hypoplastic cerebellum, and brainstem (81). Loss-of-function variation in PXDNL was associated with aberrant left-right patterning (laterality defects) in the developing heart of the human embryo (82). This phenotype seems to be logical based on the high PXDNL expression in the human heart. Another recent finding which further strengthens the importance of PXDNL in the human heart is a report about a patient who has a heterozygous mutation in PXDNL (and in two other genes: *FKBP1B* and *SCN9A*) and exhibited palpitations and syncope. They prepared induced pluripotent stem cell cardiomyocytes (hiPSC-CMs) from the patient's fibroblasts and examined them with electrophysiological methods that revealed irregular spontaneous activity. After cloning the construct which codes the mutated PXDNL, they cotransfected it with *SCN5A*-WT or with *KCND3*-WT. The first cotransfection resulted in a lower  $I_{Na}$  (inward sodium current) density compared to PXDNL-WT cotransfection. The second one resulted in higher  $I_{to}$  (transient outward potassium current) density compared to PXDNL-WT (83).

#### 1.5 The structure of basement membranes

The basement membrane is a unique extracellular matrix protein complex usually between an epithelium or endothelium monolayer and the underlying connective tissue (84) (**Figure 6**). It is noteworthy that the basement membrane is also present around

skeletal-, cardiac-, and smooth muscle cells (85). Even neuromuscular synapses contain collagen IV (86), a significant component of basement membranes, and the connective tissue capsule surrounding fat cells is also rich in these molecules. There are four major components of the basement membranes: collagen IV, laminin, nidogen/entactin, and perlecan. Collagen IV and laminin are respectively self-assembled into superstructures. These networks are connected via bridges formed by nidogen/entactin proteins and perlecans. There are also many other protein components of the basement membranes, for example, fibulin, agrin, type XV collagen, type XVIII collagen, and osteonectin (84, 87). Various physiological roles are addressed to basement membranes, like orientating and organizing cells during tissue development, providing mechanical support to cells, outside-in signaling. It can work as a selective barrier during filtration (kidney), and it is also important in the guidance of cell differentiation, proliferation, and migration (84, 88). Collagen IV is a nonfibrillar collagen that gives around 50% of the basement membrane proteins. In mammals, six different genes encode the various alpha chains (alpha1-6). These isoforms are highly conserved among vertebrates (89). Each chain comprises an N-terminal 7S domain, a triple-helical collagenous domain, and a C-terminal noncollagenous globular NC1 domain. Three alpha chains (in specific combinations like  $\alpha1\alpha1\alpha2$ ,  $\alpha3\alpha4\alpha5$ , and possibly  $\alpha1\alpha1\alpha5$ ,  $\alpha1\alpha2\alpha5$ ,  $\alpha5\alpha5\alpha6$ ) self-assemble into a protomer, then secreted from the cells. The protomers form a sheet-like network extracellularly: four protomers connect via disulfide bridges and lysine crosslinks through the 7S domains. Two protomers are also interconnected via sulfilimine bonds formed between adjacent NC1 domains (43). This unique covalent bond formation is catalyzed by PXDN (40).



**Figure 6. Schematic representation of cells with the underlying basement membrane.**

## 2. Objectives

PXDN is a multidomain protein with an active heme peroxidase domain. Its only known biological function is the catalysis of crosslink formation between collagen IV molecules in the basement membrane. PXDN goes through posttranslational proteolysis *in vitro* mediated by proprotein convertase enzymes. This will result in the cleavage of the C-terminal vWFC domain.

Our goal was to establish an *in vitro* model to study PXDN and the effect of certain features of PXDN (proprotein convertase processing, trimerization, localization) on the localization, processing, and crosslinking activity of the protein. We also wanted to know whether the proprotein convertase processing of PXDN can happen *in vivo* or not and to prove that the proprotein convertase that mediates the proteolysis is furin. We also wanted to explore whether the peroxidase activity is necessary for the processing and investigate the possible proprotein convertase mediated cleavage of PXDNL.

To achieve these goals, we established cell culture and animal model systems to answer our questions.

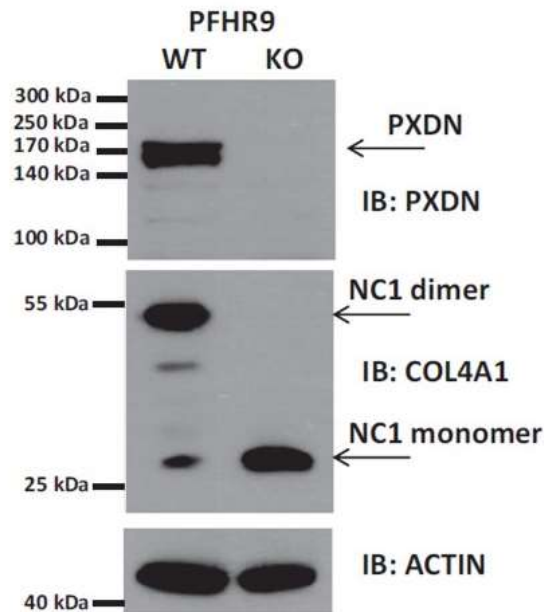
We made the following objectives:

1. Establishment of a PXDN knockout PFHR-9 cell line (KO PFHR-9) with the CRISPR-Cas9 technique.
2. Development of a mouse model with CRISPR-Cas9 technique, which expresses an N-terminally hemagglutinin (HA)-tagged PXDN (HA-PXDN).
3. Identification of the proprotein convertase which processes PXDN.
4. Studying the crosslinking activity, proprotein convertase processing, and localization of various double-tagged PXDN constructs.
5. Investigating the role of peroxidase activity in the proteolytic processing of PXDN and PXDNL.

### 3. Results

#### 3.1 Establishment of a PXDN knockout PFHR-9 cell line (KO PFHR-9) with the CRISPR-Cas9 technique

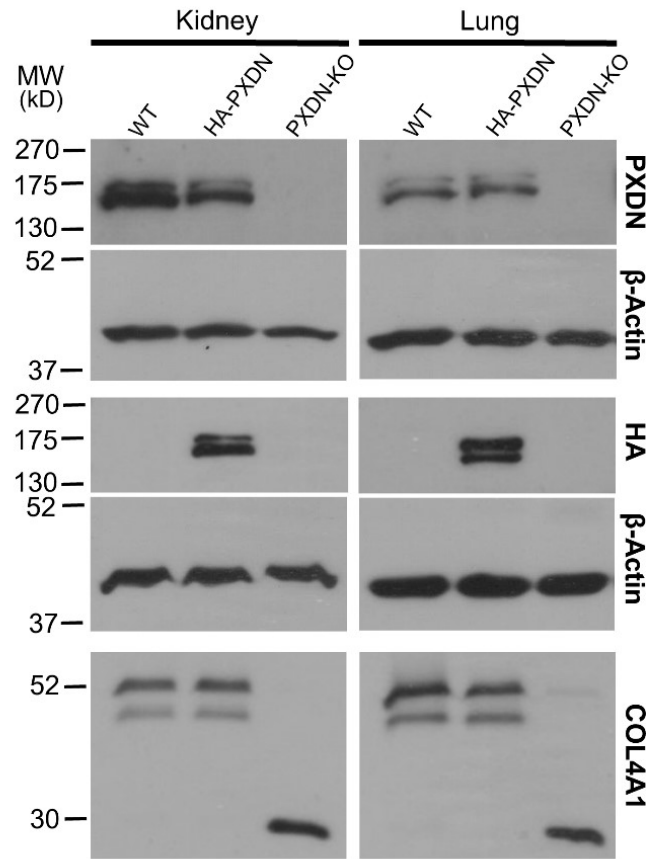
We decided to use the mouse embryonic cancer cell line, PFHR-9, as a model system for our *in vitro* experiments. Based on previous publications, we knew that this cell produces a large amount of ECM rich in collagen IV and expresses PXDN (40). In PFHR-9 cell lysates, we could detect the crosslinked NC1 dimers and uncrosslinked NC1 monomers with collagenase digestion and western blot assay. Furthermore, through the detection of crosslinked dimers, we could monitor the activity of PXDN. To study different PXDN constructs with a heterogenous expression, we created a PXDN deficient PFHR-9 cell line. This result was my contribution as a coauthor to the article of Sirokmány *et al.* (90). This KO PFHR-9 cell line was made with the help of the CRISPR-Cas9 technique (91). We verified the *pxdn* gene modification with Surveyor mismatch analysis and sequencing. We showed the absence of PXDN protein (**Figure 7. upper panel**) with western blot experiments and the lack of NC1 dimers in the KO PFHR-9 compared to the WT PFHR-9 cells with collagenase digestion assay and western blot analysis (**Figure 7. middle panel**). All these experiments confirmed the successful modification of the cell line.



