Electronic Supplementary Information:

Semi-synthesis of a protease-activatable collagen targeting probe

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S1. General

Unless stated otherwise, all chemicals and reagents were commercially obtained and used without further purification. (4-carboxylmethyl) thiophenol (MPAA), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), rat tail collagen type I (C7661) and human collagen type I from human placenta (C7774) were purchased from Sigma. Oregon Green 488 NHS ester (O-6147) and Alexa Fluor 568 NHS ester (A-20003) were obtained from Invitrogen. UV-Vis spectra were recorded on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) using ε_{280} = 31,400 $M^{-1}cm^{-1}$ for wt CNA35 and ε_{280} = 32,980 $M^{-1}cm^{-1}$ for CNA35-MMP-MESNA and CNA35-MMP-AB_3 respectively. Primers were purchased from MWG (Ebersberg, Germany). LC-MS spectra were recorded on an Applied Biosystems Single Quadrupole Electrospray Ionization Mass Spectrometer API-150EX in positive mode. Reversed phase HPLC was performed on a Shimadzu LC-8A HPLC system by using a Vydac C18 column. A gradient of acetonitrile in water, both containing 0.1% TFA was used to elute the compounds. Detection was performed by an UV-detector (λ = 214 nm). Peptide synthesis was performed on a Prelude automated peptide synthesizer (Protein Technologies) using standard Fmoc chemistry. Human recombinant MMP-1 (catalytic domain, SE-180) was purchased from Enzo Lifesciences. Wt CNA35 was expressed and purified as described before.[1]

S2. Expression and purification of CNA35-MMP-MESNA

Site-directed mutagenesis was performed on the previously constructed pTWIN-CNA35-MMP-His plasmid[2] to change the N-terminal cysteine of the CNA35-encoding region into a methionine using the QuickChange Site-directed mutagenesis kit (Stratagene) and the primers: 5`-GCGAATGACATCATTGTACACAACATGCGCGGATCCGCACGAG-3` and 5`-CTCGTGCGGATCC GCGCATGTTGTGTACAATGATGT CATTCGC-3` to yield pTWIN1-CNA35-MMP-His(C→M). The sequence of the plasmid was checked to verify the correct mutation. pTWIN1-CNA35-MMP-His(C→M) was transformed into chemically competent *E. coli* Bl21 (DE3) cells. Bacteria were grown in 0.5 L LB medium containing 100 µg/ml ampicillin at 37 °C and 250 rpm until an optical density (OD_{600nm}) of 0.6-0.8 was reached. Expression of the fusion protein was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by incubation of the culture for ~20 hours at 20 °C and 250 rpm. The bacterial cells were harvested by centrifugation for 20 minutes at 10,000 x g and 4 °C. After removal of the supernatant, the cell pellet was resuspended in Bugbuster and treated with Benzonase according to the manufacturer's instructions (Novagen). The suspension was

incubated at room temperature for 20 minutes on a rotating table and subsequently centrifuged at 40,000 x g for 20 minutes at 4 °C. The supernatant was applied on a column of 10 ml chitin resin that was equilibrated with 3 column volumes of 20 mM Na-phosphate, 500 mM NaCl, pH 8.5. After loading the supernatant, the column was washed with 10 column volumes of the same phosphate buffer, followed by 3 column volumes of 20 mM Na-phosphate, 500 mM NaCl, pH 7.0. The chitin column was then incubated overnight for pH induced splicing of the N-terminal intein of the fusion protein to yield an N-terminal methionine. A washing step was applied using 10 column volumes of phosphate buffer (pH 7.0) followed by 3 column volumes of 20 mM Na-phosphate, 500 mM NaCl, 50 mM sodium 2-mercaptoethanesulfonate (MESNA) (pH 6.0). Following overnight incubation to induce splicing of the C-terminal intein of the fusion protein, proteins were eluted in 3 column volumes of 20 mM Na-phosphate, 500 mM NaCl, pH 6.0. Amicon Ultra centrifuge tubes (MWCO: 10 kDa) were used to concentrate the elution fraction. 0.5 L *E. coli* typically yielded 40 mg protein after chitin affinity purification and splicing.

