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# Proprotein Convertase Processing Enhances Peroxidasin Activity to Reinforce Collagen IV*

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**Edited by Amanda Fosang**

**The basement membrane (BM) is a form of extracellular matrix that underlies cell layers in nearly all animal tissues. Type IV collagen, a major constituent of BMs, is critical for tissue development and architecture. The enzyme peroxidasin (Pxdn), an extracellular matrix-associated protein, catalyzes the formation of structurally reinforcing sulfilimine cross-links within the collagen IV network, an event essential to basement membrane integrity. Although the catalytic function of Pxdn is known, the regulation of its activity remains unclear. In this work we show through N-terminal sequencing, pharmacologic studies, and mutational analysis that proprotein convertases (PCs) proteolytically process human Pxdn at Arg-1336, a location relatively close to its C terminus. PC processing enhances the enzymatic activity of Pxdn and facilitates the formation of sulfilimine cross-links in collagen IV. Thus, PC processing of Pxdn is a key regulatory step that contributes to its function and, therefore, supports BM integrity and homeostasis.**

The basement membrane (BM)[2] is a form of extracellular matrix (ECM) that provides structural support for nearly all animal tissues. BMs also help to provide extracellular cues for cellular adhesion, proliferation, migration, and differentiation (1). Type IV collagen is a critical constituent of basement membranes (2). Collagen IV $\alpha$ chains form triple helical protomers that self-assemble into a mesh-like network with end-to-end C-terminal associations known as NC1 hexamers. Sulfilimine (S═N) bonds between opposing methionine and lysine residues bridge the trimer-timer interface of the NC1 hexamer and thereby structurally reinforce the collagen IV network (3). The enzyme peroxidasin (Pxdn), a basement membrane-associated heme peroxidase, catalyzes the formation of sulfilimine cross-links, a process critical for BM and tissue integrity (4). For example, peroxidasin loss of function in *Drosophila* and *Caenorhabditis elegans* compromises gut architecture and leads to early lethality (4, 5). In addition to structural reinforcement, the sulfilimine cross-link also confers immune privilege to the collagen IV auto-antigen in human Goodpasture's disease, a devastating condition presenting as rapidly progressive glomerulonephritis with or without pulmonary hemorrhage. The formation of sulfilimine cross-links by peroxidasin may, therefore, protect against the development of Goodpasture's disease (6).

Peroxidasin is a member of the animal heme peroxidase superfamily along with myeloperoxidase (MPO), eosinophil peroxidase, lactoperoxidase, and thyroid peroxidase (TPO) (7, 8). Peroxidases use hydrogen peroxide ($H_2O_2$) to oxidize halide ions ($X^- = I^-$, $Br^-$, $Cl^-$) and form hypohalous acids (HOX). Pxdn preferentially generates HOBr as a reactive intermediate to form sulfilimine cross-links in collagen IV, which represents the first known function for the element bromine (4, 9). Given its critical function in the synthesis of basement membranes, it is no surprise that peroxidasin is found throughout the animal kingdom, whereas other animal heme peroxidases are restricted to vertebrates (10, 11). In addition, unlike other members of the animal heme peroxidase family, Pxdn is a multidomain protein that contains a leucine-rich region followed by four immunoglobulin-like domains, the catalytic peroxidase domain, and a C terminal von Willebrand factor type C (vWFC) domain (7, 12, 13). The non-catalytic domains are generally thought to participate in protein-protein interactions. Recent work from our group revealed that the N-terminal Ig and peroxidase domains are necessary for the formation of collagen IV cross-links (10).

In this work we find that proprotein convertases (PCs) cleave human peroxidasin (hPxdn) at Arg-1336 near its C terminus. PC processing of Pxdn enhances HOBr production, which in turn promotes the formation of sulfilimine cross-links in the collagen IV network of basement membranes. Thus, PC processing represents a key regulatory event in Pxdn biosynthesis and function.

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[2] The abbreviations used are: BM, basement membrane; ECM, extracellular matrix; Pxdn, peroxidasin; hPxdn, human peroxidasin; TPO, thyroid peroxidase; MPO, myeloperoxidase; vWFC, von Willebrand factor type C; PHG, phloroglucinol; TMB, tetramethylbenzidine; PC, proprotein convertase; PDX, alpha1-antitrypsin Portland variant.

