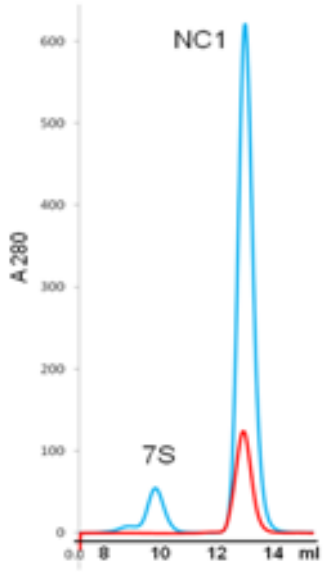
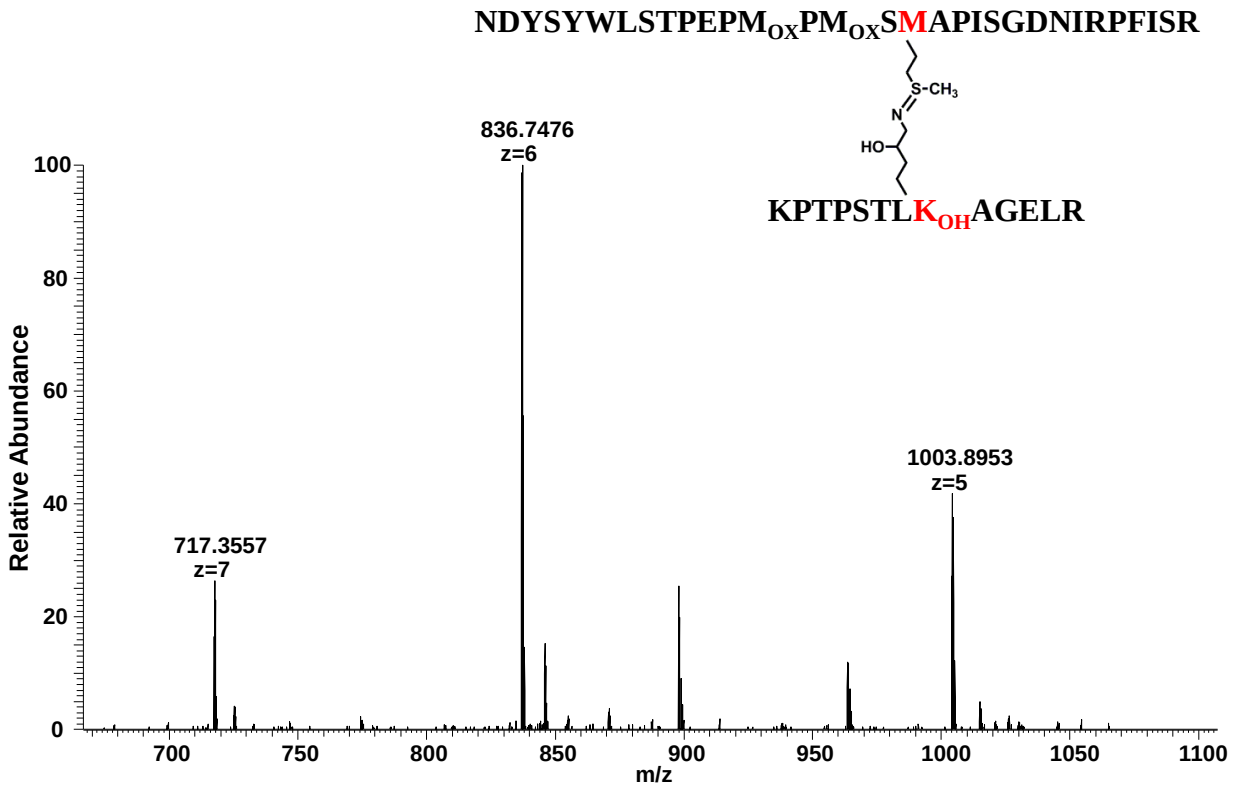
Supplementary Results

Supplementary Table 1. Proteins identified by mass spectrometry of tryptic peptides from azide labeled basement membrane proteins.

Protein	Coverage	Number of Peptides	Description
LAMA1_MOUSE	8	22	GN=Lama1, Laminin subunit alpha-1
LAMC1_MOUSE	10	16	GN=Lamc1, Laminin subunit gamma-1
CO4A2_MOUSE	4	11	GN=Col4a2, Collagen alpha-2(IV) chain
LAMB1_MOUSE	3	4	GN=Lamb1-1, Laminin subunit beta-1
B1B0C7_MOUSE	1	4	GN=Hspg2, Perlecan
Q5FWY9_RAT	10	4	GN=Col4a1, Collagen alpha-1 (IV) chain
PXDN_MOUSE	***3***	***3***	***GN=Pxdn, Peroxidasin Homolog***
Q71DI1_RAT	18	2	GN=Dcd, Dermcidin

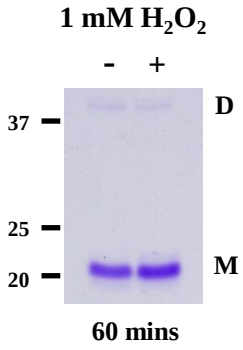


Supplementary Figure 1. Peroxidase inhibitors do not prevent assembly of the collagen IV NC1 hexamer. Collagen IV gel filtration chromatogram after collagenase digest from uncross-linked PFHR-9 basement membrane deposited in the presence of 50 μM phloroglucinol *(blue)*. 7S is the larger, N-terminal collagenase resistant fragment of collagen IV and is not the focus of this work. The identical elution position of purified bovine placenta NC1 hexamer (*red*) suggests that collagen IV protomers continue to form hexamers without sulfilimine cross-links.

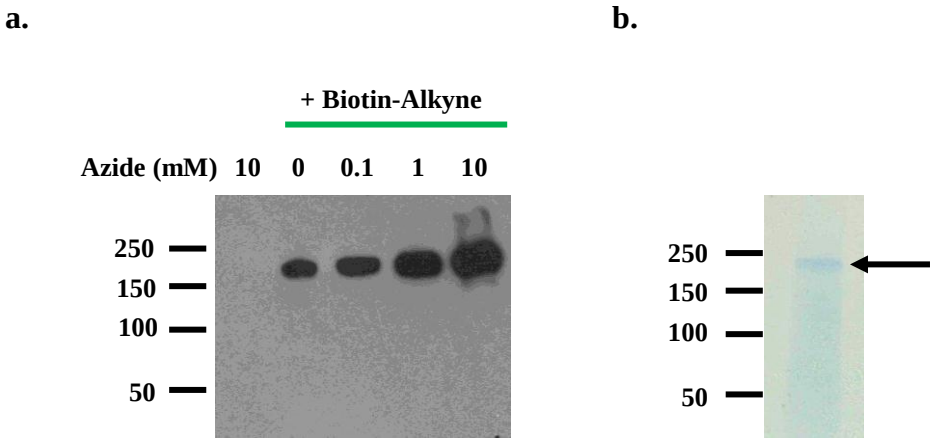


Z	Theoretical mass	Observed ion (m/z)	Observed peptide mass	Difference (theo – obs)
+5		1003.8953	5014.4375	
+6		836.7476	5014.4388	
+7		717.3557	5014.4353	
	5016.446		5014.4372	2.008

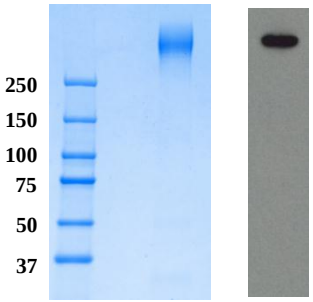
Supplementary Figure 2. Recovered cross-linked collagen IV contains sulfilimine bond. PFHR-9 basement membrane deposited in the presence of 10 mM potassium iodide was isolated and sulfilimine cross-links were reconstituted *in vitro* by adding 1 mM H_2O_2. Cross-linked NC1 dimers identified with SDS-PAGE and Coomassie blue staining were excised for trypsin digest and mass spectroscopic analysis. The sulfilimine cross-linked peptide was identified by the mass of 5014.4372 characteristically 2 amu less than the theoretical mass. M_{OX} represents methionine sulfoxide.