S3. Synthesis

FmocCys(tButhio)GlyGly-OH. The title compound was prepared on an automated peptide synthesizer following the Fmoc standard peptide synthesis protocol on 200 μmol scale. 2-Chlorotrityl chloride resin was used to obtain a C-terminal carboxylic acid after cleavage. For the first coupling, 2.0 equivalents of Fmoc-Gly-OH were dissolved in DCM and added to the resin followed by 2.0 equivalents of DIPEA. The mixture was agitated at room temperature for 5 minutes. 3.0 Equivalents of DIPEA were added and the mixture was agitated for an additional hour. MeOH (1 mL/g resin) was added and shaking was continued for 15 minutes. The resin was drained and washed with DCM (3x), NMP (3x) and DCM (3x). Subsequent amino acids were added using double couplings for 20 minutes each in NMP with 4.0 equivalents of activated ester. Cleavage from the resin was performed by treatment with TFA/H_2O/TIS (v/v/v 95:2.5:2.5). The mixture was concentrated, dissolved in acetonitrile and lyophilized. ESI-MS: Calc. $C_{26}H_{32}N_3O_6S_2H^+$: m/z 546.7; Found: m/z 546.9.

$(tBuO)_3$-FmocCys(tButhio)GlyGly (2). A solution of aminotriester **1** (96 mg, 0.23 mmol) in DMF (1 mL) was added to a solution of FmocCys(tButhio)GlyGly-OH (157 mg, 0.29 mmol) and DIPEA (67 mg, 0.52 mmol) in DMF (1 mL). This mixture was cooled in an ice bath before the addition of HATU (81 mg, 0.29 mmol) as a solid. The reaction mixture was allowed to gradually warm to RT. After 12 hours, the reaction mixture was diluted with DCM (20 mL) and the organic layer was washed with sat. $NaHCO_3$ (3 x 20 mL) and H_2O (20 mL). The reaction mixture was further purified by flash chromatography using a gradient elution of DCM to DCM containing 6% MeOH. The title compound was obtained as a white solid (174 mg, 80%). ^1H NMR ($CDCl_3$, 400 MHz): δ 7.76 (d, 2H, J = 7.3 Hz), 7.61 (d, 2H, J = 7.69 Hz), 7.40 (t, 2H, J = 7.3 Hz), 7.31 (t, 2H, J = 7.3 Hz), 7.08 (bs, 1H), 7.02 (bs, 1H), 6.43 (s, 1H), 5.95 (d, 1H), 4.49-4.42 (m, 2H), 4.23 (t, 1H, J = 6.96 Hz), 4.03-3.89 (m, 1H), 3.86 (d, 2H, J = 5.9 Hz), 3.82 (d, 2H, J = 5.5 Hz), 3.10-3.08 (m, 2H), 2.20 (t, 6H, J = 7.3 Hz), 1.97 (t, 6H, J = 7.7 Hz), 1.42 (s, 27H),

1.34 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.1, 171.1, 168.9, 168.0, 156.6, 143.8, 141.5, 127.9, 127.3, 125.3, 120.2, 80.9, 67.5, 57.9, 55.0, 48.7, 47.2, 43.7, 43.6, 41.6, 30.0, 29.9, 28.2. IR (cm^{-1}): ν 3284, 2975, 1727, 1679, 1635, 1520, 1451, 1392, 1366, 1316, 1248, 1147, 1102, 1034, 947, 846, 758, 740. MALDI-TOF-MS: Calc. $C_{48}H_{70}N_4O_{11}S_2Na^+$: m/z 965.5; Found: m/z 965.4.