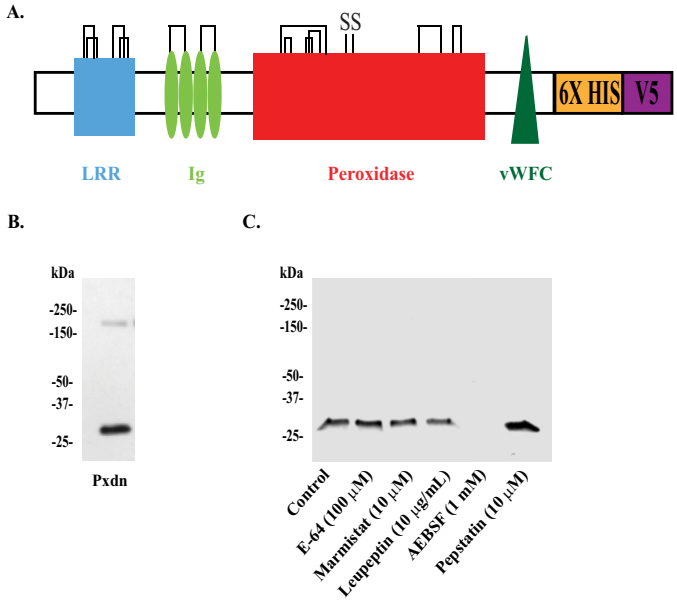



FIGURE 1. **Heterologously expressed human peroxidasin is proteolyzed.** *A*, schematic depiction of recombinant hPxdn with leucine repeat-rich (*LRR*), immunoglobulin (*Ig*), peroxidase, and vWFC domains. $His_6$ and V5 epitope tags were added to the C terminus. Validated intramolecular disulfide bridges are shown as *connected lines*, while suggested intermolecular disulfides are labeled *S* (8, 18). *B*, immunoblot of purified, recombinant hPxdn. *C*, immunoblot of ammonium sulfate-precipitated media under reducing conditions from HEK293 cells stably transfected with Pxdn and cultured without (control) or with indicated protease inhibitors. AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride) at 1 mM was cytotoxic. Blots were probed with anti-V5 antibody and are representative of three independent experiments.

## Results

*hPXDN Is a Substrate for PC Processing*—In our earlier work (4) we attempted to affinity-purify recombinantly expressed hPxdn using a C-terminal $His_6$ tag (Fig. 1*A*). Unfortunately, an ~30-kDa protein predominated the affinity-purified product instead of the predicted 170-kDa full-length Pxdn protein (Fig. 1*B*). We initially hypothesized that the protein may undergo nonspecific proteolytic degradation at the C-terminal end, creating a 30-kDa fragment that is captured by affinity chromatography. To test this hypothesis, we treated peroxidasin-expressing cells with several protease inhibitors to suppress proteolysis. None of these inhibitors resulted in the appearance of the expected full-length product (Fig. 1*C*). Rather than nonspecific proteolysis, we then considered whether Pxdn may undergo physiologic proteolytic processing during biosynthesis. Many secretory proteins are synthesized as inactive precursors that in addition to signal peptide cleavage undergo post-translational processing to become biologically active (14). A family of $Ca^{2+}$-dependent subtilisin-like secretory serine proteases known as PCs are often responsible for this form of proteolytic processing (15). To explore this possibility, a PC site prediction algorithm (ProP 1.0 Server) was used to determine whether Pxdn possessed any potential PC sites that often consist of a core (K/R)$X_n$(K/R) sequence ($X$ = any amino acid; $n$ = 0, 2, 4, or 6) (14, 16). A RGRR motif with predicted cleavage at the C-terminal Arg-1336 was found immediately preceding the vWFC domain, a location consistent with the observed 30-kDa C-terminal fragment. To test whether PCs process Pxdn, we treated Pxdn-expressing cells with a PC inhibitor, decanoyl-RVKR-chloromethyl ketone and found that these cells predominantly produce the expected full-length Pxdn product (Fig. 2*B*). To verify these findings, Pxdn-expressing cells were co-transfected with a protein PC inhibitor, α-1 antitrypsin Portland variant (PDX), and these cells also produced full-length Pxdn (Fig. 2*C*) (17). Taken together, these data suggest that Pxdn undergoes PC processing, which releases a 30-kDa C-terminal fragment.

PC processing of secreted proteins can occur intracellularly often in the Golgi or extracellularly at the cell surface (16). Immunoblotting of cell lysates revealed a predominance of full-length Pxdn in contrast to the media fraction where the C-terminal 30-kDa fragment is in excess. These data suggest that PC processing of Pxdn primarily occurs extracellularly (Fig. 2*D*).

In their discovery of Pxdn in *Drosophila*, Fessler and co-workers (13) found that Pxdn is a disulfide-linked homotrimer and hypothesized that the vWFC domain may act as an oligomerization domain. Recent work with hPxdn has confirmed these findings (8, 18). PC processing of hPxdn allowed us to investigate the oligomerization status and disulfide linkage of the vWFC domain, as these questions remained unexamined. Under non-reducing conditions, the larger 150-kDa Pxdn fragment was replaced by a much slower migrating presumed trimer band. However, the mobility of the C-terminal 30-kDa fragment was unchanged, suggesting this fragment lacks intermolecular disulfide bridges (Fig. 3*A*). If the vWFC domain promotes homotrimerization, one might expect that the C-terminal vWFC fragment may itself be a non-covalent oligomer. Gel filtration chromatography of the C-terminal fragment revealed an apparent molecular mass of 146 kDa suggesting an oligomer, although higher order than the predicted trimer (Fig. 3, *B* and *C*).

*Proprotein Convertases Cleave Human Peroxidasin at Arg-1336*—Analysis of the hPxdn protein sequence directly after the predicted PC recognition site suggests that the sequence of the proteolytically cleaved fragment (hPxdn-PCF) will begin with SLEFSYQ (Fig. 4*A*). To confirm the predicted site for PC processing of Pxdn and the sequence of the resulting hPxdn-PCF, the C-terminal 30-kDa fragment was submitted for N-terminal sequencing. Edman sequencing revealed the sequence immediately after Arg-1336 at the C-terminal end of the RGRR predicted PC site (Fig. 4*B*). To further confirm this site, the RGRR sequence was mutated to RGAA, which would not be amenable to PC processing. As predicted, the RGAA mutant of hPxdn (hPxdn-RGAA) was not cleaved and did not produce the 30-kDa C-terminal fragment (Fig. 4*C*).