**Figure 7. Characterization of PXDN KO PFHR-9 cells.** On the upper blot, the double band of PXDN is visible in the WT PFHR-9 cell at the appropriate molecular weight, representing the unprocessed and the proprotein convertase cleaved form of the protein (the lowest panel is the loading control). The middle panel shows the absence of crosslinked NC1 dimer in the KO PFHR-9 sample detected with an Alpha1 collagen IV isoform-specific antibody. The blots are representative of three independent experiments.

### **3.2 Development of a mouse model with CRISPR-Cas9 technique, which expresses an N-terminally hemagglutinin (HA)-tagged PXDN (HA-PXDN)**

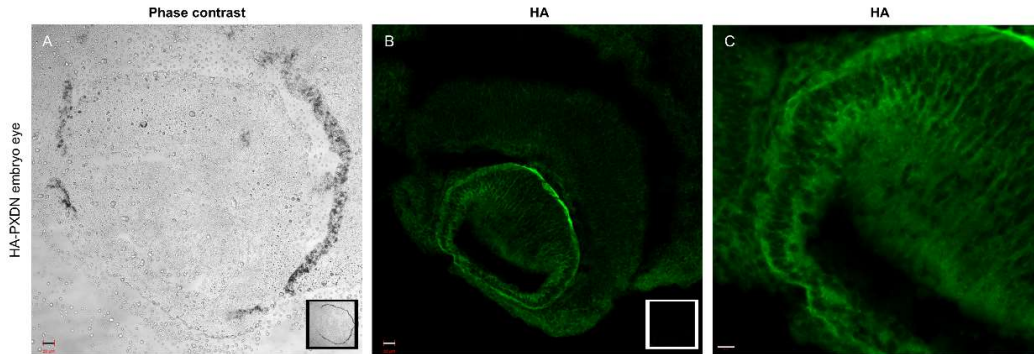
To study PXDN's localization, function, and *in vivo* proprotein convertase processing in mice, we have devised a novel knockin animal model. The mouse expresses a hemagglutinin (HA)-tagged PXDN (HA-PXDN). We achieved this modification using the CRISPR-Cas9 technique, and the HA epitope tag-encoding nucleotide sequence was introduced at the N-terminus of the encoded PXDN right after the secretory signal. We predicted that if proprotein convertase processing of PXDN occurs *in vivo* in the HA-PXDN mice, we will detect two HA-specific bands around 170 kD. As we can see in **Figure 8.**, we detected these bands in the kidney and lung of the HA-PXDN mice. This observation was confirmed with a PXDN-specific polyclonal antibody as well, in the same organs in WT and HA-PXDN mice (**Figure 8.**). The specificity of the polyclonal antibody was proved by the PXDN knockout mouse kidney and lung samples. The HA-tag did not alter the function of PXDN, as we could not observe any phenotypic changes in mice which expressed the modified gene in a hetero- or homozygous manner. On the lowest blot of **Figure 8.** we can also see that the crosslinking activity of PXDN was unaffected by the HA-tag. These experiments allowed us to conclude that proprotein convertase processing of PXDN can happen *in vivo*. With the help of this newly developed animal model, we also attempted to examine the localization of PXDN in mouse organs. We chose for this purpose the developing mouse eye prepared from fetuses between day 12.5-14.5 of gestation. This seemed to be a good choice based on previous observations related to the importance of PXDN in mouse eye development (56, 57).



**Figure 8. Development of a hemagglutinin epitope (HA)-tagged PXDN expressing mouse model.** Western blot analysis of kidney and lung samples derived from WT, HA-PXDN knockin, and PXDN-KO mice. Most upper panels show the double bands of PXDN in the WT, and HA-PXDN animals detected with a PXDN-specific polyclonal antibody. This result is confirmed with an HA-tag-specific antibody as well. The upper band probably represents the unprocessed PXDN, the lower one, the proprotein convertase cleaved form of PXDN. Under the PXDN and HA blots, we can see the corresponding loading controls. At the lowest panel, we can see that the crosslinking activity of PXDN is normal in the HA-PXDN animal compared with the WT. Meanwhile, the crosslinked NC1 dimers are absent in the PXDN-KO organs. The NC1 dimers and monomers are detected with an Alpha1 collagen IV isoform-specific antibody. The blots are representative of three independent experiments.

Immunostaining of HA-PXDN revealed an intense PXDN signal in the embryonic eye of the HA-PXDN fetus. The staining was the most intense in the lens, where we could

observe a network-like pattern inside the lens and strong labeling at the borders of the lens (**Figure 9.**). There was no PXDN-specific staining in the WT embryo's eye (**Figure 9. inserts**).



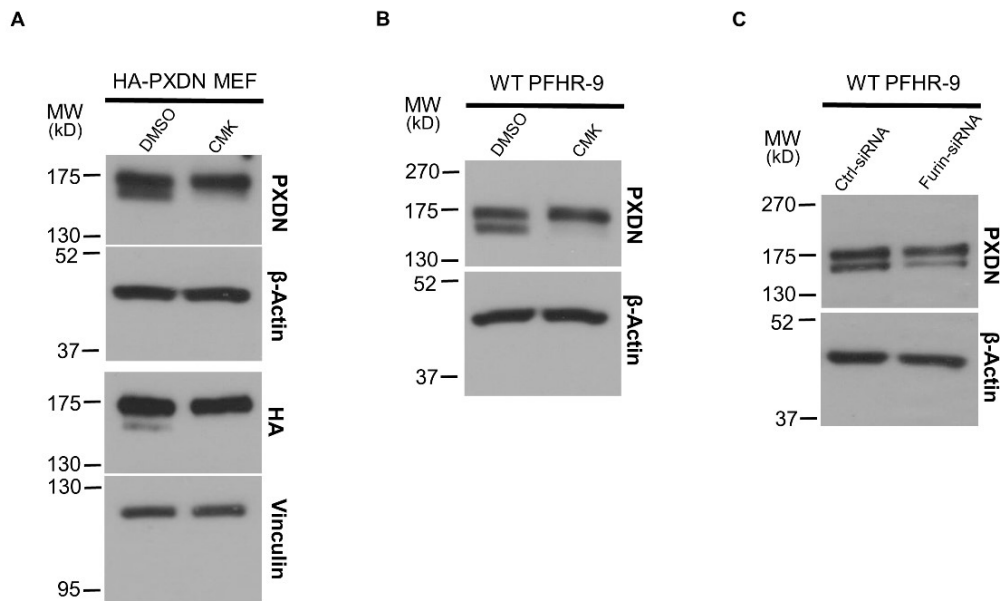
**Figure 9. Immunostaining of HA-PXDN in the developing mouse eye.** (A) Phase contrast image of an embryo eye taken with 20x objective from an HA-PXDN fetus. The inserted photo in the right corner of the picture is taken from a WT eye. The dark signal around the eye represents the pigmented layer of the retina. Bar indicates 20  $\mu\text{m}$ . (B) Immunostaining of HA-PXDN with HA-specific antibody shows specific staining in the lens, around the cells of the lens, and at the border of the lens. The inserted photo in the right corner of the picture is taken from a WT eye. Here we can not see a specific signal. Bar indicates 20  $\mu\text{m}$ . (C) Immunostained HA-PXDN embryo eye taken with 40x objective, highlighting the PXDN signal localization in a basement membrane-like structure. Bar indicates 10  $\mu\text{m}$ .

### 3.3 Identification of the proprotein convertase which processes PXDN

Although we detected double bands of PXDN in WT PFHR-9 cells and the kidney and lung samples of WT and HA-PXDN mice, we could not say for sure that these bands represent the unprocessed and proteolytically processed forms of PXDN. To prove our point, we prepared a primary cell culture from HA-PXDN mice embryos (mouse embryonic fibroblast, MEF) between days 12.5-14.5 of gestation. In this cell culture model, we could study the effect of proprotein convertase inhibitor Decanoyl-Arg-Val-Lys-Arg-Chloromethylketone (CMK), which is used to block furin/proprotein convertases (92). We can see in **Figure 10.A** that CMK treatment of the HA-PXDN MEF decreased the lower PXDN-specific band's intensity, representing the proteolytically



processed form compared to the vehicle (DMSO) treated cells. Both PXDN-specific and HA-specific antibodies confirmed this finding. We also further studied the processing of PXDN in WT PFHR-9 cells. Treatment of the cells with CMK decreased the lower PXDN-specific band's intensity in the tumor cell line as well (**Figure 10.B**). CMK is an inhibitor of furin, but to make sure that the proprotein convertase which is responsible for the PXDN's cleavage is furin, we conducted experiments with furin-specific siRNA on the PFHR-9 cells. Treatment with the furin-specific siRNA could reduce the processing of PXDN (**Figure 10.C**). With the siRNA experiment, we confirmed that furin has a role in the proteolytic cleavage of PXDN.