$(OH)_3$-FmocCys(tButhio)GlyGly (3). The title compound was prepared from $(tBuO)_3$-FmocCys(tButhio)GlyGly (135 mg, 0.14 mmol) after 10 hours stirring at RT in formic acid. After removal of formic acid in vacuo, a colorless solid (100 mg, 90%) was obtained. The product was used without further purification. ^{1}H NMR (CD_3OD, 400 MHz): δ 8.11 (s, 1H), 7.79 (d, 2H, J = 7.3 Hz), 7.68 (d, 2H, J = 7.7 Hz), 7.38 (t, 2H, J = 7.3 Hz), 7.31 (t, 2H, J = 6.6 Hz), 7.20 (s, 1H), 4.49-4.34 (m, 2H), 4.24 (t, 1H, J = 6.9 Hz), 3.94-3.80 (m, 1H), 3.80 (s, 2H), 3.79 (s, 2H), 3.24-2.95 (m, 2H), 2.27 (t, 6H, J = 7.3 Hz), 2.03 (t, 6H, J = 7.0 Hz), 1.33 (s, 9H). ^{13}C NMR (CD_3OD, 100 MHz): δ 177.3, 174.0, 172.3, 171.0, 158.9, 145.4, 142.8, 129.0, 128.4, 126.5, 121.1, 68.5, 59.1, 56.4, 50.1, 49.5, 44.3, 44.0, 42.9, 30.9, 30.5, 29.4. IR (cm^{-1}): ν 2960, 2477, 1647, 1452, 1332, 1166, 1106, 1042, 978, 760, 741. MALDI-TOF-MS: Calc. $C_{36}H_{46}N_4O_{11}S_2Na^+$: m/z 797.3; Found: m/z 797.6.

tBoc-AO-PEG-N_3 (5). tBoc-aminooxyacetyl N-Hydroxysuccinimide ester (tBoc-AO-OSu (**4**)[3], 500 mg, 2.4 mmol) was dissolved in DCM (5 mL). To this solution dry DIPEA (1.2 ml, 6.8 mmol) and NH_2-PEG-N_3 (800 mg, 2.3 mmol) were added. After stirring for 22 hours at room temperature, the product was purified by a DOWEX MONOSPHERE 55Å anion exchange column (in MeOH). Evaporation of the solvent and drying in vacuo resulted in the title compound as a yellow oil (192 mg, 40%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.91 (bs, 1H), 7.75 (bs, 1H), 4.27 (s, 2H), 3.62-3.31 (m, 28H), 1.42 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 168.7, 156.9, 82.0, 75.4, 70.1, 50.2, 38.5, 27.7. IR (cm^{-1}): ν 2871, 2102, 1660, 1251, 1099, 946, 850, 773. MALDI-TOF-MS: Calc. $C_{21}H_{41}N_5O_{10}H^+$:
m/z 546.3; Found: m/z 546.3.

tBoc-AO-PEG-NH_2 (6). tBoc-AO-PEG-N_3 (303 mg, 0.58 mmol) was dissolved in MeOH (20 mL) under an inert atmosphere. Pd/C (10% Pd, 60 mg) in MeOH (10 mL) was added followed by H_2. After stirring for 3 hours at room temperature the reaction mixture was filtered through a short plug of Celite to remove the Pd/C. Evaporation under reduced pressure yielded the product as a yellow oil (272 mg, 95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.74 (bs, 1H), 4.27 (s, 2H), 3.57-3.41 (m, 28H), 1.41 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 169.3, 157.6, 82.3, 70.6, 41.8, 39.0, 28.3. IR (cm^{-1}): ν 3301, 2871, 1725, 1667, 1548, 1458, 1368, 1350, 1285, 1253, 1110, 951, 851. MALDITOF- MS: Calc. $C_{21}H_{43}N_3O_{10}H^+$: m/z 498.3; Found: m/z 498.4.