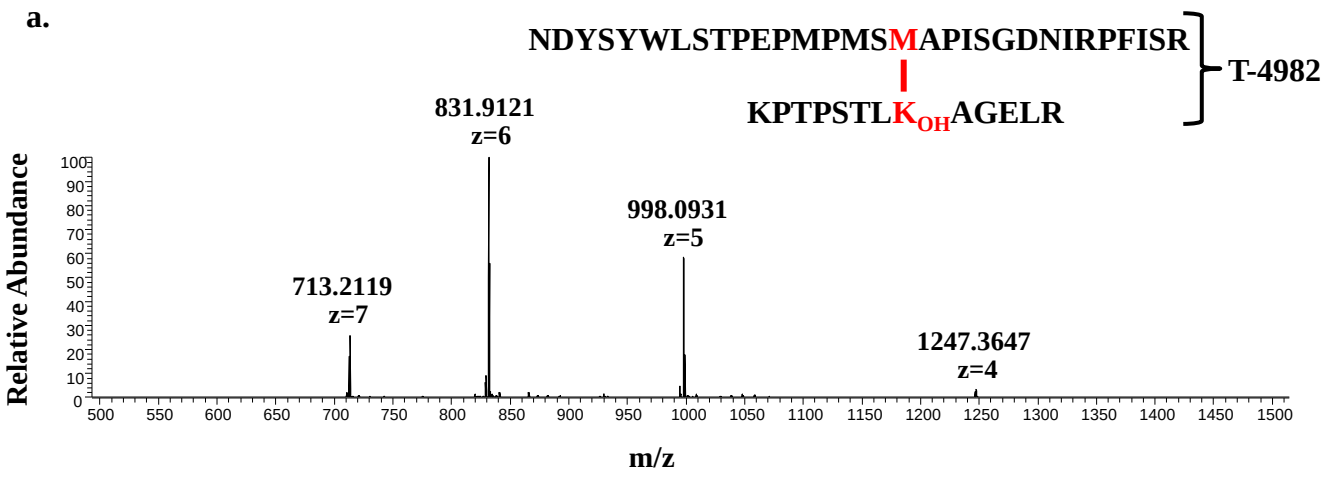
Supplementary Figure 3. H_2O_2 does not directly form sulfilimine cross-links in collagen IV. Uncross-linked PFHR-9 basement membrane was first extracted with 2M guanidine chloride and then incubated with or without 1 mM H_2O_2 for 1 hour. Guanidine extraction eliminated cross-linking activity even with sufficient H_2O_2. Gel is representative of 4 independent experiments.

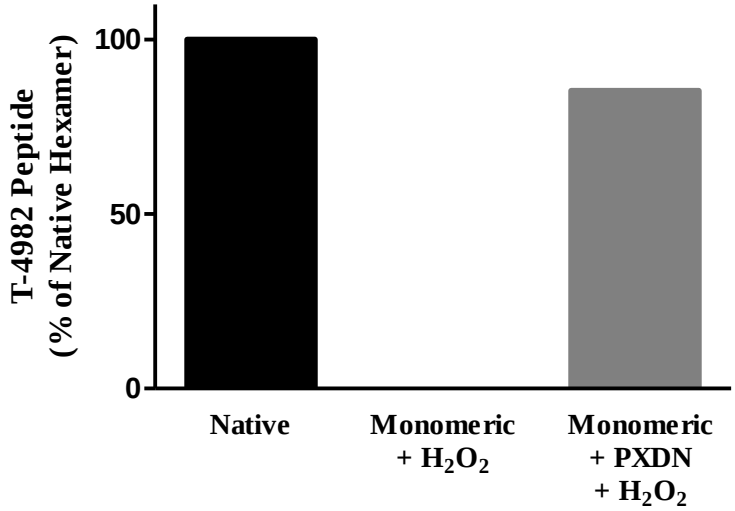


Supplementary Figure 4. Azide labeling reveals peroxidasin as a basement membrane peroxidase. (a) Isolated PFHR-9 basement membrane was incubated with 1 mM H_2O_2 and the indicated concentrations of azide for 1 hour. Basement membrane proteins were solubilized and underwent click biotinylation as described in the Methods. Streptavidin blotting of these proteins revealed dose-dependent azide incorporation with increasing azide concentration. Biotinylation did not occur in the absence of biotin-alkyne reagent, but some background was present in the absence of azide. **(b)** Affinity purification of biotinylated proteins using streptavidin-agarose with subsequent SDS-PAGE revealed a single prominent band (*arrow*) which was excised for trypsin digestion and mass spectroscopic identification. The most commonly identifed peptides by mass spectrometry are shown in Supplementary Table 1.

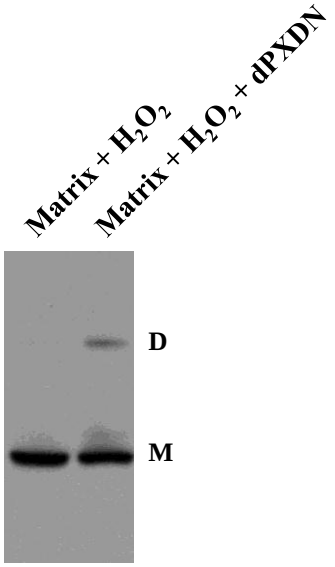


Supplementary Figure 5. Purification of human peroxidasin from stably transfected HEK293 Cells. HEK293 cells were transfected with pCDNA-V5-His-TOPO (Life Technologies) containing the human peroxidasin coding sequence provided by Dr. Miklos Geizst (Semmelweis University, Budapest, Hungary) using standard calcium-phosphate precipitation. G418 resistant clones were isolated, screened for peroxidasin expression, and a highly productive clone was selected for purification. Conditioned media was collected and human peroxidasin was purified using ammonium sulfate precipitation, ion-exchange chromatography, and density ultracentrifugation as described in the Methods. Purified protein was electrophoresed within a 5% polyacrylamide gel under denaturing conditions without reducing agent, stained with Coomassie blue (*left panel*), and immunoblotted with rabbit polyclonal anti-human peroxidasin antibody (*right panel*) generated against the peptide IREKLKRLYGSTLNI. Similar to *Drosophila* peroxidasin, the purified protein is a disulfide-linked trimer around 450-500 kD.