*Proprotein Convertase Processing Enhances Pxdn Activity*—PC processing of catalytic proteins often activates or enhances the activity of the target protein. To investigate whether PC processing of Pxdn affects catalytic activity, recombinantly expressed wild type hPxdn and hPxdn-RGAA were purified. Both proteins exhibited similar heme incorporation based on $A_{415}/A_{280}$ ratios, but the RGAA mutant demonstrated reduced peroxidase activity by tetramethylbenzidine (TMB) oxidation and diminished HOBr production (Fig. 5). However, the biologic significance of Pxdn catalytic activity lies in the production of HOBr as a reactive intermediate to form sulfilimine cross-links in the collagen IV network. Moreover, although

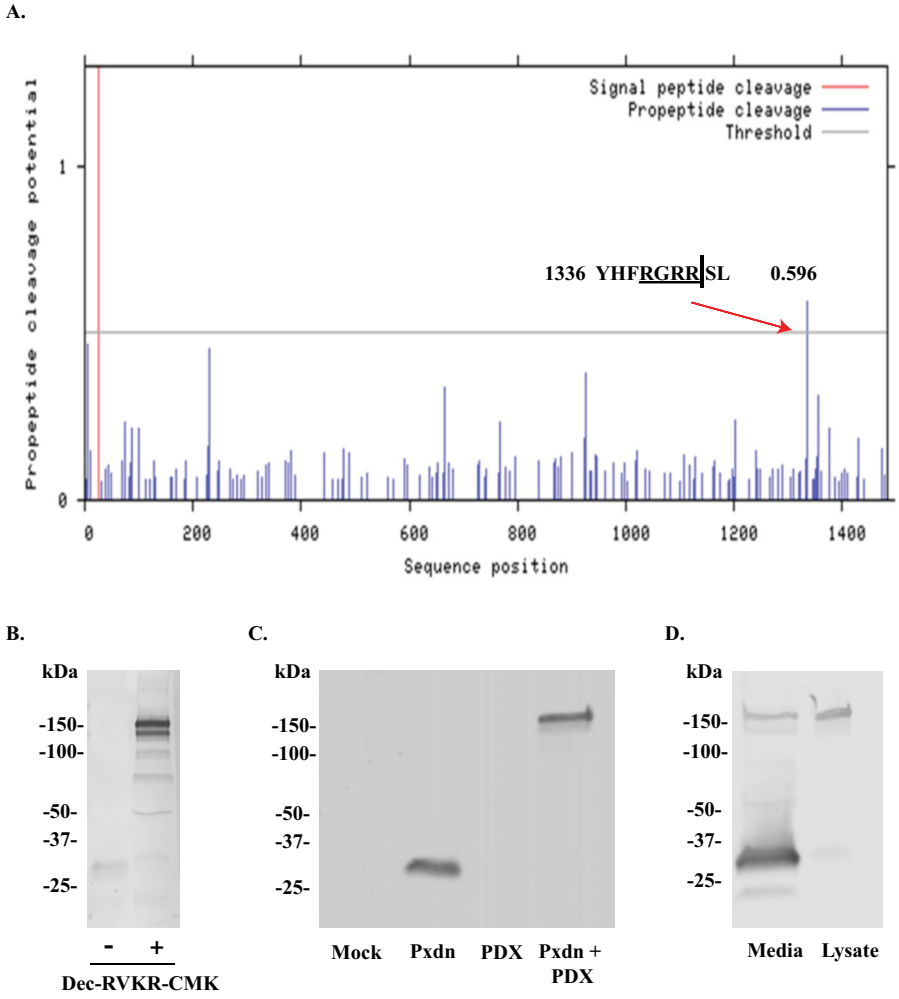



FIGURE 2. **Pxdn undergoes PC processing.** *A*, proprotein convertase cleavage site prediction results for Pxdn using the ProP 1.0 server (14). *B*, immunoblot of ammonium sulfate precipitated media under reducing conditions from HEK293 cells stably producing Pxdn that were either cultured without (−) or with (+) 100 $\mu$M decanoyl-RVKR-chloromethyl ketone (*Dec-RVKR-CMK*) proprotein convertase inhibitor. *C*, immunoblot of ammonium sulfate-precipitated media under reducing conditions from HEK293T cells transiently transfected with vector only (*Mock*), Pxdn, the PC inhibitor $\alpha$1-antitrypsin Portland variant (*PDX*), or co-transfected with Pxdn and PDX (*Pxdn + PDX*). *D*, immunoblot of cell lysate and ammonium sulfate-precipitated media under reducing conditions from HEK293 cells stably expressing Pxdn. Blots were probed with anti-V5 antibody and are representative of three independent experiments.

diminished without PC processing, residual catalytic activity could sufficiently cross-link collagen IV. Using CRISPR-Cas9 gene editing, the Pxdn gene was abrogated in the endodermal PFHR9 cell line, a previously characterized system that produces large quantities of cross-linked collagen IV and basement membrane. As expected, in the absence of Pxdn, the collagen IV network was devoid of sulfilimine cross-links. Pxdn was then reintroduced into these knock-out cells either as wild type hPxdn or as hPxdn-RGAA. The number of sulfilimine cross-links per hexamer was diminished in the RGAA mutant compared with wild type hPxdn (Fig. 6, *A* and *B*). These data suggested that PC processing of Pxdn is physiologically required for efficient sulfilimine bond formation. However, to further confirm that unprocessed Pxdn, even at high levels, is functionally deficient, we utilized the "overlay assay" where stably transfected cells overexpressing wild type or the mutant hPxdn-RGAA were plated on top of an uncross-linked collagen IV matrix from PFHR9 cells. Despite overexpression, the mutant hPxdn-RGAA protein remained less efficient at forming sulfilimine cross-links in collagen IV (Fig. 6, *C* and *D*). Taken together, these data suggest that PC processing of hPxdn enhances enzymatic and functional activity.