$(tBoc\text{-}AO)_3$-FmocCys(tButhio)GlyGly (7). A solution of $(OH)_3$-FmocCys(tButhio)GlyGly (**3**) (80 mg, 0.10 mmol), tBoc-AO-PEG-NH_2 (**6**) (169 mg, 0.34 mmol) and DIPEA (107 mg, 0.83 mmol) in DCM (3 mL) was cooled in an ice bath before the addition of PyBOP (177 mg, 0.34

mmol) as a solid. The reaction mixture was allowed to gradually warm to RT. After 12 hours, the reaction mixture was diluted with DCM (15 mL) and the organic layer was washed with sat. $NaHCO_3$ (3 x 15 mL) and brine (15 mL). After drying and concentration, the title compound was further purified by flash chromatography using a gradient elution of 5% to 10% MeOH in DCM. The title compound was obtained as a colorless solid (145 mg, 65%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.40 (s, 3H), 8.06 (bs, 6H), 7.70 (d, 2H, J = 7.3 Hz), 7.60 (d, 2H, J = 7.3 Hz), 7.39 (t, 2H, J = 7.3 Hz), 7.31 (t, 2H, J = 7.3 Hz), 7.23 (s, 2H), 7.14 (s, 1H), 6.51 (d, 1H), 4.53-4.41 (m, 2H), 4.36 (s, 6H), 4.21 (t, 1H, J = 5.9 Hz), 3.97-3.81 (m, 1H), 3.62-3.38 (m, 88H), 3.13-3.06 (m, 2H), 2.21 (m, 6H), 1.98 (m, 6H), 1.47 (s, 27H), 1.33 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 174.4, 171.5, 170.9, 169.6, 169.3, 157.5, 156.5, 143.7, 141.3, 127.8, 127.2, 125.0, 120.0, 77.2, 75.6, 70.4, 70.2, 70.1, 69.5, 69.4, 66.9, 58.5, 55.1, 48.4, 47.1, 44.0, 43.3, 41.9, 39.6, 39.0, 31.0, 30.4, 29.8, 28.1. MALDI-TOF-MS: Calc. $C_{99}H_{169}N_{13}O_{38}S_2Na^+$: m/z 2236.1; Found: m/z 2236.2.

(tBoc-AO)$_3$-Cys(tButhio)GlyGly (8). (tBoc-AO)$_3$-FmocCys(tButhio)GlyGly (**7**) (115 mg, 0.051 mmol) was dissolved in 20% v/v piperidine in DCM (4 mL) and stirred for 1.5 hours at room temperature. The reaction mixture was diluted with DCM (20 mL), washed with phosphate buffer containing 1M NaCl pH 5.5 (5 x 20 mL), dried on $MgSO_4$ and filtered. After evaporation of the solvent under reduced pressure, the product was obtained as a yellow oil (86 mg, 83%). ESI-MS: Calc. $C_{84}H_{159}N_{13}O_{36}S_2H^+$: m/z 1992.4; Found: m/z 1992.6.

(AO)$_3$-Cys(tButhio)GlyGly (9). To a mixture of cooled TFA containing 2.5% v/v H_2O and triisopropylsilane (5 mL) was added (tBoc-AO)$_3$-Cys(tButhio)GlyGly (**8**) (43 mg, 0.022 mmol). After stirring for 1 hour at 0 ºC the solvents were evaporated under reduced pressure. The resulting mixture was dissolved in H_2O (10 mL) and washed with Et_2O (4 x 10 mL). The solvent volume was reduced by freeze drying and the resulting solution was purified by preparative RP-HPLC using a gradient of 5-35% acetonitrile in H_2O (both containing 0.1% TFA) over 60 minutes. Freeze drying resulted in a viscous, colourless oil (10.4 mg, 30%). ESI-MS: Calc. $C_{69}H_{135}N_{13}O_30S_2H^+$: m/z 1692.0; Found: m/z 1691.4.

(AO)$_3$-CysGlyGly (10). (AO)$_3$-Cys(tButhio)GlyGly (**9**) (5.0 mg, 3 μmol) was dissolved in 1 mL 50 mM sodium phosphate buffer pH 6.5 containing 1% v/v 2-mercaptoethanol. After three hours at room temperature, the product was purified by RP-HPLC using a gradient of 5-25% acetonitrile in H_2O (both containing 0.1% TFA) over 10 minutes (3.5 mg, 70%). ESI-MS: Calc. $C_{65}H_{127}N_{13}O_{30}SH^+$: m/z 1603.9; Found: m/z 1603.5.