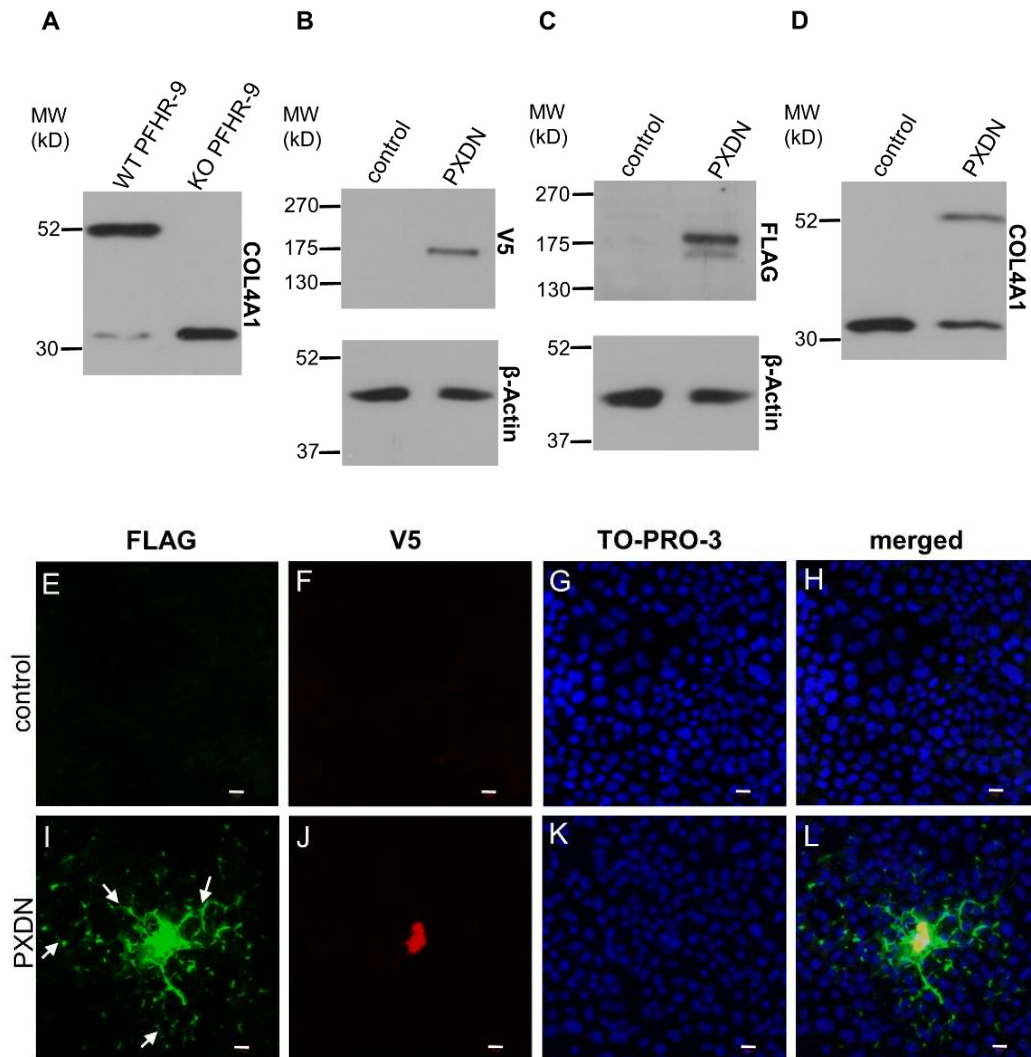


**Figure 10. Inhibition of furin activity with proprotein convertase inhibitor and furin-specific siRNA in cell culture.** (A) HA-PXDN mouse embryonic fibroblasts (HA-PXDN MEF) were treated with CMK (furin/proprotein convertase inhibitor) or vehicle (DMSO). The CMK treatment significantly decreased the lower band's intensity which represents the cleaved form of PXDN. This decrease was confirmed with PXDN-specific and HA-specific antibodies as well. Under the PXDN and HA blots, we can see the corresponding loading controls. (B) CMK treatment of WT PFHR-9 cells decreased the proteolytic cleavage of PXDN. (C) Treatment of WT PFHR-9 cells with furin-specific siRNA significantly reduced the cleavage of PXDN compared to the control siRNA-

treated sample. This confirmed the role of furin in the proteolytic processing of PXDN. The blots are representative of three independent experiments.

### **3.4 Studying the crosslinking activity, proprotein convertase processing, and localization of various double-tagged PXDN constructs**

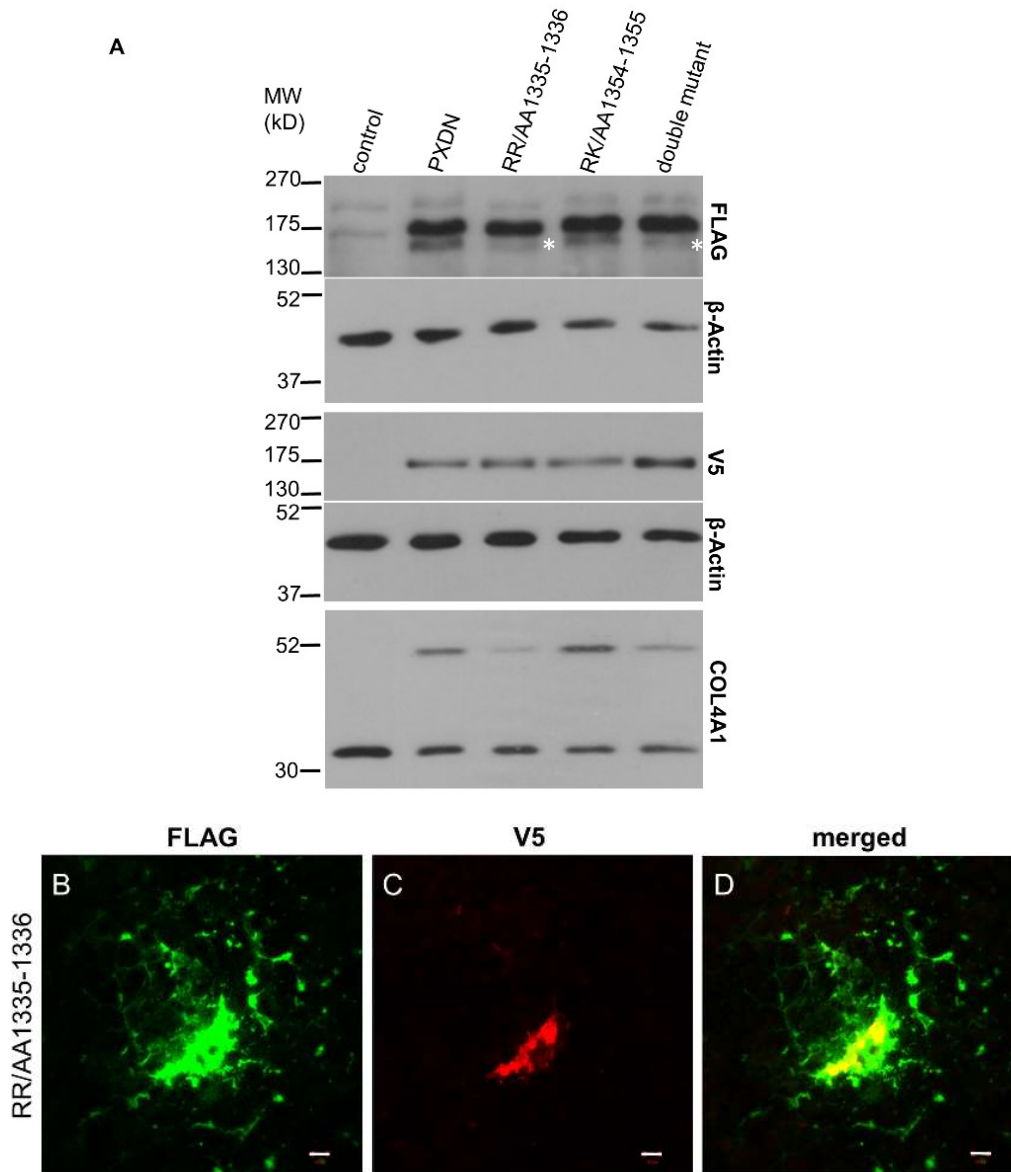
To study the behavior of different PXDN constructs in a cell culture model, we used the previously described KO PFHR-9 cells. In this model, the cells produce an uncrosslinked collagen IV network compared with WT PFHR-9 cells (**Figure 11.A**). To further investigate PXDN proprotein convertase cleavage, we planned a double-tagged PXDN construct with a FLAG-tag at the N-terminus after the signal peptide and at the C-terminus with a V5-tag. The schematic drawings of all the constructs that we used in the following experiments can be seen in **Figure 16**. In **Figure 11.B** we can see that if we transfect the KO PFHR-9 cells with this double-tagged, full-length PXDN coding plasmid, the V5 antibody will detect a single band around the predicted molecular weight of PXDN. Meanwhile, if we use the FLAG antibody, we can see a double band at that molecular weight (**Figure 11.C**). In the latter case, the lower band represents the cleaved form of PXDN. The transfected cells' crosslinking activity was rescued (**Figure 11.D**), which proved that the newly developed double-labeled PXDN is functional. Our labor previously investigated PXDN localization with a C-terminally labeled PXDN construct, and we observed a cell-associated PXDN signal (39). Based on the knowledge that PXDN processing occurs close to the C-terminus, we thought that probably the cell-associated PXDN signal of the C-terminally tagged construct represented only the unprocessed form of PXDN. To figure out whether the cleaved protein can appear in a different localization, we transfected the KO PFHR-9 cells with the double-tagged PXDN, and consecutively we immunostained with both anti-FLAG-, and anti-V5-antibodies. In **Figure 11.J**, we can observe an intracellular, cell-associated V5 signal. In **Figure 11.I** the FLAG signal, besides being cell-associated it is also extracellularly located around the neighboring cells in a network-like structure. These FLAG signals can be detected even far away from the transfected cell (indicated by white arrows).