## Discussion

In this work we found that PCs proteolytically process peroxidasin near its C terminus, thereby releasing a large, catalytically active fragment and a smaller fragment consisting primarily of the C-terminal vWFC domain. This processing of Pxdn enhances catalytic activity and promotes the formation of sulfilimine cross-links in the collagen IV network of BM. Thus, PC processing of Pxdn represents a key regulatory step in the biosynthesis and function of Pxdn to support BM and tissue integrity. Expectedly, the peroxidasin-collagen IV-sulfilimine bond triad is conserved throughout the animal kingdom, where the evolution of basement membranes is thought to be a key step in the development of multicellular tissues. The multidomain structure of peroxidasin is mostly conserved in *Bilateria*, whereas there is a distinct lack of the C-terminal vWFC domain in *Cnidaria* (11). Interestingly, we find that a PC cleavage site between the catalytic and vWFC domains is predicted in nearly

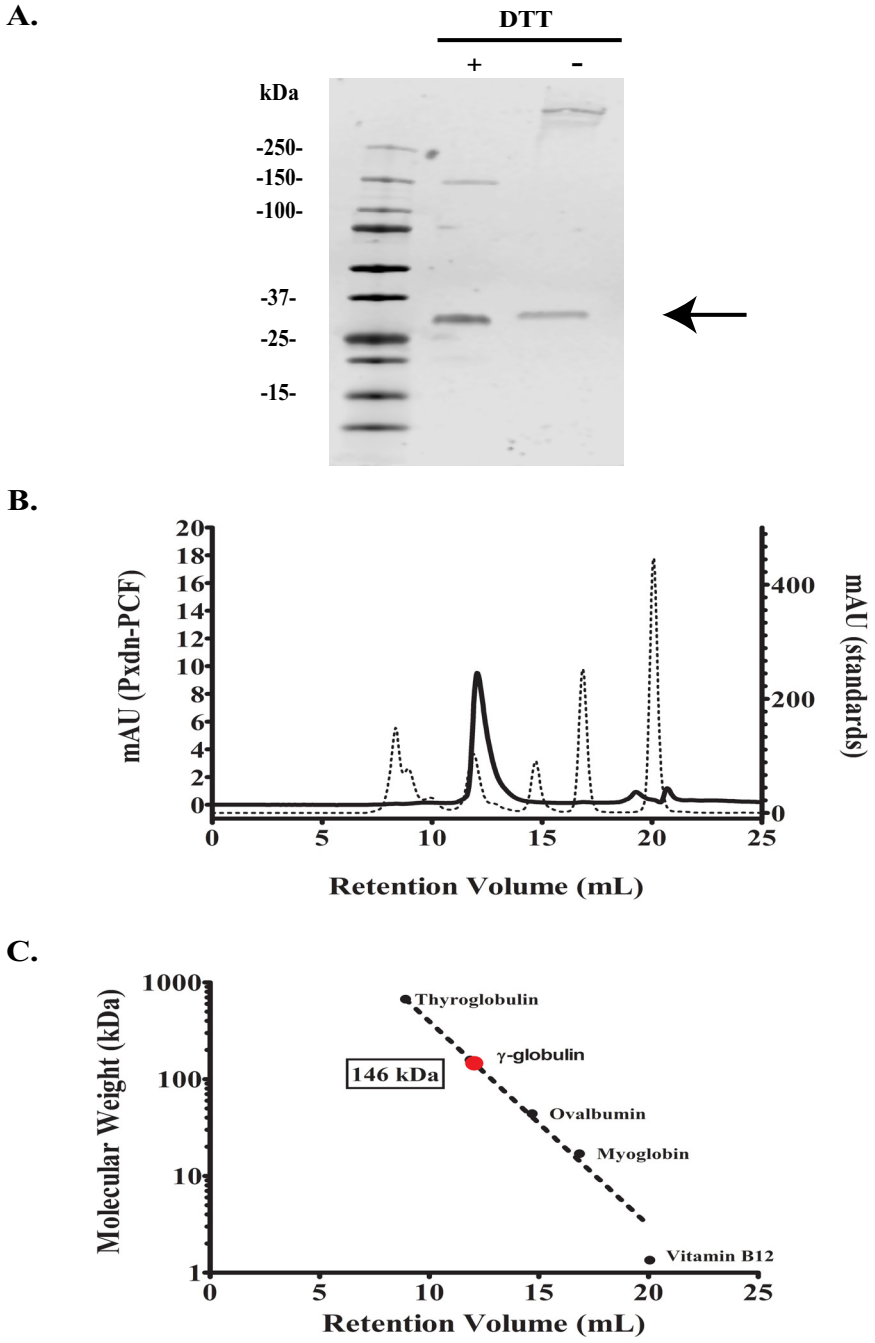
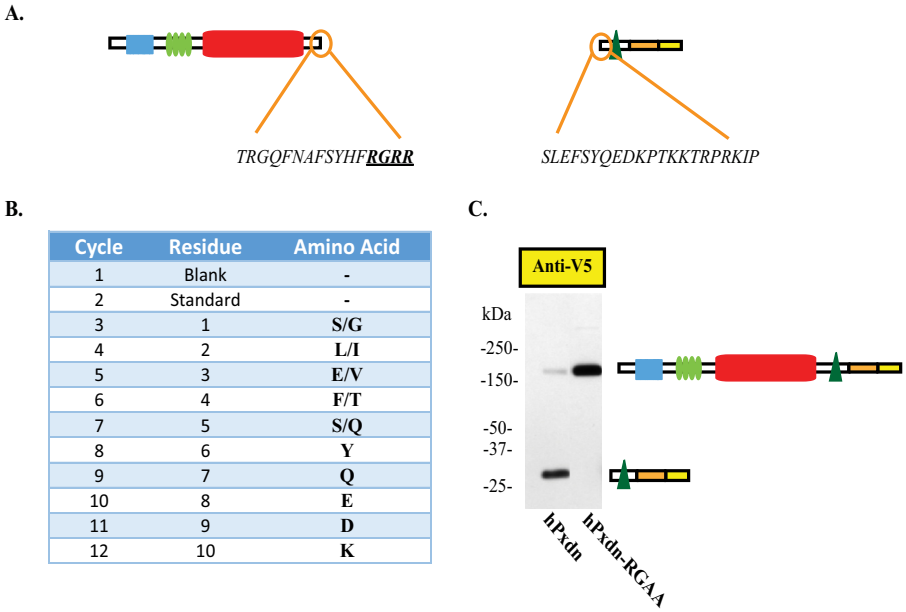