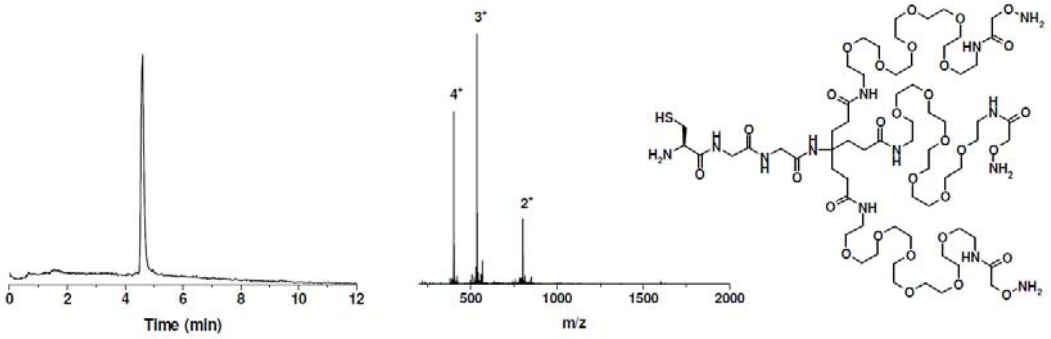

Figure S1 *LC-MS characterization of the orthogonally modified dendron. The total ion current and the m/z spectrum are shown (calculated mass: 1602.9 Da; found mass: 1602.6 Da).*

S-(GPO)$_5$. Peptide synthesis was performed on an automated peptide synthesizer (Prelude, Protein technologies) following the standard Fmoc peptide synthesis protocol on a 50 µmol scale of Rink amide resin. Each amino acid was allowed to couple two times 20 minutes. The peptide was cleaved from the resin using a mixture of TFA/H_2O/TIS (v/v/v 96:2:2). After stirring for 2 hours at room temperature the mixture was filtered and cold Et_2O (40 mL) was added. The mixture was kept for 2 hours at -30 °C. The precipitate formed after centrifugation was dissolved in H_2O and lyophilized. Purification was performed using preparative RP-HPLC using a gradient of 5-22% acetonitrile in H_2O (both containing 0.1% TFA) over 36 minutes. Freeze drying resulted in the pure peptide (56%). ESI-MS: Calc. $C_{63}H_{94}N_{17}O_{22}H^+$: m/z 1440.7; Found: m/z 1440.5.

Glyoxyl-(GPO)$_5$. S-(GPO)$_5$ (13 mg, 0.009 mmol) was dissolved in 0.01 M sodium phosphate buffer pH 7.0 (2 mL) to which $NaIO_4$ (2.9 mg, 0014 mmol) was added. The mixture was allowed to react at room temperature for 15 minutes and was subsequently purified by preparative RP-HPLC using a gradient of 2-18% acetonitrile in H_2O (both containing 0.1% TFA) over 41 minutes. This resulted in the title compound with a yield of 78%. ESI-MS: Calc. $C_{62}H_{88}N_{16}O_{22}H^+$: m/z 1409.6; Found: m/z 1409.2.

S4. Native chemical ligation and oxime ligation to obtain pro-CNA35

10 was dissolved in dH_2O to obtain a stock solution of 10 mM. To perform native chemical ligation, a 3-fold molar excess of cys-AB_3 was added to CNA35-MMP-MESNA in 20 mM sodium phosphate, 500 mM NaCl, pH 6.0 at a protein concentration of ~350 µM. MPAA and TCEP were added to final concentrations of 50 mM and 2 mM, respectively. The pH was adjusted to ~6.5 with 1 M sodium phosphate, pH 9.5. After overnight incubation at room temperature, the ligation mixture was buffer exchanged to 10 mM HBS, pH 7.4 to remove MPAA and TCEP by repeated concentration and dilution using an Amicon Ultra-4 centrifugal concentrator (MWCO: 10 kDa). The reaction mixture was purified to remove minor degradation products and the excess of **10** using nickel affinity chromatography by applying it to a column of 3 ml HisBind resin charged with $NiSO_4$. The column was washed with 10 column volumes of