**Figure 11. Characterization of a double-labeled PXDN construct in KO PFHR-9 cells.** (A) KO PFHR-9 cells produce uncrosslinked collagen IV, on the blot, the upper band represents the crosslinked NC1 dimers, the lower band is the uncrosslinked NC1 monomer. In the case of WT PFHR-9 cells, we can detect dimers and monomers with an Alpha1 collagen IV specific antibody. Meanwhile, in the KO PFHR-9 cells, we can only see the uncrosslinked monomers in the collagenase digested protein sample. (B) Detection of the double-tagged PXDN in KO PFHR-9 cells with the help of C-terminally located V5-tag. (C) Detection of the double-tagged PXDN with the N-terminally placed FLAG-tag in KO PFHR-9 cells. The upper band is the unprocessed the lower one is the proteolytically processed form of PXDN. (D) The double-tagged PXDN construct can

rescue the crosslinking activity in the KO PFHR-9 cells. **(E-H)** Immunostaining of untransfected KO PFHR-9 cells (control) represents the absence of FLAG-**(E)** and V5 **(F)** signals. **(G)** Nuclear staining with TO-PRO-3 (blue) and the merged picture **(H)** proves the presence of the cells on the coverslip. **(I-L)** Immunostaining of double-tagged PXDN transfected KO PFHR-9 cells. **(I)** The FLAG signal (green) appears both in a cell-associated and extracellular network-like localization (arrows). **(J)** The V5 signal (red) is only present at the cell-associated localization. **(K)** TO-PRO-3 (blue) staining shows the presence of nuclei. **(L)** On the merged photo, we can observe the partial colocalization of V5- and FLAG signals in a cell-associated manner; meanwhile, only the FLAG signal appears in a net-like pattern around the cells. The bar indicates 10  $\mu\text{m}$ . The blots and immunostainings are representative of three independent experiments.

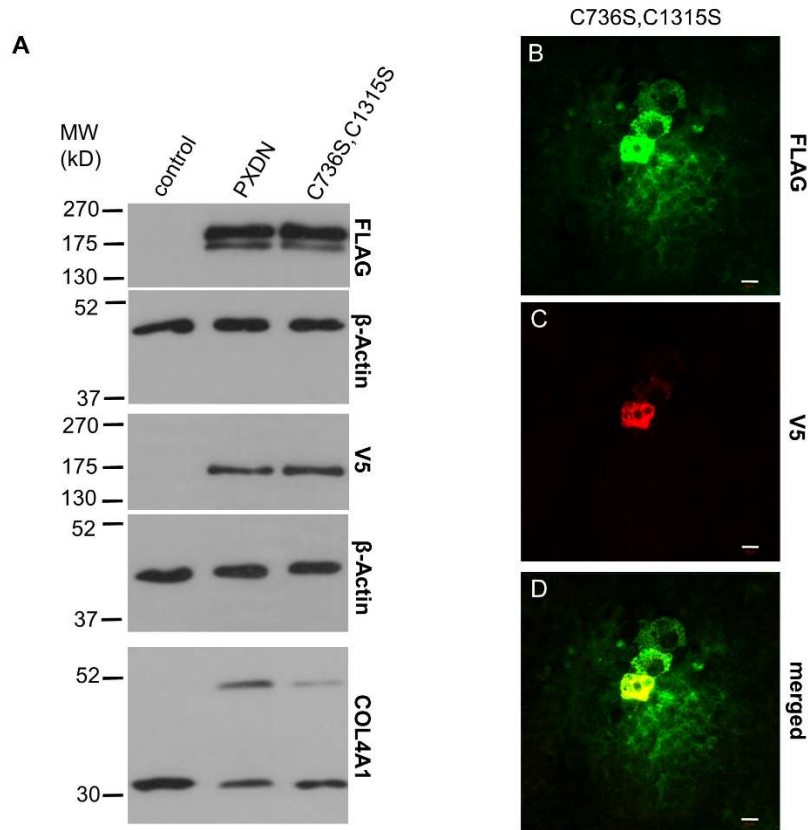
One of our goals was to examine with our newly developed tools how the proprotein convertase processing of PXDN can influence the crosslinking activity- and localization of PXDN. With ProP 1.0 proprotein cleavage prediction software, we found two potential sites for cleavage. One predicted site, Arg1336 was the same as what Colon *et al.* have identified before (53). The software indicated another highly probable proteolysis site at Lys1355. We introduced mutations in the full-length, double-tagged PXDN construct. Arg1335 and Arg1336 were changed to alanines in one plasmid (RR/AA 1335-1336), and Arg1354 and Lys1355 were mutated to alanines in another construct (RK/AA 1354-1355). Finally, we also made a double mutant construct that carries both mutations. In **Figure 12.A** upper panel, we can see that transfection of the RR/AA 1335-1336 construct into KO PFHR-9 cells resulted in reduced proteolytic processing of PXDN (the lower band intensity is way smaller than, the higher one compared with the unmutated construct detected with FLAG antibody), the RK/AA 1354-1355 didn't decrease the processing, and the double mutant behaved similarly to the RR/AA 1335-1336 construct. In **Figure 12.A** lowest panel we can see, that the collagen IV crosslinking activity was reduced in the case of the RR/AA 1335-1336 and the double mutant construct transfection. This points to the conclusion that proteolytic processing of PXDN may have a role in regulating the crosslinking activity of PXDN. Next, we examined the effect of mutations on the localization of PXDN. We saw that the mutant PXDN also could be deposited extracellularly in a net-like pattern (**Figure 12.B**), but importantly the V5 signal was still just cell-associated. It could not be detected in the network around the cells (**Figure 12.C**).



**Figure 12. Investigation of PXDN's proteolytic processing.** (A) Western blot analysis of KO PFHR-9 cells transfected with double-tagged, unmutated PXDN, or with double-tagged, proprotein convertase target site mutated PXDN constructs (RR/AA 1335-1336, RK/AA 1354-1355, double mutant). The upper panel shows that the lower FLAG-specific band's intensity is less in the RR/AA 1335-1336 and the double mutant (indicated with asterisks), proving the importance of the 1336Arg in the proteolytic processing. Under the FLAG and V5 blots, we can see the corresponding loading controls. On the lowest panel, we can observe that decreased proteolytic processing of PXDN also reduces its collagen IV crosslinking activity in the KO PFHR-9 cells. (B-D) Immunostaining of the

RR/AA 1335-1336 mutant expressing cells. **(B)** FLAG signal (green) appears both in a cell-associated and extracellular localization. **(C)** The V5 signal (red) is only present in association with the cell. **(D)** The merged picture of the two signals shows partial colocalization. The bar indicates 10  $\mu\text{m}$ . The blots and immunostainings are representative of three independent experiments.