FIGURE 3. **The C-terminal fragment of peroxidasin (*Pxdn-PCF*) released by proprotein convertase processing is a non-covalently linked oligomer.** *A*, immunoblot of ammonium sulfate-precipitated media from HEK293 cells stably transfected with hPxdn. The migration of the proteolytically processed C-terminal fragment is unchanged with the addition of 50 mM dithiothreitol (*DTT*; *arrow*). Blots were probed with anti-V5 antibody. *B*, gel filtration chromatography elution profile based on absorbance at 280 nm (*mAU*) of proteolytically processed C-terminal fragment of Pxdn (Pxdn-PCF; *solid line*) and molecular mass standards (*dashed line*) run successively on GE S200 gel filtration column (GE Healthcare). *C*, estimation of molecular weight of PXDN-PCF using gel filtration chromatography retention volume of molecular weight standards (*black*) and PXDN-PCF (*red*).

all *Bilateria*, adding further credence to the importance of this processing in regulation of Pxdn function (Fig. 7). Because Pxdn produces a powerful, potentially damaging oxidant in the form of HOBr, the processing of Pxdn extracellularly may activate its catalytic activity to act upon its collagen IV substrate while minimizing collateral oxidative damage.

Based on this and prior work, a model for Pxdn biosynthesis may be proposed. Pxdn monomers may initially trimerize through the vWFC domain, which allows for cysteines throughout the remainder of the molecule to approach one another and form disulfide linkages to covalently consolidate the homotrimeric state (7, 13, 16). The vWFC domain itself, although cysteine-rich, remains a noncovalent trimer with either free cysteines or cysteines forming intramolecular disulfide bridges. Temporally close to extracellular secretion, PCs cleave Pxdn releasing the vWFC domain oligomer. This acti-




| Cycle | Residue | Amino Acid |
|---|---|---|
| 1 | Blank | - |
| 2 | Standard | - |
| 3 | 1 | S/G |
| 4 | 2 | L/I |
| 5 | 3 | E/V |
| 6 | 4 | F/T |
| 7 | 5 | S/Q |
| 8 | 6 | Y |
| 9 | 7 | Q |
| 10 | 8 | E |
| 11 | 9 | D |
| 12 | 10 | K |

FIGURE 4. **Proprotein convertases cleave peroxidasin at Arg-1336.** *A*, schematic representation of the predicted Pxdn fragments that result upon proprotein convertase proteolytic processing. *B*, N-terminal Edman degradation sequencing cycles of the C-terminal fragment of Pxdn released after PC processing (Alphalyse Inc., Palo Alto, CA). *C*, immunoblot of ammonium sulfate-precipitated media under reducing conditions from HEK293 cells transiently transfected with hPxdn or hPxdn-RGAA and probed with anti-V5 antibody. Shown is a blot representative of three independent experiments.