bind buffer (20 mM Tris, 500 mM NaCl, 5 mM imidazole, pH 7.9), followed by 6 column volumes of wash buffer (20 mM Tris, 500 mM NaCl, 15 mM imidazole, pH 7.9). The column was then flushed with 6 column volumes of elution buffer (20 mM Tris, 500 mM NaCl, 1M imidazole, pH 7.9). Finally, the elution fraction was concentrated and buffer exchanged to HBS to remove the imidazole by repeated concentration and dilution using Amicon Ultra centrifuge tubes (MWCO: 10 kDa). Approximately 55% of the amount of protein applied to the column was collected in the elution fraction.

The eluted proteins were further buffer exchanged to 100 mM anilinium acetate, pH 4.5. Since the protein concentration in anilinium acetate could not be determined, an estimated 20-fold molar excess of glyoxyl-$(GPO)_5$-NH_2 was added and the reaction mixture was incubated overnight at 37 °C. After incubation, the ligation mixture was buffer exchanged to HBS and the excess of glyoxyl-$(GPO)_5$-NH_2 was removed by repeated concentration and dilution using an Amicon centrifugal tube (MWCO: 10 kDa).

S5. Purification of proCNA35 using collagen affinity chromatography

Rat tail collagen type I (20 mg) was dissolved in 20 mM acetic acid to a final concentration of 2.7 mg/ml. NHS-activated Sepharose Fast Flow resin (50 ml suspension) was centrifuged for 5 minutes at 3700 rpm and 4 °C, after which the supernatant was removed. Subsequently, the resin was washed 5 times with 1 mM HCl (cold) by mixing the resin with the HCl solution, centrifuging it for 5 minutes at 3700 rpm and 4 °C and removing the supernatant again. The rat tail collagen type I solution was diluted in 0.2 M $NaHCO_3$, 500 mM NaCl, pH 8.3 to 25 ml and added to the resin. After mixing thoroughly, pH was set to 7.5 and the resin was incubated for 2.5 hours on a rotating table at RT. The mixture was centrifuged again for 5 minutes at 3700 rpm and 4 °C and the supernatant was removed. Next, the resin was washed with 30 ml 0.1 M Tris-HCl, pH 8.5 and 30 ml 0.1 M acetate pH 4.5, respectively. This two step wash procedure was repeated 3 times. Finally, the resin was resuspended in HBS (10 mM Hepes, 135 mM NaCl, pH 7.4), poured into a column and connected to a BioRad FPLC system.

The column was first equilibrated with 3 column volumes of HBS. The mixture of pro-CNA35, partially functionalized and unfunctionalized CNA35 was loaded on the column and afterwards the column was washed with 7-8 column volumes of HBS to remove unbound protein. Collagen binding proteins were eluted using 5 column volumes of 50 mM NaOH, 500 mM NaCl. The flow through during the washing and elution steps was collected in fractions of 5 mL. Fractions showing a UV signal at 280 nm were analyzed using SDS-PAGE. The fractions containing unbound protein were pooled and concentrated using Amicon Ultra centrifuge tubes (MWCO: 10 kDa). The concentration of proCNA35 was determined using the BCA protein assay kit (Thermo Scientific) according to the manufacturer's instructions. Reversed phase HPLC was performed on a Vydac C4 protein column with a mobile phase of acetonitrile/H_2O with 0.1% formic acid. LC-MS spectra were measured on a Thermo Finnigan LCQ Deca XP Max in positive mode. The spectra were deconvoluted using MassLynx software.

S6. Digestion of pro-CNA35 with MMP-1

Pro-CNA35 was buffer exchanged to 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5 using Amicon Ultra centrifuge tubes (MWCO: 10 kDa) and diluted to a final concentration of ~23 µM. 127 units of recombinant MMP-1 were added per 1 nmol of pro-CNA35 and the reaction was incubated at 37 °C. Samples were taken at time points t = 0, t = 15 minutes, t = 30 minutes, t = 45 minutes, t = 1 hour, t = 2 hours and t = 3 hours and the reaction in the samples was stopped by the addition of 20 mM ethylenediaminetetraacetic acid (EDTA) and subsequently analyzed using SDS-PAGE and SPR collagen binding assays.