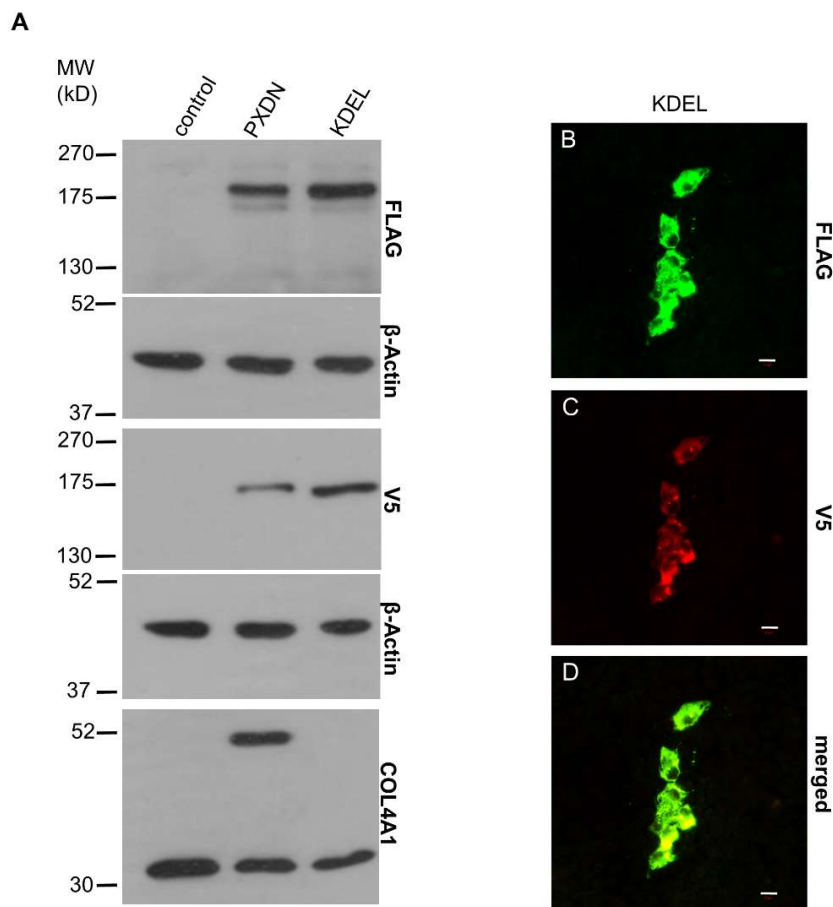
To further investigate the preconditions of PXDN's proteolytic processing, we also examined the behavior of a PXDN mutant that is unable to form oligomers. Previously we identified two cysteine residues (Cys736 and Cys1315) necessary for creating disulfide bridge coupled PXDN trimers (39). Transfection of KO PFHR-9 cells with the trimerization mutant form of PXDN (C736S, C1315S) resulted in a slightly reduced cleavage of PXDN compared with the wild type PXDN transfected KO cells (**Figure 13.A**). We have already described that this mutant has less crosslinking activity (**Figure 13.A the lowest panel**) (39). Localization of the trimerization mutant PXDN showed a more diffuse extracellular staining. We could not observe the net-like pattern of the FLAG signal (**Figure 13.B**). The V5-signal (**Figure 13.C**) was cell-associated as we saw it with the other constructs.



**Figure 13. Analysis of the effect of PXDN oligomerization on the proteolytic processing and localization of the protein. (A)** Western blot experiments with the trimerization mutant PXDN (C736S, C1315S) shows slightly reduced proteolytic cleavage detected with the FLAG antibody (upper panel). Under the FLAG and V5 blots, we can see the corresponding loading controls. On the lowest panel, we see that the monomeric PXDN form has less collagen IV crosslinking activity than the wild type PXDN. **(B-D)** Immunostaining of the trimerization mutant PXDN transfected KO PFHR-9 cells. **(B)** The FLAG-signal (green) appears in a cell-associated and diffuse extracellular pattern, lacking the previously seen network-like structure. **(C)** The V5 signal (red) is only cell-associated. **(D)** On the merged picture, the cell-associated FLAG-, and V5-signals are colocalized. The bar indicates 10  $\mu$ m. The blots and immunostainings are representative of three independent experiments.

The cleavage of proteins by proprotein convertases can happen at different intracellular sites, for example, in the trans-Golgi network, at other points of the secretory pathway, or on the external surface of the plasma membrane (93). We wanted to know where the

cleavage of PXDN occurs. Is it an extracellular or an intracellular event? We created a mutant of PXDN that cannot leave the cell because of an ER-retention signal (KDEL) at the C-terminus of the protein. The ER-retention mutant PXDN (KDEL) expression in KO PFHR-9 cells led to the accumulation of cell-associated, unprocessed PXDN (**Figure 14.A**). On the lowest panel of **Figure 14.A** we can see that this modification of PXDN destroyed the crosslinking activity of the enzyme. Analysis of the immunostained ER-retention mutant expressing KO PFHR-9 samples with FLAG- and V5 antibodies proved that this retention signal at the C-terminus prevented the secretion of PXDN and led to its intracellular accumulation (**Figure 14.B-D**).



**Figure 14. Analysis of the ER-retention mutant PXDN (KDEL) in KO PFHR-9 cells.**

(A) Western blot experiments of KDEL mutant show that the mutant form is less processed than the wild type PXDN (upper panel, increased intensity of upper FLAG-specific band in the KDEL mutant), the V5 signal is more prominent in the case of the KDEL mutant, probably because of the accumulation of the mutated protein in the cell.



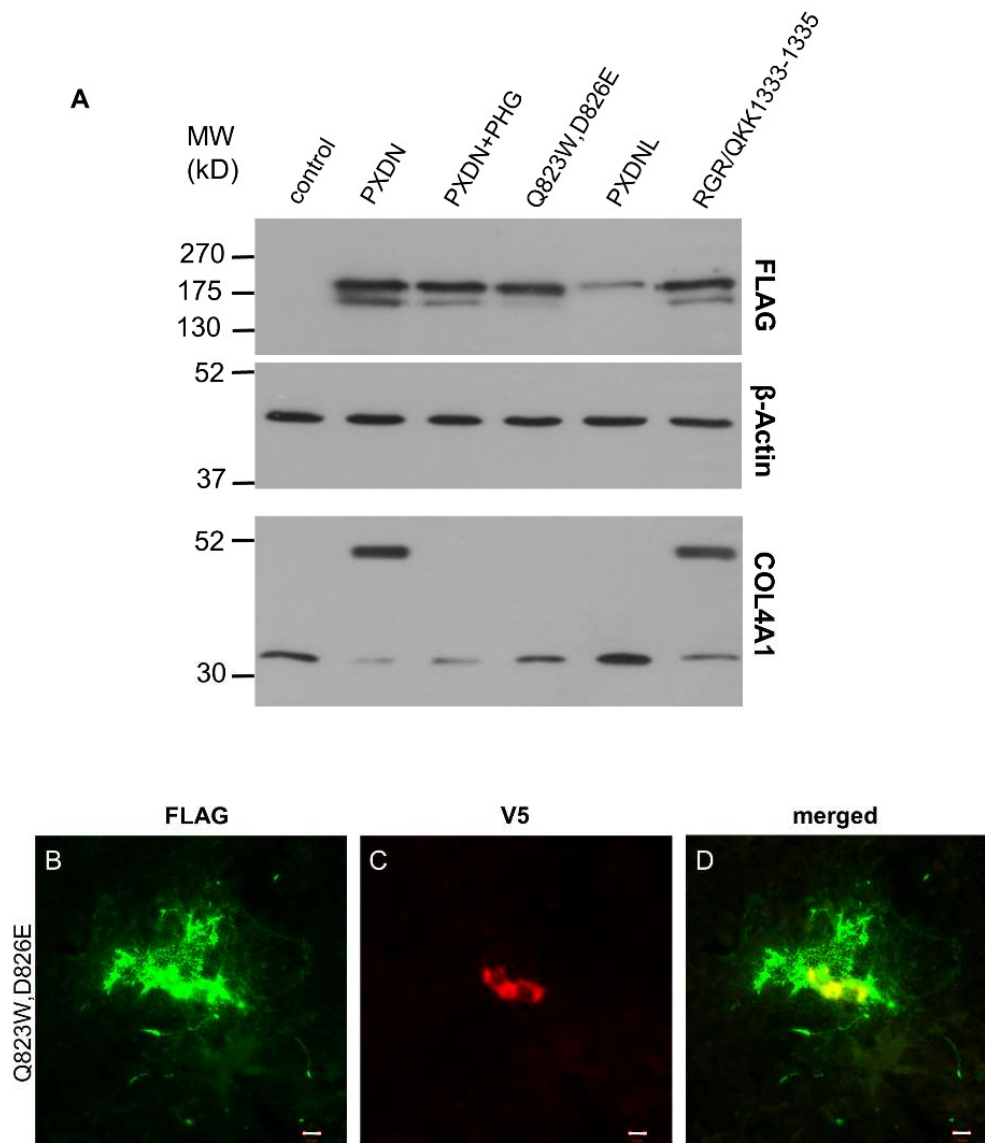
Under the FLAG and V5 blots, we can see the corresponding loading controls. At the lowest panel, we can see that the KDEL mutant has no collagen IV crosslinking activity. **(B-D)** Immunostaining of the KDEL mutant shows that both FLAG signal (green) and V5 signals (red) are cell-associated and colocalized. There is no FLAG signal extracellularly. The bar indicates 10  $\mu\text{m}$ . The blots and immunostainings are representative of three independent experiments.