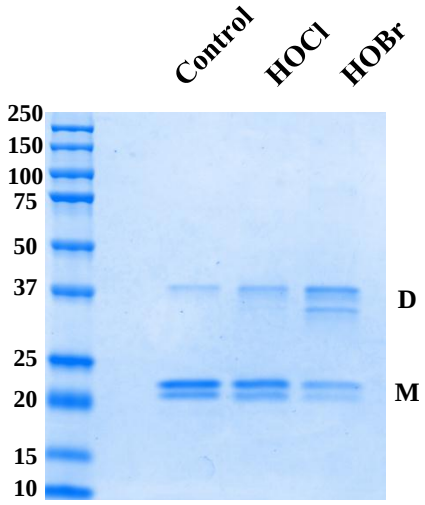




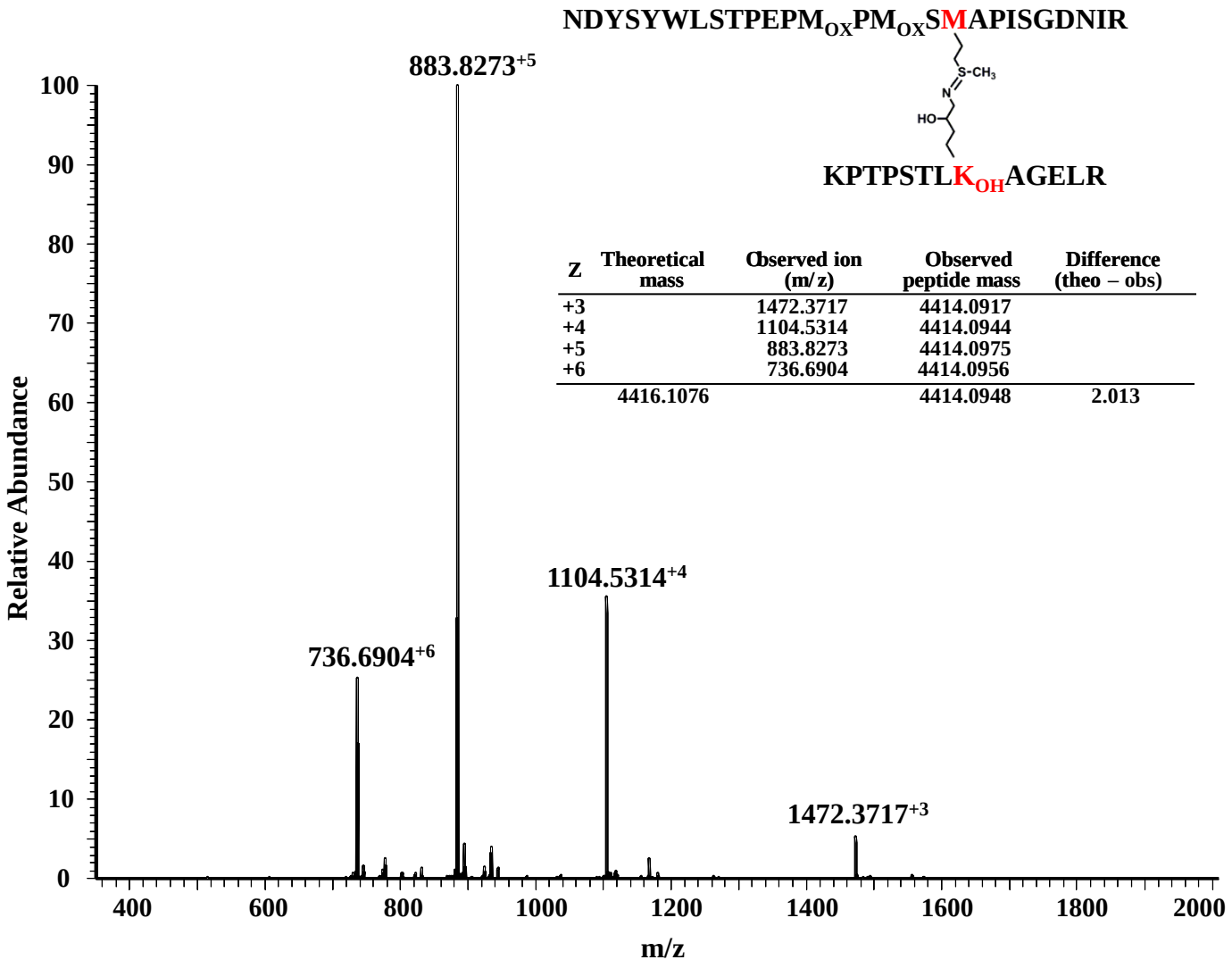
Supplementary Figure 6. Mass spectrometry directly demonstrates that peroxidasin forms sulfilimine bonds in isolated collagen IV NC1 hexamer. (a) Representative MS spectrum showing the +7, +6, +5, and +4 ions for the peptide complex T-4982 formed upon incubation of monomeric NC1 hexamer with peroxidasin and 10 μM H_2O_2 for 1 hour at 37°C. **(b)** The extent of sulfilimine cross-linking in native PFHR-9 collagen IV NC1 hexamer, H_2O_2 (10 μM) reacted monomeric hexamer, and peroxidasin (+H_2O_2) reacted hexamer was determined by normalizing the area of the m/z 831.9 ion peak to the area of an unrelated NC1 peptide peak. Values were expressed as a % of native PFHR-9 hexamer. No cross-linked peptide was detected with H_2O_2 alone.



Supplementary Figure 7. *Drosophila* peroxidasin catalyzes cross-links within PFHR-9 collagen IV network. PFHR-9 basement membrane formed in the presence of phloroglucinol to create an uncross-linked collagen IV network was incubated with 10 μM H_2O_2 for 1 hour at 37°C with or without *Drosophila* peroxidasin (dPXDN) purified from Kc7e10 cells. Reaction was stopped with 50 μM phloroglucinol, basement membrane was collagenase digested, and the solubilized NC1 hexamer was immunoblotted with anti-collagen IV α2 rat monoclonal antibody (H22, 1:2000; Shigei Medical Research Institute). Modest formation of cross-linked collagen IV was observed only when peroxidasin was included in the reaction.

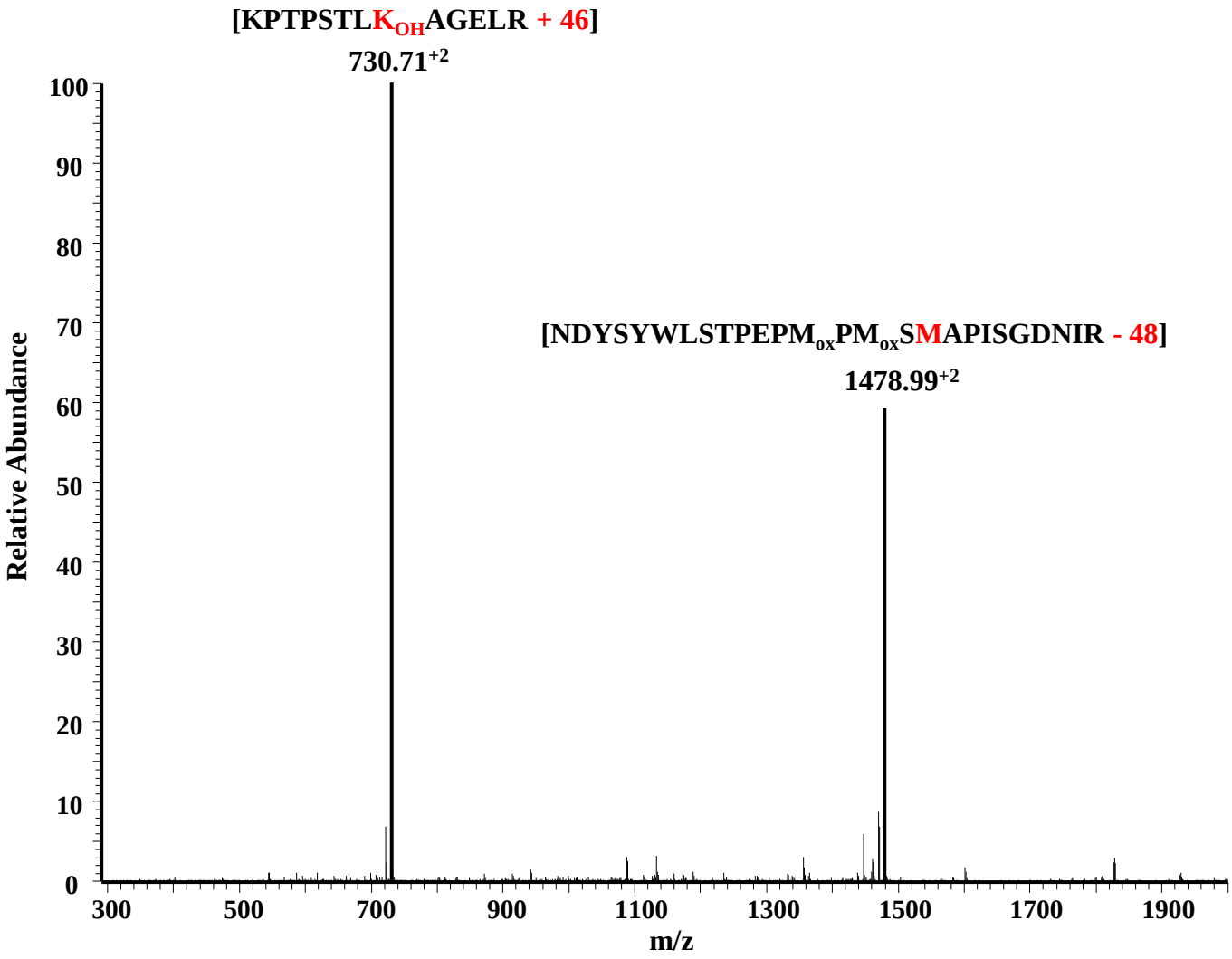


Supplementary Figure 8. Hypohalous acids form sulfilimine bonds in collagen IV. 500 nM collagen IV NC1 hexamer (3 μM potential cross-links) was incubated alone (control) or with 5 μM hypochlorous (HOCl) or hypobromous acid (HOBr) for 30 minutes at 37ºC. SDS-PAGE and Coomassie blue staining revealed the formation of cross-linked NC1 dimeric subunits in hypohalous acid treated reactions compared to control.