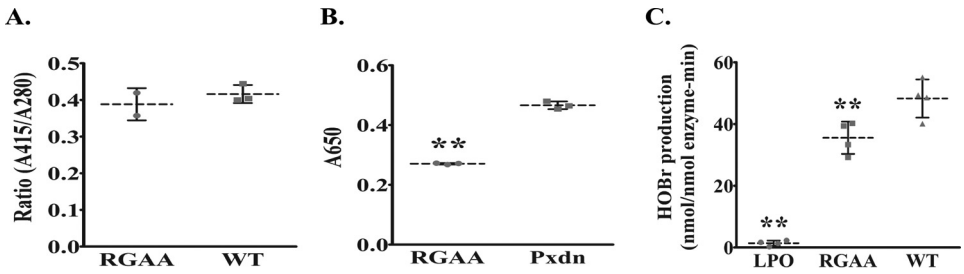



FIGURE 5. **Proprotein convertase proteolysis of peroxidasin enhances enzymatic activity.** *A*, ratio of absorbance at 415 nm and 280 nm for both Pxdn and Pxdn-RGAA demonstrating equal heme incorporation. *B*, peroxidase activity as determined by TMB oxidation measured as absorbance at 650 nm ($A_{650}$). *C*, HOBr production expressed as nmol of HOBr generated/nmol of enzyme/min measured in 1× PBS supplemented with 100 $\mu$M NaBr for wild type hPxdn (*WT*) and mutant hPxdn-RGAA (*RGAA*). In all panels individual data points are displayed with mean (*dotted line*) and S.D. Data were analyzed using *t* test or analysis of variance followed by post hoc pairwise comparisons with Bonferroni's correction for multiple comparisons (**, $p \leq 0.01$ compared with WT Pxdn).

vates the nearby catalytic domain to form HOBr as an intermediate to create sulfilimine cross-links in collagen IV. A caveat to this model is that gel filtration chromatography of the released C-terminal fragment containing the vWFC domain revealed an oligomer of nearly five subunits rather than the expected three subunits (Fig. 3*C*). This may reflect a larger hydrodynamic radius such that the trimeric C-terminal fragment anomalously elutes as a larger oligomer. Alternatively, the C-terminal vWFC domain may be a dimer of two trimers or a hexamer that forms either before or after proteolytic processing with a slightly smaller than expected hydrodynamic radius. Lastly, after PC processing, trimeric C-terminal fragments may dissociate and reorganize into pentamers.

PC processing of secreted proteins is ubiquitously present in nearly all mammalian tissues and, therefore, plays a critical role in normal physiology and disease. In regard to extracellular matrix homeostasis, PC processing activates several proteases, such as matrix metalloproteases, and also promotes the polymerization of structural proteins (15, 16). Whereas most processing events occur on the N-terminal end of proteins, several extracellular matrix proteins, such as fibrillin, collagen V, and now peroxidasin, undergo C-terminal processing (19, 20). Besides Pxdn, PCs also cleave other animal heme peroxidases, such as MPO and TPO. Unlike Pxdn, PC processing of MPO and TPO targets the N terminus and primarily affects protein trafficking rather than catalytic activity (21, 22).

Taken together, our work begins to shed light on the regulation of Pxdn function and identifies PC processing as an activation step to target HOBr efficiently toward collagen IV and minimize oxidative damage. Future mechanistic work will focus on the molecular mechanism of this regulation, which may involve the vWFC domain of Pxdn acting as a negative regulator of its catalytic domain.

## Experimental Procedures

*Site-directed Mutagenesis*—Full-length human peroxidasin was used as the starting construct (23). The PC recognition sequence within Pxdn, RGRR, was mutated to RGAA using QuikChange mutagenesis as previously described (24).

*Cell Culture*—Human embryonic kidney 293 and 293T cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium/F-12 medium

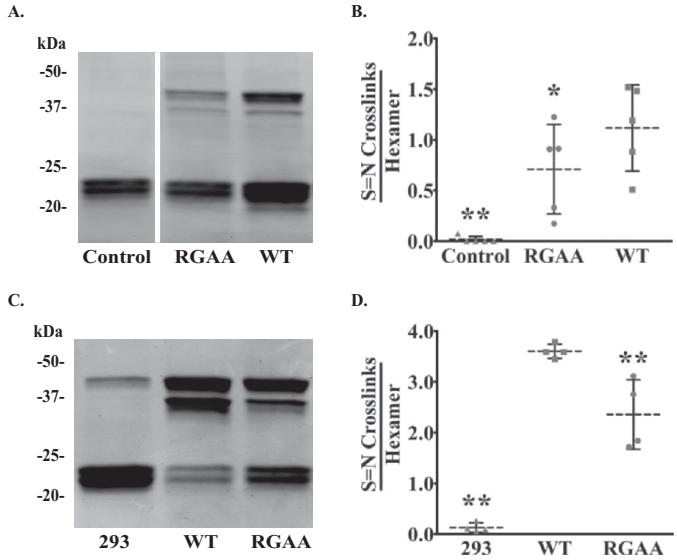



FIGURE 6. **Proprotein convertase processing of peroxidasin enhances the formation of sulfilimine cross-links in collagen IV.** *A* and *B*, CRISPR generated Pxdn knock out PFHR-9 cells (*Control*) were rescued by stable transfection of either wild type (*WT*) or the mutant hPxdn-RGAA (*RGAA*). The matrix containing collagen IV network was isolated 5 days post confluency. The number of cross-links per collagen IV NC1 hexamer was determined using densitometric quantitation of the dimeric and monomeric subunits from SDS-PAGE of the collagenase-digested matrix. *C* and *D*, untransfected HEK293 cells or HEK 293 cells stably transfected with either wild type (WT) or the mutant hPxdn-RGAA (*RGAA*) were plated on PFHR-9 uncross-linked matrix, and the underlying matrix was isolated 24 h after overlay for analysis of collagen IV sulfilimine cross-link content. The number of cross-links per collagen IV NC1 hexamer was determined using densitometric quantitation of the dimeric and monomeric subunits from SDS-PAGE of the collagenase-digested matrix. In all panels individual data points are displayed with mean (*dotted line*) and S.D. Data were analyzed using analysis of variance followed by post hoc pairwise comparisons with Bonferroni's correction for multiple comparisons (*, $p \leq 0.05$; **, $p \leq 0.01$ compared with WT Pxdn).