### **3.5 Investigating the role of peroxidase activity in the proteolytic processing of PXDN and PXDNL**

PXDN is an active peroxidase enzyme. We were interested in whether the peroxidase activity has any effect on the proteolytic processing of PXDN. This question can be important because, as we saw, the unprocessed PXDN has less collagen IV crosslinking activity. Some circumstances can reduce the enzymatic activity of the protein, such as thiocyanate ion. An other relevance of this question to be raised is PXDNL. We know that PXDNL is highly homologous to PXDN, possessing the same domain structure, still because of the change of some critical amino acid residues in the active center of the peroxidase domain, it doesn't have detectable peroxidase activity (74). Our approach to answer this question was first applying a known peroxidase inhibitor, phloroglucinol (PHG). This drug can inhibit the collagen IV crosslinking activity of PXDN (40). In **Figure 15.A** we can see that the PHG treatment reduced the proteolytic processing of the wild type PXDN and on the lowest panel of **Figure 15.A** we can see that it also decreased the crosslinking activity (by this, we proved that the drug treatment was effective) of PXDN. Next, we transfected the KO PFHR-9 cells with a peroxidase activity-mutant PXDN construct. In this plasmid, two critical amino acid residues are mutated (Gln823Trp and Asp826Glu), which results in a diminished peroxidase activity (39). In **Figure 15.A** upper panel, we can see that the peroxidase activity-mutant PXDN's (Q823W, D826E) proteolytic processing is reduced, compared to the wild type PXDN the intensity of the lower FLAG-specific band is less intense. We can also see on the lowest blot that this mutation prevents the crosslinking of the collagen IV NC1 domains. We analyzed the localization of this construct and observed a similar pattern in the FLAG- and V5 signals as in the case of the wild type PXDN (**Figure 15.B-D**). We often detected the extracellular FLAG signal in smaller areas around the cell, and the signal was less organized in a network.

We were interested in the possible proteolytic processing of PXDNL. We used the same previously mentioned software to search for potential cleavage sites in the PXDNL sequence. We found a candidate site at Arg1319. The sequence of this site was different from the one in PXDN. It was interesting that after this site, the C-terminal part of PXDNL is just one amino acid longer than in the case of PXDN (144 amino acids in PXDNL and 143 amino acids in PXDN).

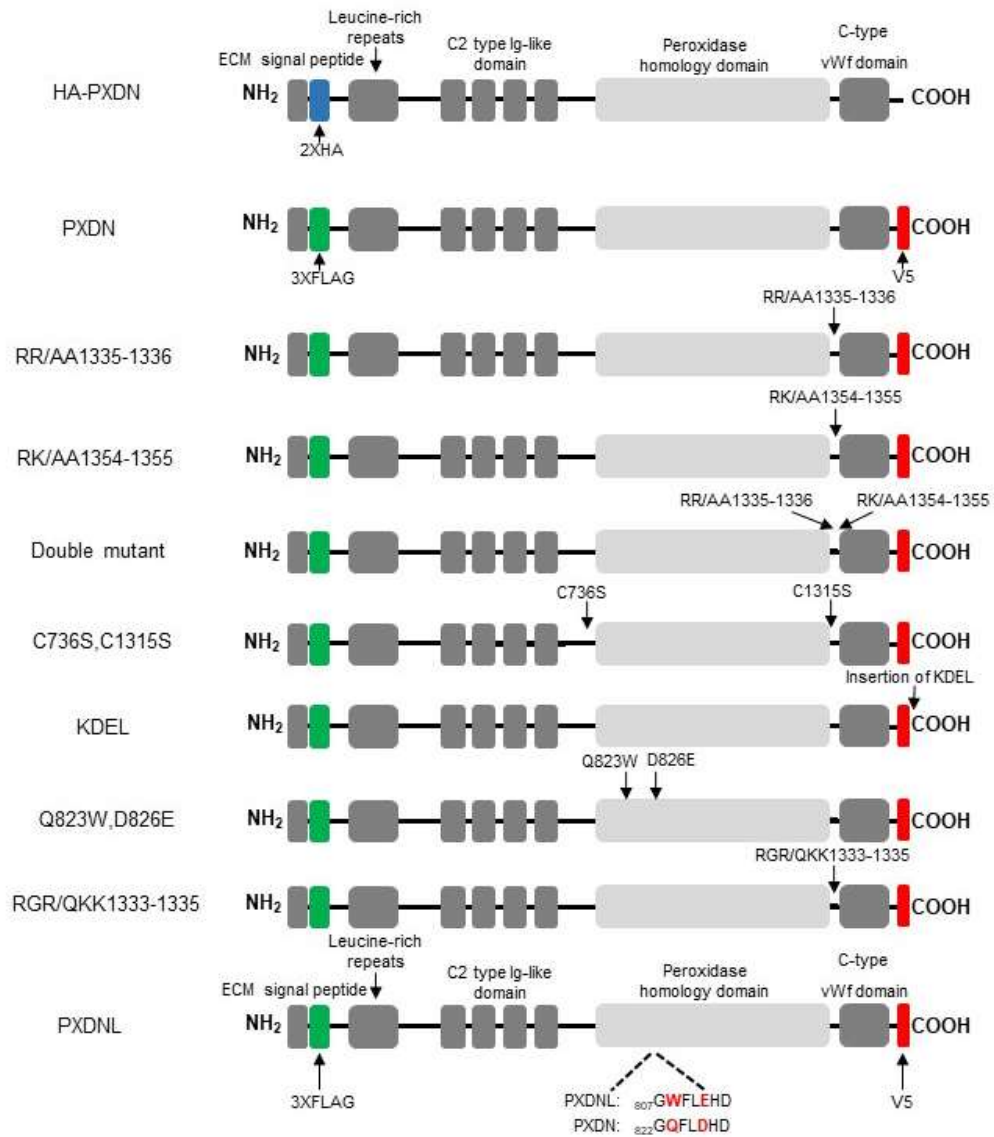
We created a double-tagged (FLAG-tag at the N-terminus, V5-tag at the C-terminus) full-length PXDNL coding construct to study the proteolytic processing of PXDNL. We can see in **Figure 15.A** that if we transfect the KO PFHR-9 cells with this plasmid (PXDNL), there is no processing, we cannot observe the lower FLAG-specific band even after prolonged exposure, and as we expected, this construct is unable to crosslink collagen IV molecules (**lowest blot of Figure 15.A**). We hypothesized that this unsuccessful cleavage of PXDNL might result from the inability of the protease enzyme to recognize the potential cleavage site. To test this idea, we created another PXDN mutant in which the efficiently working proprotein convertase recognition site is replaced with the one in the PXDNL sequence. This PXDN mutant (RGR/QKK1333-1335) was effectively cleaved in the KO PFHR-9 cells, and of course, it was able to crosslink the collagen IV molecules (**Figure 15.A lowest blot**). This result combined with the one observed with PHG treatment and with the Q823W, D826E mutant PXDN suggest that peroxidase activity relates to the posttranslational proteolytic processing of PXDN.