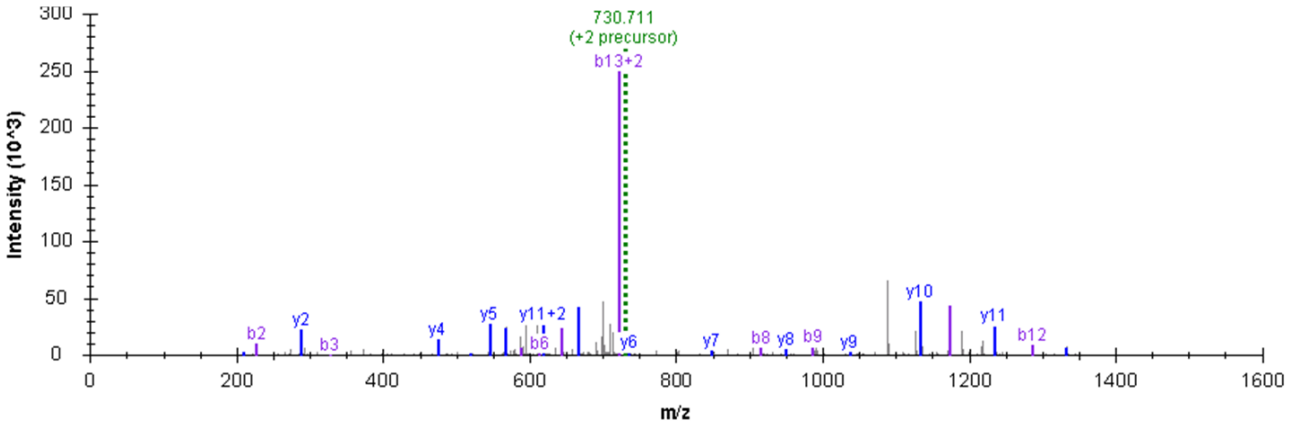
Z	Theoretical mass	Observed ion (m/z)	Observed peptide mass	Difference (theo – obs)
+3		1472.3717	4414.0917	
+4		1104.5314	4414.0944	
+5		883.8273	4414.0975	
+6		736.6904	4414.0956	
	4416.1076		4414.0948	2.013

Supplementary Figure 9a. HOBr forms sulfilimine cross-links in collagen IV NC1 hexamer as demonstrated by mass spectrometry. Representative MS spectrum of tryptically digested NC1 hexamer after reaction with HOBr showing the +6, +5, +4, and +3 ions for the sulfilimine cross-linked peptide complex. The peptide was found to have a mass of 4414.0958 which is 2 amu less than the theoretical mass of the individual peptides, a characteristic of the sulfilimine cross-link. M_{OX} represents methionine sulfoxide.

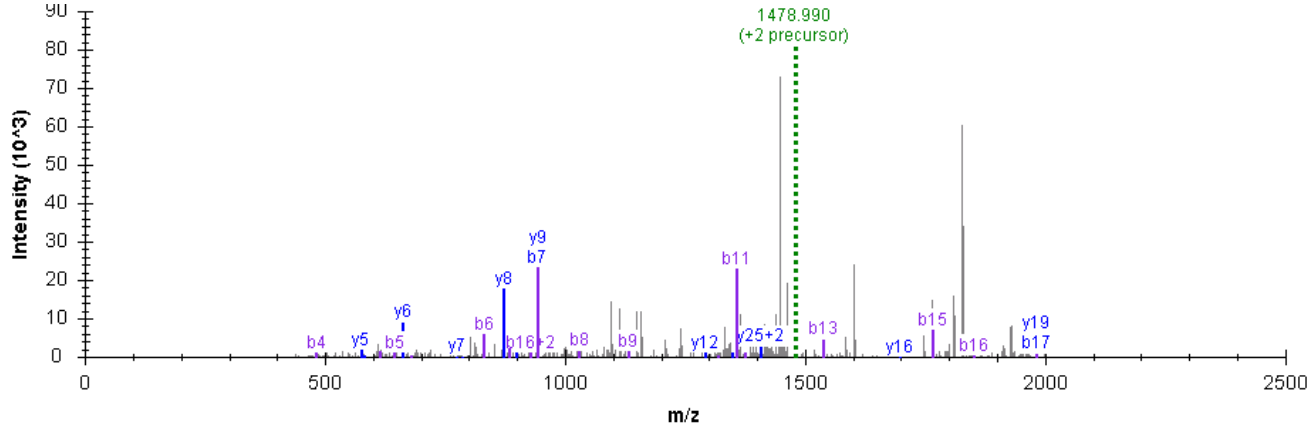


Supplementary Figure 9b. CID MS^2 fragmentation of m/z 883.8273^{+5} ion of the HOBr reacted collagen IV sulfilimine cross-linked peptide. This spectrum shows the CID fragmentation of the quintuple-charged m/z 883.8273 ion, which generates two main fragments. m/z 730.71 ion corresponding to the hydroxylysine-containing peptide plus 46 amu and m/z 1478.99 ion corresponding to the methionine containing peptide losing 48 amu upon fragmentation. M_{OX} stands for methionine sulfoxide.