(Sigma) containing 5% fetal bovine serum. Stable transfections were done by using the calcium phosphate precipitation method and 6 $\mu$g of plasmid DNA with both the native hPxdn and mutant hPxdn-RGAA construct. Clones resistant to 250 $\mu$g/ml G418 (Mediatech, Manassas, VA) were isolated and expanded. Stably transfected clones were screened for protein expression from serum-free medium by Western blotting using anti-V5 antibody (Life Technologies). Transient transfections were done using Lipofectamine LTX reagent and 2.5 $\mu$g of plasmid DNA following the manufacturer's protocol (Life Technologies). The medium was collected 24 h post transfection and analyzed by anti-V5 immunoblotting.

*Protein Purification*—Recombinant hPxdn was purified as previously described (4). Recombinant hPxdn-RGAA and hPxdn-PCF proteins were purified from conditioned media collected from stably transfected HEK293 cells by affinity chromatography on a HiTrap TALON affinity column on an ÄKTApurifier HPLC system (GE Healthcare) according to the manufacturer's instructions and eluted with 100 mM imidazole. The resulting peak fractions were combined, then precipitated with 40% ammonium sulfate and reconstituted in cetyltrimethylammonium bromide (CTAB) buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 0.2% CTAB) with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml of leupeptin, and 1 $\mu$g/ml of pepstatin). The concentration of purified protein was determined using the bicinchoninic acid (BCA) assay (ThermoFisher Scientific Pierce).

*Gel Electrophoresis and Western Blotting*—Purified proteins and cell culture medium were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% gradient gels (Bio-Rad) then transferred to nitrocellulose membranes, blocked with 5% nonfat milk in 1× TBS with 0.05% Tween 20 for 1 h, and probed overnight with anti-V5 antibody (Life Technologies) at 1:5000 dilution. Bound antibody was detected using anti-mouse secondary antibodies conjugated with alkaline phosphatase or horseradish peroxidase followed by detection with colorimetric or luminescent substrates.

*Determination of Relative Heme Incorporation*—To determine the relative incorporation of heme for each construct, UV-visible spectrums of both wild type hPxdn and the mutant hPxdn-RGAA were read, and the ratio of the peak absorbance at 415 nm and 280 nm was calculated.

*Peroxidase Activity Assays*—Purified hPxdn and mutant hPxdn-RGAA concentrations were determined using BCA protein assay, equal amounts of protein were added to a 96-well microtiter plate in triplicate along with TMB liquid substrate (Sigma), and the absorbance was read at 650 nm ($A_{650}$) using a SpectraMax 190 plate reader (Molecular Devices LLC, Sunnyvale, CA).

*Measurement of Hypobromous Acid Production*—Hypobromous acid (HOBr) production was determined as previously described (4). Briefly, hypobromous acid standard solutions were freshly prepared by combining 80 mM NaOCl with 90 mM KBr and adjusting the pH to 7.4 after 1 min. The HOBr concentration was quantified using $A_{329} = 332\ \text{M}^{-1}\text{cm}^{-1}$ at alkaline pH. Equimolar amounts of hPxdn and hPxdn-RGAA were combined with 5 mM taurine in 1× PBS + 100 $\mu$M NaBr, and reactions were initiated with the addition of 50 $\mu$M $H_2O_2$, and allowed to proceed for 10 min. Reactions were stopped with the addition of 20 $\mu$g/ml bovine liver catalase (Sigma). After 5–10 min, ¼ volume of developing buffer (0.4 M sodium acetate, pH 5.4, 2 mM tetramethylbenzidine, 10% dimethylformamide) was added, and $A_{650}$ was measured. HOBr standards were used to calculate concentrations from $A_{650}$ measurements.

*Stably Transfected Cell Overlay Assay for Cross-link Determination*—PFHR-9 cells were cultured as previously described (10). Briefly, cells were grown at 10% $CO_2$ for 5 days post-confluency in the presence of 50 $\mu$M phloroglucinol to inhibit peroxidasin-mediated sulfilimine bond formation (4). The PFHR-9 cells were then removed using two detergent extractions with hypotonic buffer (10 mM Tris-Cl, pH 7.5, + 0.1 mM $CaCl_2$ + 0.1% bovine serum albumin) + 1% Triton-X, followed by 2 washes with hypotonic buffer + 0.1% sodium deoxycholate. The remaining matrix was extracted with 4 M guanidine hydrochloride + 50 mM Tris-Cl, pH 7.5, and then washed extensively with 1× PBS. These PFHR-9 matrix plates were either stored under sterile conditions at −80 °C or used immediately.