**Figure 15. Investigation of the influence of peroxidase activity on the proteolytic processing of PXDN and PXDNL.** (A) Pharmacological inhibition of wild type PXDN transfected KO PFHR-9 cells with phloroglucinol in 50  $\mu$ M decreased the cleavage of PXDN (less intense lower FLAG-specific band compared with the PXDN transfected untreated sample). In the peroxidase-mutant PXDN construct (Q823W, D826E) transfected cells, the cleavage of PXDN was also reduced. PXDNL is not cleaved based on our experiments, although it poses a possible cleavage site for proprotein convertase enzymes. On the other hand, the cleavage site imported from PXDNL in PXDN can be

cleaved efficiently (RGR/QKK1333-1335). Under the FLAG blot, we can see the corresponding loading control. On the lowest blot, we can see that PHG treatment inhibits the crosslinking activity of PXDN, the peroxidase activity mutant PXDN construct is also unable to form crosslinks, PXDNL, which doesn't have peroxidase activity, cannot form dimers of NC1 domains, and the RGR/QKK1333-1335 mutant PXDN can crosslink the monomers because it has the active peroxidase domain. **(B-D)** Immunostaining of Q823W, D826E transfected KO PFHR-9 cells show similar FLAG- and V5-specific staining than in the case of wild type PXDN transfected cells. The FLAG signal extracellular pattern is slightly different from what we can observe in the wild type PXDN stainings. The bar indicates 10  $\mu$ m. The blots and immunostainings are representative of three independent experiments.

In **Figure 16**, we can see the designs of all the PXDN and PXDNL constructs we used in the previously described experiments.



**Figure 16.** Schematic pictures of the PXDN and PXDNL constructs we used in the experiments.

#### 4. Discussion

In basement membranes the unique sulfilimine bond formation between collagen IV molecules is catalyzed by PXDN (40). During the oxidative crosslinking of the NC1 domains Br<sup>-</sup> is oxidized to HOBr (45). The collagen IV network-stabilizing sulfilimine bond and the enzyme, PXDN which catalyzes its formation, seem essential in the organogenesis during the evolution of multicellular animals (55). There are still many open questions related to this protein. One of them is the source of H<sub>2</sub>O<sub>2</sub> for the collagen IV crosslinking. We tried to investigate this issue with the help of several genetically modified mouse model systems. There are many examples of cooperation between Nox/Duox enzymes and animal heme peroxidases, so we wondered whether the NADPH oxidases could provide the H<sub>2</sub>O<sub>2</sub> for PXDN. We examined the crosslinking activity in organs derived from Nox4 knockout mice (90). This idea was encouraged because Nox4 is expressed in cells usually surrounded by basement membranes (3, 94). We didn't find a decreased crosslinking activity in the tested organs, proving that Nox4 is not the source of H<sub>2</sub>O<sub>2</sub>. We also analyzed the crosslinking activity in a mouse strain that lacks functional p22<sup>phox</sup> protein (90). This protein is required for Nox1, Nox2, Nox3, Nox4 activity (95, 96). Testing this mouse model allowed us to investigate the possible involvement of several Nox isoforms. In these experiments, we again found intact crosslinking activity, excluding the role of these enzymes from the H<sub>2</sub>O<sub>2</sub> production (90). Dual oxidases are also expressed in epithelial cells (97, 98), so they could be a good H<sub>2</sub>O<sub>2</sub> source for PXDN. We analyzed the collagen IV crosslinking in DuoxA1 and DuoxA2 double knockout mice and Duox1 KO mice. In both model animals, we found a proper crosslinking activity (90). To further investigate the H<sub>2</sub>O<sub>2</sub> source for PXDN, we used the PFHR-9 cell culture model and created a PXDN KO PFHR-9 cell line. In the WT PFHR-9 cells, we tried several oxygen concentrations for 24 hours and analyzed the NC1 dimer/monomer ratios (during these experiments, the KO PFHR-9 was used as a control). In conclusion, we saw that the crosslinking activity of PXDN was undisturbed by hypoxic treatments (90). With the help of the WT and KO PFHR-9 cells, we also investigated the mitochondria as a possible ROS source for crosslinking. Different approaches were established, like diphenyliodonium (DPI) treatment of the cells or targeting heterologously expressed catalase to the cytosol and mitochondrial matrix. In none of these experiments did we find impaired crosslinking activity (90). Taken together our findings, we couldn't identify the

ROS source for PXDN, but we could exclude many possible sources (Nox/Duox enzymes, mitochondria). During my Ph.D work, we tried to get to know more about PXDN and PXDNL proteins. As a part of this work, we created a KO PFHR-9 cell line. This cell line allowed us to study the proteolytic processing of PXDN and PXDNL, the crosslinking activity, and the localization of PXDN. As was mentioned above, this cell-based experimental system also contributed to the study of PXDN's H<sub>2</sub>O<sub>2</sub> source.

We developed a new mouse model, the HA-PXDN mouse. This mouse expresses an N-terminally HA-tagged PXDN. This tag doesn't disturb the biological function of PXDN. We didn't see the characteristic phenotype of the PXDN knockout mouse in the case of HA-PXDN animals. Also, we found undisturbed crosslinking activity in these knockin mice. With the help of the newly developed mouse, we proved that the proteolytic cleavage of PXDN could happen *in vivo*, and we could immunostain PXDN in the developing embryonic eye by using an HA-specific antibody. This was impossible before using the PXDN KO mouse as a negative control because of the ASD these animals display during their development (56).

From the HA-PXDN mice, we could prepare a primary cell culture, the HA-PXDN MEF cells. In the cell culture, we applied the furin/proprotein convertase inhibitor CMK, which effectively reduced the lower molecular weight form of HA-PXDN. This confirmed that the two bands of PXDN, which we detected in the organs of the HA-PXDN mouse, are the unprocessed and the proteolytically processed forms of PXDN. To support this data, we repeated the CMK treatment on PFHR-9 cells and found similar results. Furthermore, we also treated the PFHR-9 cells with furin-specific siRNA, which decreased the processing as well. This data is fundamental because it highlights that from the proprotein convertase enzymes furin is the one, which has a role in PXDN's cleavage.

We gained information from the KO PFHR-9 cells about the processing, crosslinking activity, and localization of PXDN, and how these are affected by specific structural changes in the protein. To address these problems, we made a double-tagged PXDN construct (N-terminally FLAG-tagged and C-terminally V5-tagged). If this plasmid was expressed in the KO PFHR-9 cells, it could rescue the crosslinking activity, and we could immunostain it as well with the help of the epitope-specific antibodies. Both tags were detected intracellularly, but only the FLAG tag was present extracellularly in a net-like

pattern. This pointed out that probably the cleaved form of PXDN (which doesn't have the V5 tag) can be built in the ECM.

Modification of the double-tagged PXDN with site-directed mutagenesis allowed us to study the possible proteolysis site in PXDN. We confirmed that Arg 1336 is the site where it occurs. This is the same place that was reported previously by Colon *et al.* (53). The mutation didn't completely abolish the proteolytic processing of PXDN, and it reduced the crosslinking activity. The processed mutated construct was detected extracellularly, but again just with FLAG-specific antibody. This further confirmed that the unprocessed form doesn't integrate into the ECM.

From a long time ago, we know that the *Drosophila* PXDN is present in the forms of oligomers (26). Our laboratory proved that trimerization also occurs in human PXDN. We identified the cysteine residues, which are responsible for the disulfide bridge formation (cysteine 736, cysteine 1315) (39). We studied the effect of oligomerization on the processing, crosslinking activity, and localization of PXDN. The trimerization mutant PXDN (C736S, C1315S) was slightly less processed than the WT PXDN and had a smaller crosslinking activity. Furthermore, its localization was also more diffuse extracellularly than in the case of the WT PXDN. Thus, it suggested that the oligomerization might be an important event for PXDN's integration into the ECM.

To explore the place of PXDN proteolytic processing, we created a PXDN construct attached with an ER-retention signal (KDEL). This mutant was less processed, had no crosslinking activity, and its localization was entirely intracellular. These results pointed out that the processing probably happens after the ER and the crosslinking of collagen IV also occurs after PXDN leaves the ER. Thus, the crosslinking site may be a cell surface-associated "hot spot" [38] or somewhere extracellular.