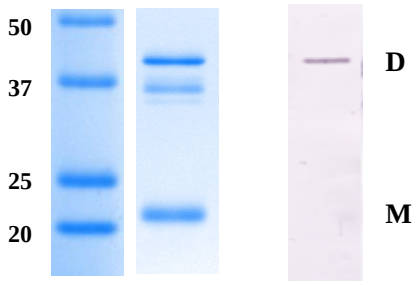
KPTPSTL[K_{OH}+46]AGELR

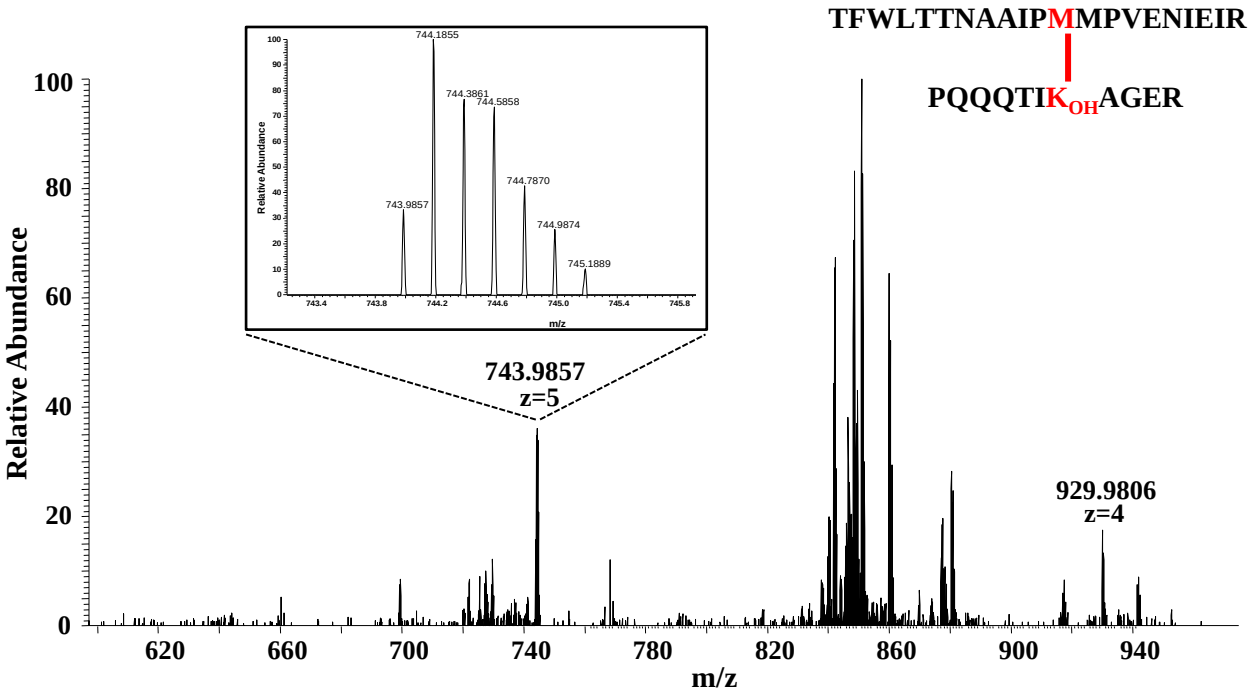


NDYSYWLSTPEPM$_{OX}$PM$_{OX}$S[M-48]APISGDNIR



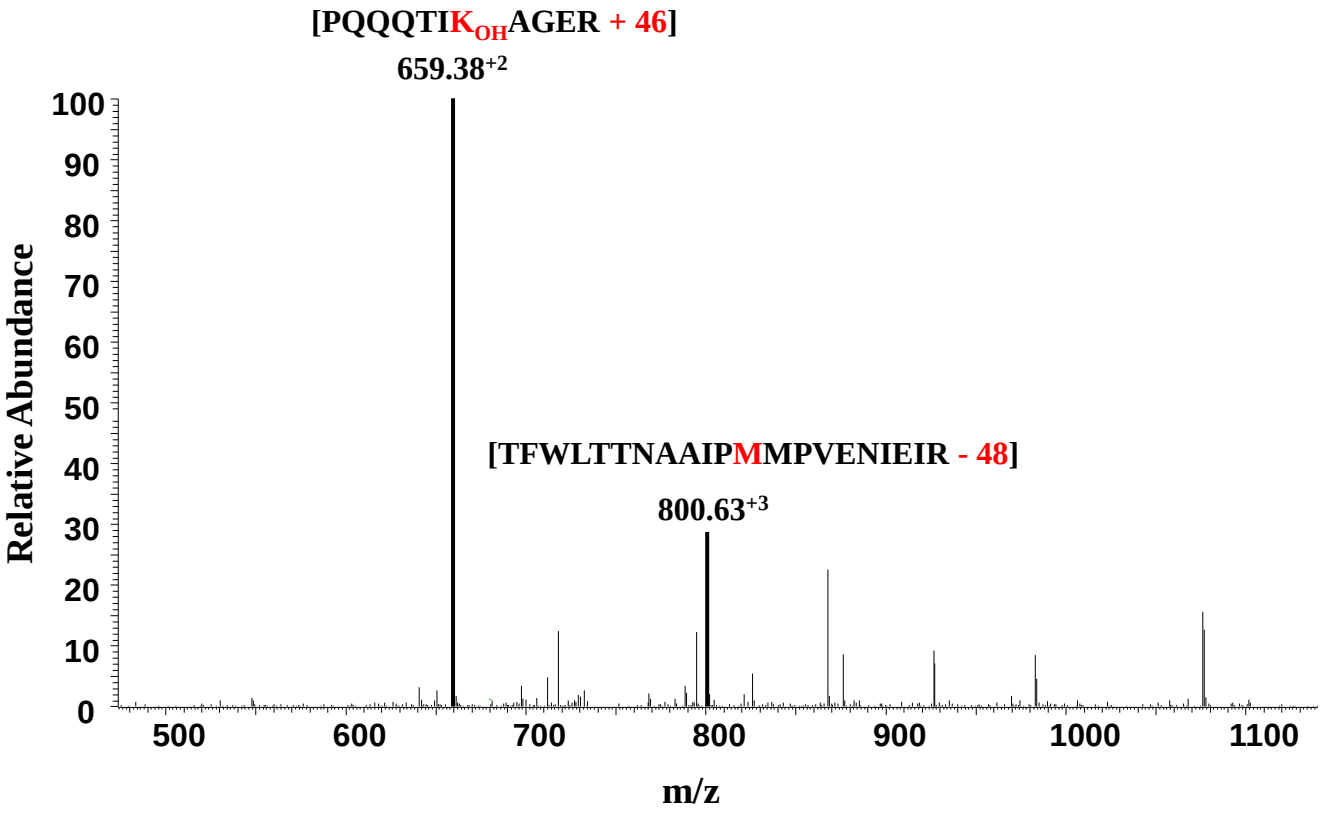
Supplementary Figure 9c. CID MS^3 fragmentation spectra of the MS^2 ions from HOBr reacted collagen IV NC1 hexamer. (*Upper panel*) The spectrum from the MS^2 730.71 ion shows the b- and y-series of ions which are consistent with the sequence shown on top where the hydroxylysine (red) carries an additional 46 amu (SCH_2). (*Lower panel*) The spectrum from the MS^2 1478.99 ion shows the b- (purple) and y- (blue) series of ions which are consistent with the peptide sequence containing methionine losing 48 amu from its side-chain as a result of gas-phase fragmentation.



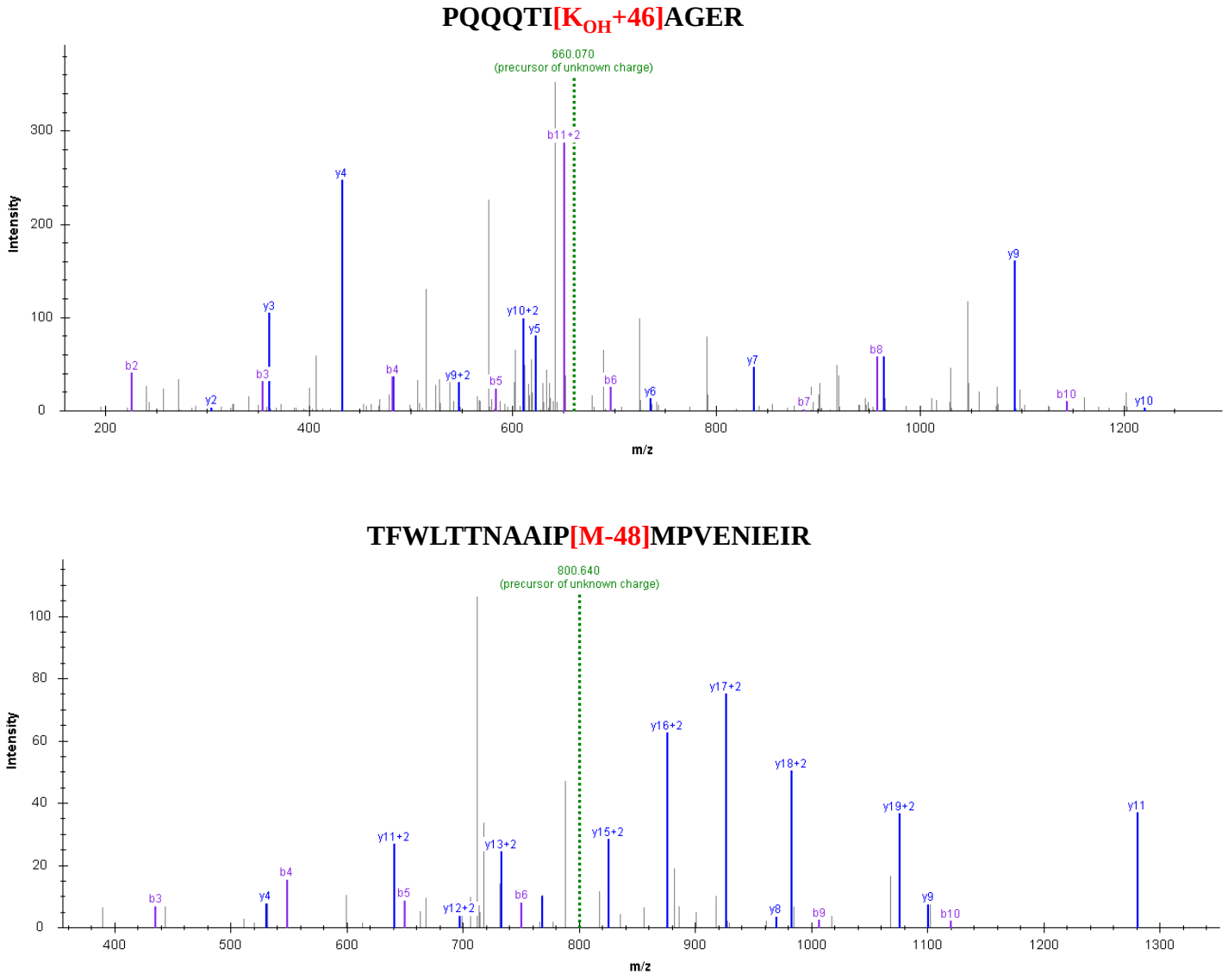


Z	Theoretical mass	Observed ion (m/ z)	Observed peptide mass	Difference (theo – obs)
+5		743.9857	3714.8895	
+4		929.7286	3714.8832	
	3716.9018		3714.8865	2.0153

Supplementary Figure 10a. *Drosophila* collagen IV NC1 hexamer is sulfilimine cross-linked. (*Upper panel*) SDS-PAGE and Coomassie blue staining of purified collagen IV NC1 hexamers from *Drosophila* larvae immunoblotted with mouse monoclonal anti-*Drosophila* collagen IV antibody (6G7; 1:500) to confirm protein identity. (*Lower panel*) High resolution spectrum of tryptic peptides from purified *Drosophila* collagen IV NC1 hexamer. The labeled peaks represent the quintuple and quadruple charged ions derived from the peptide complex shown. The inset shows the isotopic envelope and the monoisotopic peak at m/z 743.9857. The table shows that the difference between the observed and calculated mass for the peptide complex is 2 hydrogens as expected for a sulfilimine bond.



Supplementary Figure 10b. CID MS^2 fragmentation of m/z 743.9857^{+5} ion. This spectrum shows the CID fragmentation of the quintuple-charged ion m/z 743.9857 generates two fragments: m/z 659.38 which corresponds to the hydroxylysine-containing peptide plus 46 amu and m/z 800.63 which corresponds to the methionine containing peptide losing 48 amu upon fragmentation.



Supplementary Figure 10c. CID MS^3 fragmentation spectra of the MS^2 ions from *Drosophila* collagen IV NC1 hexamer. (*Upper panel*) The spectrum from the MS^2 659.58 ion shows the b- and y-series of ions which are consistent with the sequence shown on top where the hydroxylysine (red) carries an additional 46 amu (SCH_2). (*Lower panel*) The spectrum from the MS^2 1478.99 ion shows the b- (purple) and y- (blue) series of ions which are consistent with the peptide sequence containing methionine losing 48 amu from its side-chain as a result of gas-phase fragmentation.