S7. SPR measurements

The SPR measurements were performed on a BIAcore T100 (GE Healthcare). Human collagen type I and the collagen mimic were immobilized on a CM5 chip using standard EDC/NHS protocols. Human collagen type I was immobilized to a final immobilization level of ~1500 RU. The collagen mimic with a lysine at the focal point of the scaffold was prepared as previously described and immobilized to a final level of ~100 RU.[2, 4] All binding experiments were performed at 25 °C using HBS-EP (HBS containing 3 mM EDTA and 0.05% v/v Surfactant P20) containing 1 mM DTT as a running buffer. The SPR measurements were performed at a flow rate of 5 µl/min. The protein was injected at a concentration of 1 µM for 200 s followed by a dissociation phase of 200 s. Regeneration of the chips was performed by two injections of 10 mM glycine-HCl, pH 1.5 for 30 seconds. Aspecific binding and buffer effects were taken into account by subtracting the simultaneous response from a reference surface only blocked with ethanolamine.

S8. Fluorescent labeling of wt CNA35 and proCNA35

WtCNA35 and proCNA35 were fluorescently labeled with Alexa Fluor 568 and Oregon Green 488, respectively, via amine coupling by buffer exchange to 0.1 M $NaHCO_3$, pH 8.3 and subsequent addition of a 10-fold molar excess of Alexa Fluor 568 NHS ester or Oregon Green®488 NHS ester from a stock solution in DMF. The labeling reaction was performed at a protein concentration of ~150 µM for wtCNA35 and ~60 µM for proCNA35. The concentration and labeling efficiency for wtCNA35 was determined by measuring the absorbance at 280 nm and 578 nm. This resulted in a labeling ratio of ~1.6 dye molecules per protein for wtCNA35. For proCNA35, the concentration and labeling efficiency was determined by using the BCA protein assay kit (Thermo Scientific) for protein concentration using wt-CNA35 to create a standard curve and measuring the absorbance at 496 nm. This resulted in a labeling ratio of ~3.9 dye molecules per proCNA35 protein.

S9. Collagen imaging in pig pericardial tissue

Collagen imaging experiments were performed by incubation of pig pericardium tisue in 1 ml 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5 containing 0.5 µM of both wtCNA35-AF568 and proCNA35-OG488 for 3 hours at room temperature, in either absence or presence of 1.75 µg/ml (1840 units/ml) recombinant MMP-1. Pig pericardium was incubated in a 24-wells plate on a rotating table, protected from light. After incubation, tissues were washed for 15 minutes in 1

ml HBS. Imaging was performed on an inverted Zeiss Axiovert 200 microscope coupled to an LSM 510 Meta (Carl Zeiss, Germany) confocal laser scanning microscope using a 20x objective. Imaging of the tissues stained with both labeled proteins was performed by excitation of wtCNA35-AF568 using a helium-neon laser (543 nm) with the corresponding PMT defined as 565-615 nm. Pro-CNA35-OG488 was again excited using an argon laser (488 nm) and the corresponding PMT was defined as 500-530 nm.

S10. References

1. K. N. Krahn, C. V. C. Bouten, S. van Tuijl, M. A. M. J. van Zandvoort and M. Merkx, *Anal. Biochem.*, 2006, **350**, 177-185.
2. M. Breurken, E. H. M. Lempens and M. Merkx, *ChemBioChem*, 2010, **11**, 1665-1668.
3. S. Foillard, M. O. Rasmussen, J. Razkin, D. Boturyn and P. Dumy, *J. Org. Chem.*, 2008, **73**, 983-991.
4. M. Breurken, E. H. M. Lempens, R. P. Temming, B. A. Helms, E. W. Meijer and M. Merkx, *Bioorg. Med. Chem.*, 2011, **19**, 1062-1071.