During our work, we also found that the peroxidase activity of PXDN also plays a role in furin-mediated processing. Inhibition of the peroxidase activity with PHG decreased the cleavage of the protein, and the peroxidase domain mutated form of PXDN (which doesn't have peroxidase activity) was even less processed compared to the PHG treated WT PXDN. This finding suggests that before the furin processing, maybe a quality control system works and checks whether PXDN has the peroxidase activity or not. Furthermore, we saw that the furin site mutated PXDN has less crosslinking activity, so



somehow the cleavage of PXDN enhances its enzymatic activity. Which might indicate that it wouldn't make sense for the cell to process the PXDN if it lacks the peroxidase activity and therefore it doesn't proceed with it. The deeper mechanism of this possible "quality control" mechanism is honestly still unknown to us. There are data about furin's possible inhibition by H<sub>2</sub>O<sub>2</sub>. In a publication, it was proposed that H<sub>2</sub>O<sub>2</sub>-mediated oxidation of furin decreases its Ca<sup>2+</sup>-binding capacity, thereby decreases its activity (99). So our theory is that the reduced peroxidase activity could lead to the accumulation of H<sub>2</sub>O<sub>2</sub>, which could then oxidize furin, leading to lower furin activity with reduced PXDN processing. The intact catalytic activity as a precondition of heme peroxidase maturation was already described in MPO, where the heme-binding was proved to be an essential step in the maturation of the enzyme (100). Furin is interesting from other aspects as well. As our knowledge is emerging about COVID-19, we know that one of the most critical variations in the sequence of SARS-CoV-2 S protein is an insertion of positively charged amino acids at the S1/S2 site. This insertion creates a cleavage site for furin-like proteases (101-104). The proteolytic cleavage ensures virus entry into the cell by activation of the S protein. So altogether, the furin cleavage increases the pathogenicity of the virus.

PXDNL is highly homologous to PXDN, and we were curious whether this protein can be proteolytically processed or not. This question was interesting because due to amino acid changes in its peroxidase domain, it cannot bind heme and doesn't have peroxidase activity (74). We created a double-tagged PXDNL (N-terminally FLAG-tagged and C-terminally V5-tagged), transfected it into KO PFHR-9 cells, and found that PXDNL is not processed at all. The result was surprising because the cleavage site prediction software found a possible sequence motif for proteolysis. Importing this motif into PXDN instead of its furin-cleavage site allowed us to see whether the motif itself is working in other proteins or not. According to our results, the sequence imported from PXDNL into PXDN was recognized and cleaved by furin. This result further supports our idea about the importance of peroxidase activity in furin-mediated processing. On the other hand, it can be interesting whether PXDNL processing can occur *in vivo* in the heart. If not, can this somehow influence the interactions of PXDNL with other possible molecular partners?

In summary, our observations made us get many steps closer to understanding the behavior and biological relevance of PXDN and PXDNL proteins.

## 5. Conclusions

Based on our experiments, we can make the following conclusions:

1. We successfully created a PXDN knockout PFHR-9 cell line (KO PFHR-9) with the CRISPR-Cas9 technique. The cells don't have detectable PXDN production with Western blot. They produce an uncrosslinked collagen IV network.
2. We developed a new mouse model with the CRISPR-Cas9 technique, which expresses an N-terminally hemagglutinin (HA)-tagged PXDN (HA-PXDN). With the help of this animal, we proved that proprotein convertase processing of PXDN could occur *in vivo*. Furthermore, in the developing eye of this mouse strain, we studied the localization of PXDN.
3. In a primary cell culture (MEF), which we prepared from the HA-PXDN mice embryos, we could decrease the processing of PXDN with a furin/proprotein convertase inhibitor (CMK). In PFHR-9 cells, we pharmacologically inhibited the proteolytic cleavage of PXDN. With furin-specific siRNA treatment of PFHR-9 cells, we could identify that furin has a role in the proprotein convertase processing of PXDN.
4. We studied the crosslinking activity, proprotein convertase processing, and localization of a double-tagged wild type, proprotein convertase site mutated, trimerization, and ER-retention mutant PXDN in KO PFHR-9 cells. We confirmed that the proteolysis site is at 1336Arg and that the cleavage site mutant PXDN has lower crosslinking activity. With immunostaining, we proved that only the processed form of PXDN could be built into the ECM. The trimerization mutant processing was slightly decreased, and the ER-retention mutant was less processed and didn't have crosslinking activity.
5. We found that in the case of the pharmacological inhibition of PXDN's peroxidase activity with PHG, the processing of PXDN is reduced. We saw decreased processing in the peroxidase activity mutant PXDN as well. We created a double-tagged PXDNL and examined the processing of this construct. According to our experiments, PXDNL is not cleaved in KO PFHR-9 cells even though it possesses a potential cleavage site for proprotein convertase enzymes.

## 6. Summary

PXDN and PXDNL belong to the peroxidase-cyclooxygenase superfamily. Both of them share a similar domain structure, which is special amongst the animal heme peroxidases. The unique feature of these proteins is the combination of a peroxidase domain with other conserved motifs usually present in ECM proteins (LRRs, C2 type Ig-like domain, vWFC domain) and mediate protein-protein interactions. PXDN's only known physiological function is the crosslinking of NC1 domains between adjoining collagen IV molecules. The reaction requires  $\text{Br}^-$  and  $\text{H}_2\text{O}_2$ . The source of  $\text{H}_2\text{O}_2$  is currently unknown. We excluded the different Nox/Duox enzymes as a possible source, and our initial data also indicate that the mitochondria are most probably not the source either. We created a new animal model, a new cell culture model, and a new "PXDN construct model" in our work. These new tools helped to prove that PXDN proteolytic processing can occur *in vivo*, and the protease that has role in it is the furin. The new mouse model can be beneficial in studying PXDN localization. We confirmed that the site of proteolysis is at 1336Arg. With the help of heterogeneous expression of different PXDN constructs in KO PFHR-9 cells, we characterized the relevance of PXDN oligomerization, and the place of the furin processing. Our experiments also revealed a new insight into PXDN and PXDNL maturation mechanisms. We found that peroxidase activity might be a prerequisite of furin processing. In the case of pharmacological inhibition of peroxidase activity, or inactivating mutation of the peroxidase domain, PXDN's furin-mediated processing is significantly reduced. The endogenous PXDNL doesn't have peroxidase activity due to critical amino acid residue changes in the peroxidase domain, and it is not processed at all in a heterologously expressed system.

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## 8. Bibliography of the candidate's publications

The Ph.D. thesis is based on the following publications:

1. **Kovács, Hajnal A.**; Lázár, Enikő ; Várady, György; Sirokmány, Gábor; Geiszt, Miklós

Characterization of the Proprotein Convertase-Mediated Processing of Peroxidasin and Peroxidasin-like Protein

ANTIOXIDANTS 10: 10 Paper: 1565, 17 p. (2021)

IF (2020): 6,312

2. Sirokmány, G; **Kovács, HA**; Lázár, E; Kónya, K; Donkó, Á; Enyedi, B; Grasberger, H; Geiszt, M

Peroxidasin-mediated crosslinking of collagen IV is independent of NADPH oxidases

REDOX BIOLOGY 16 pp. 314-321., 8 p. (2018)

IF: 7,793

Other publications which are related to the Ph.D thesis:

3. Zana, M; Peterfi, Z; **Kovacs, HA**; Toth, ZE; Enyedi, B; Morel, F; Paclet, MH; Donko, A; Morand, S; Leto, TL; Geiszt, M

Interaction between p22(phox) and Nox4 in the endoplasmic reticulum suggests a unique mechanism of NADPH oxidase complex formation.

FREE RADICAL BIOLOGY AND MEDICINE 116 pp. 41-49., 9 p. (2018)

IF: 5,657

4. Lázár, E; Péterfi, Z; Sirokmány, G; **Kovács, HA**; Klement, E; Medzihradzky, KF; Geiszt, M

Structure-function analysis of peroxidasin provides insight into the mechanism of collagen IV crosslinking

FREE RADICAL BIOLOGY AND MEDICINE 83 pp. 273-282., 10 p. (2015)

IF: 5,784

5. Péterfi, Z; Tóth, ZE; **Kovács, HA**; Lázár, E; Sum, A; Donkó, Á; Sirokmány, G; Shah, AM; Geiszt, M

Peroxidasin-like protein: A novel peroxidase homologue in the human heart

CARDIOVASCULAR RESEARCH 101: 3 pp. 393-399., 7 p. (2014)

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