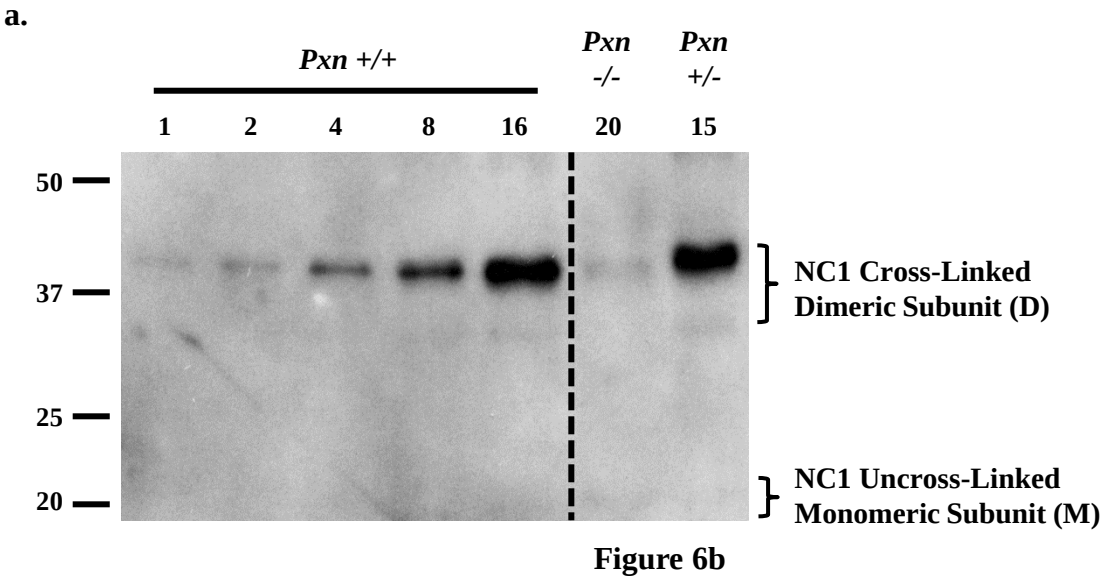
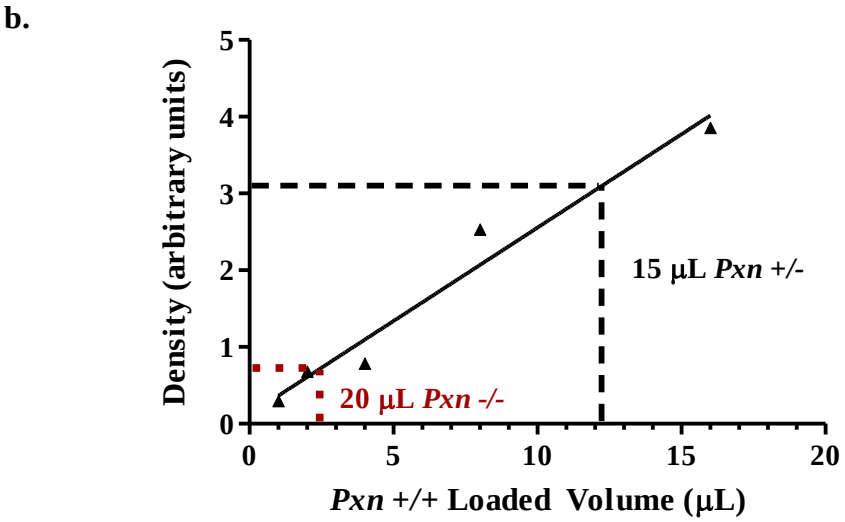
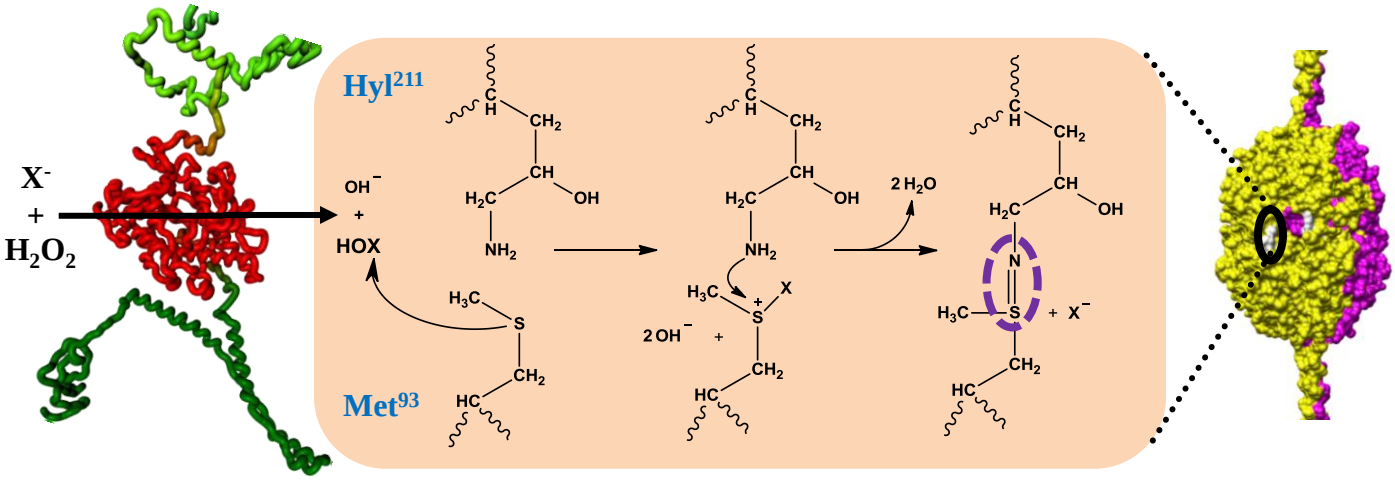


Figure 6b



Supplementary Figure 11. Collagen IV NC1 immunoreactivity is decreased in *Pxn* -/- larvae. **(a)** Immunoblot of collagenase solubilized basement membrane from *Pxn* +/+, *Pxn* +/-, and *Pxn* -/- third instar larvae using rabbit polyclonal anti-*Drosophila* collagen IV antibody at 1:2500. Numbers above lanes indicate loaded volume in µL after equalization of protein concentration using bicinchoninic assay to determine protein concentration of respective deoxycholate soluble fractions. The dotted line indicates the location of cropping for Figure 6b. This part of the image was also flipped for the figure. **(b)** Density of *Pxn* +/+ immunoreactive bands from (a) was determined as the area under the curve of the intensity versus migration distance profile of each lane and plotted (filled triangles) as a function of loaded volume to define a standard curve by linear regression ($R^2 = 0.96$; GraphPad Prism version 5.04, GraphPad Software, San Diego, CA). 1 µL band density was defined as the lower detection limit of the assay. The density of the *Pxn* -/- and *Pxn* +/- collagen IV NC1 immunoreactivity was determined in a similar fashion and then used to delineate an equivalent amount of *Pxn* +/+ loaded volume. The immunoreactive band density of 20 µL of *Pxn* -/- material was equal to 2.37 µL of *Pxn* +/+ which along with the 58:42 ratio of cross-linked to uncross-linked NC1 suggests that total collagen IV NC1 content in Pxn -/- flies is about 20.4% of *Pxn* +/+ flies. Similarly, 15 µL of Pxn +/- material equals 12.29 µL of Pxn +/+ loaded volume; thus *Pxn* +/- NC1 content is 82% of *Pxn* +/+.



Supplementary Figure 12. Proposed chemical reaction scheme for formation of the collagen IV sulfilimine bond. Halide transfer from peroxidasin generated hypohalous acid to methionine 93 (Met^{93}) of collagen IV forms a halosulfonium cation intermediate. This intermediate is attacked by the nearby hydroxylysine 211 (Hyl^{211}) amino group to yield a sulfilimine (S=N) bond (*encircled in purple*) across the NC1 trimer-trimer interface. Peroxidasin is artistically rendered on the *left* in truncated form with only its peroxidase (*red*), von Willebrand (*dark green*), and immunoglobulin (*light green*) domains, while the NC1 hexamer crystal structure (PDB ID 1T61)[51] is on the *right* with an encircled region to highlight the sulfilimine bond bridging Met^{93} and Hyl^{211} on apposed collagen IV protomers. X = Br^- or Cl^-.