HEK293 cells stably transfected with either wild type hPxdn or hPxdn-RGAA mutant construct were plated onto PFHR-9 matrix and were then incubated for 24 h in the presence of 5 $\mu$M hematin and 5 mM sodium butyrate. After incubation, the cells and underlying ECM were scraped into deoxycholate buffer

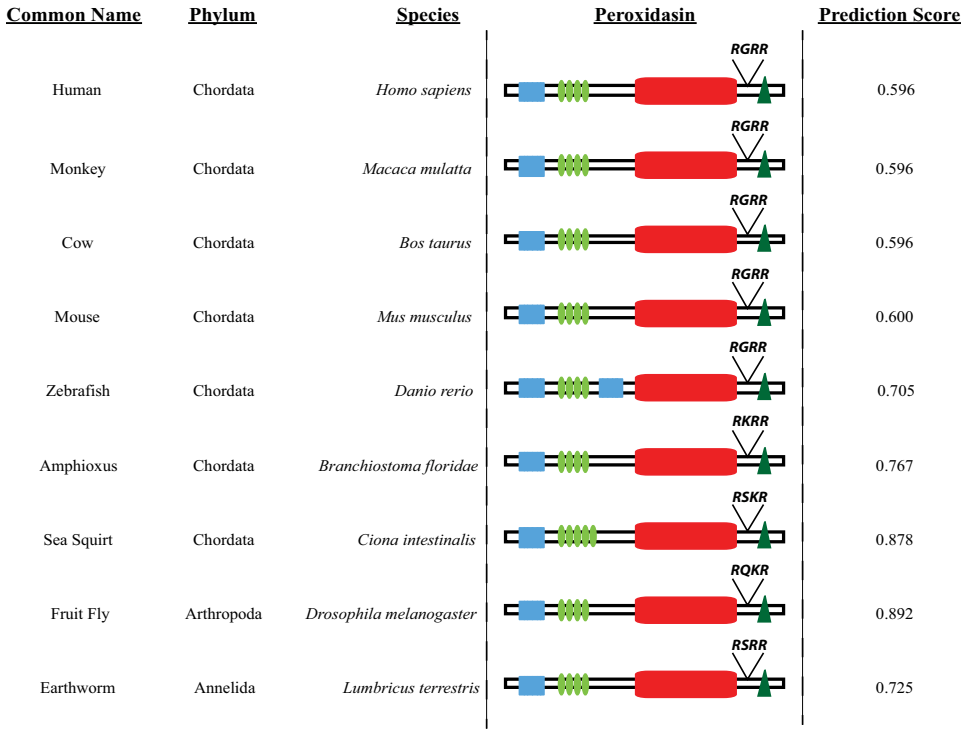



FIGURE 7. **The presence of a C-terminal proprotein convertase recognition sequence in peroxidasin is evolutionarily conserved.** Shown is a schematic representation of peroxidasin among differing species. The presence of a predicted PC recognition sequence is found across several Eumetazoa phyla. Sequence data were gathered from National Center for Biotechnology Information Reference Sequence. PC recognition site scores were predicted by the ProP 1.0 server (14).

(1% sodium deoxycholate, 10 mM Tris-Cl, pH 7.5, and 1 mM EDTA-Na) including protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml leupeptin, and 1 $\mu$g/ml pepstatin) and sonicated to eliminate viscosity. The resulting supernatant was designated as the cell lysate, and the insoluble pellet was further processed to isolate ECM. To determine sulfilimine cross-link content of collagen IV, the ECM fraction was digested with collagenase (50 $\mu$g/ml; CLSPA grade, Worthington Biochemical Corp., Lakewood, NJ) overnight at 37 °C in collagenase buffer (100 mM NaCl, 50 mM Tris-Cl, pH 7.5, 25 mM 6-aminocaproic acid, 5 mM benzamidine, 0.5 mM PMSF, and 50 $\mu$M phloroglucinol). The collagenase digest supernatant underwent SDS-PAGE under non-reducing conditions followed by Coomassie Blue staining to visualize and quantify sulfilimine cross-linked dimeric and non-cross-linked monomeric subunits. The average number of cross-links per hexamer was determined using the density of the single (D1)- and double-cross-linked (D2) and non-cross-linked (M) subunit bands: cross-links per hexamer = (6 × fraction D2) + (3 − fraction D1).

*CRISPR-mediated Knock-out*—PFHR9 cells were transfected with guide RNAs targeting the first exon of the mouse Pxdn gene and a hygromycin resistance homologous recombination vector using Lipofectamine 2000 transfection reagent and a total of 3 $\mu$g of DNA per the manufacturer's protocol (Life Technologies). Transfected cells underwent selection with 250 $\mu$g/ml hygromycin B and 0.2 $\mu$M fialuridine 48 h after transfection. Resistant clones were isolated, expanded, and screened for the presence and/or absence of the knock-out cassette via homologous recombination utilizing the primers Pxdn HR forward primer (5′-GGGGGCTGTCCCTAGATCTAT-3′) and HR reverse primer (5′-AAGGCGCAAAGCACAACTCCA-3′). The clones were also screened for the presence and/or absence of the wild type allele utilizing the primers Pxdn WT forward primer (5′-CTCCTCCGCTGCTGTTCACGC-3′) and WT reverse primer (5′-AATCGCACGTCCCGCAGACCC-3′).

*Author Contributions*—G. B. conceived the project. S. C. conducted most of the experiments, and G. B. conducted the preliminary work. Both S. C. and G. B. wrote the manuscript.

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