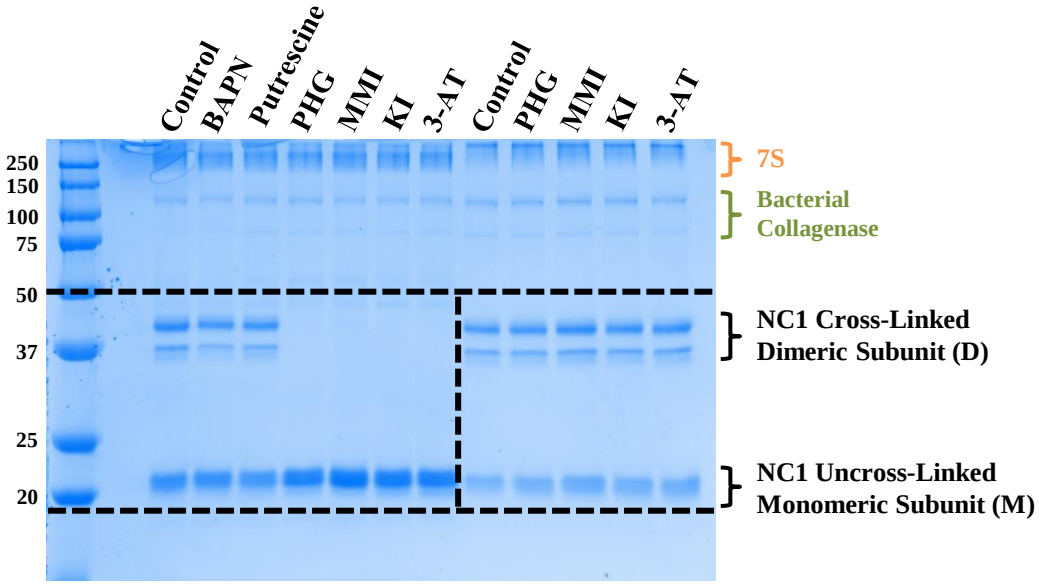
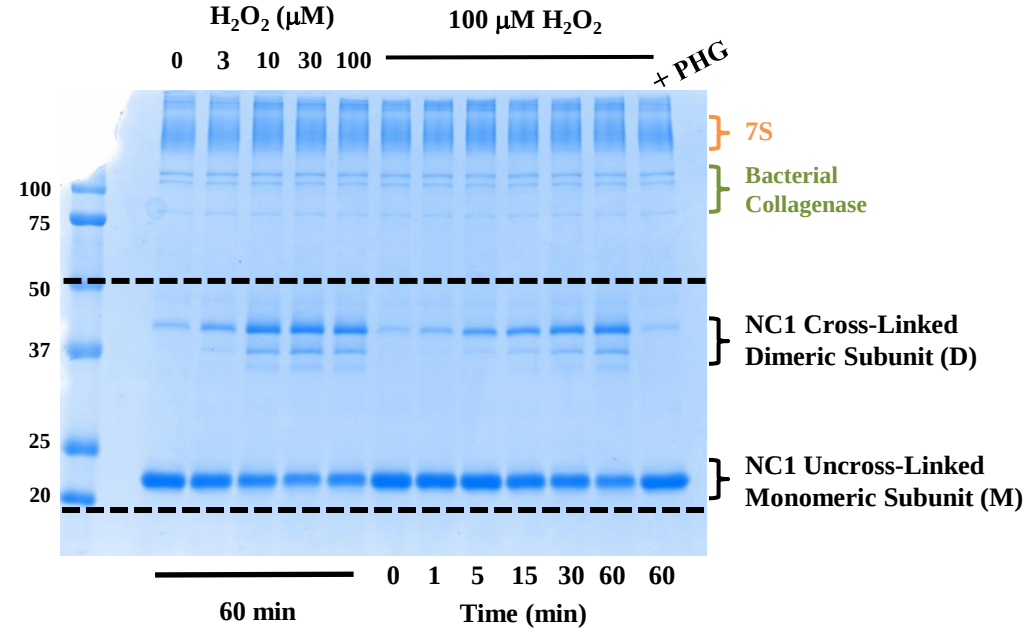
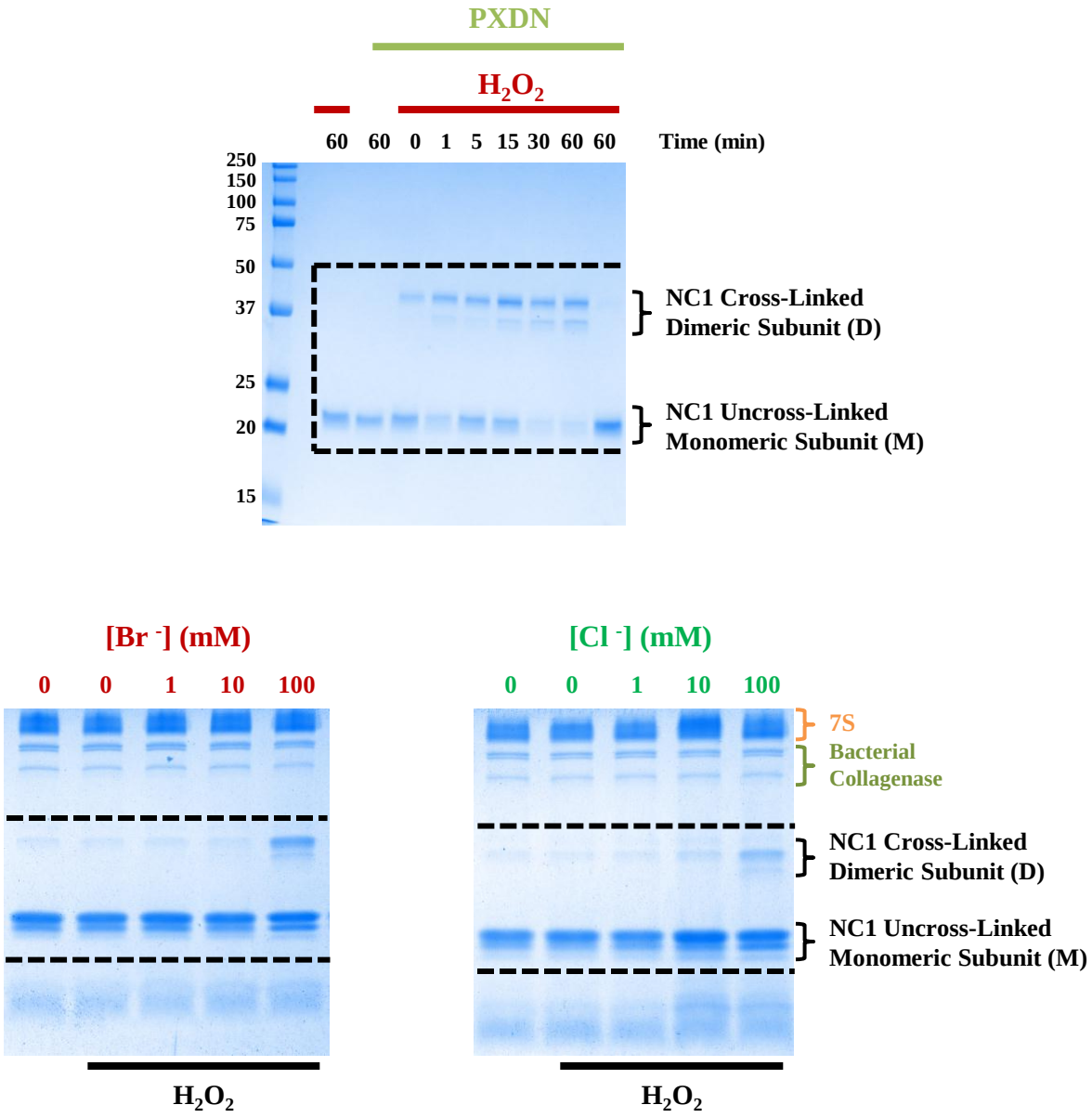


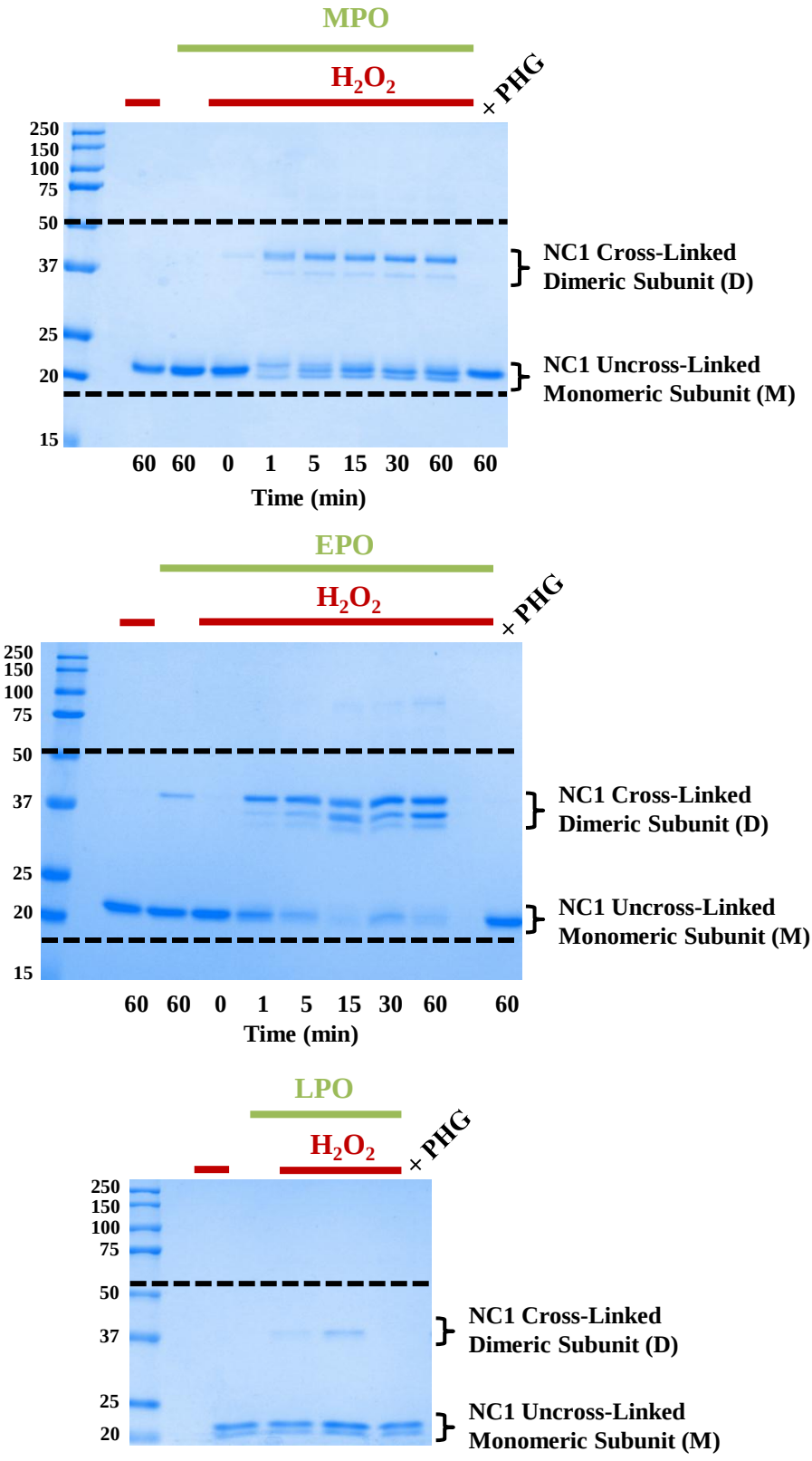
Figure 2a Figure 2b



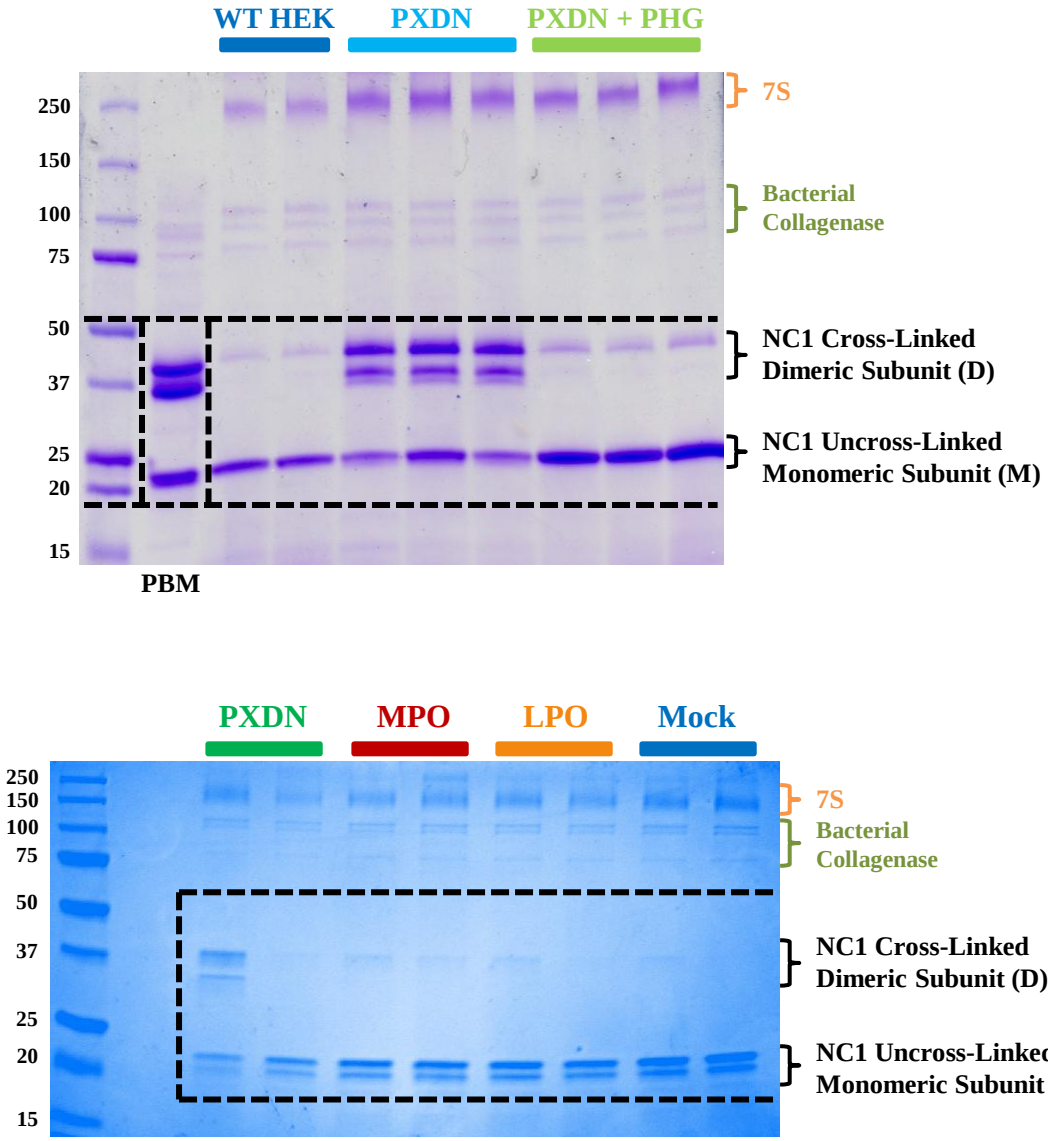
Supplementary Figure 13. Full Coomassie stained SDS-PAGE gels from Figure 2. Gels for data displayed in Figures 2a and 2b (*top*) and 2c (*bottom*). Dotted lines demarcate cropping of gel for presentation. The larger collagenase resistant N-terminal fragment known as 7S and contaminating bacterial collagenase represent the major stained bands in the cropped portion of the gel. Left most lane contains stained molecular weight markers with indicated estimated molecular weights.



Supplementary Figure 14. Full Coomassie stained SDS-PAGE gels for Figure 3. Gels for data displayed in Figures 3a (*top*) and 3b (*bottom*). Dotted lines demarcate cropping of gel for presentation. The larger collagenase resistant N-terminal fragment known as 7S and contaminating bacterial collagenase represent the major stained bands in the discarded upper portion of the gels for Figure 3b.



Supplementary Figure 15. Full Coomassie stained SDS-PAGE gels for Figure 4. Gels for data displayed in Figures 4a (*top*), 4b (*middle*), and 4c (*bottom*). Dotted lines demarcate cropping of gel for presentation. NC1 cross-linked dimeric (D) and uncross-linked monomeric (M) subunits are indicated. Left most lane contains stained molecular weight markers with indicated estimated molecular weights.



Supplementary Figure 16. Full Coomassie stained SDS-PAGE gels for Figure 5. Gels for data displayed in Figures 5b (*top*) and 5c (*bottom*). PBM represents collagen IV NC1 hexamer purified from bovine placental basement membrane. Dotted lines demarcate cropping of gel for presentation. The larger collagenase resistant N-terminal fragment known as 7S and contaminating bacterial collagenase represent the major stained bands in the discarded upper portion of the gels. NC1 cross-linked dimeric (D) and uncross-linked monomeric (M) subunits are indicated.