The FEBS Journal

FEBS PRESS science publishing by scientists

Propeptide glycosylation and galectin-3 binding decrease proteolytic activation of human proMMP-9/progelatinase B

Lise Boon[1], Estefania Ugarte-Berzal[1], Erik Martens[1], Jennifer Vandooren[1], Vasily Rybakin[1], Didier Colau[2], Monica Gordon-Alonso[3], Pierre van der Bruggen[3], Walter Stöcker[4], Christoph Becker-Pauly[5], Peter Witters[6,7], Eva Morava[8], Jaak Jaeken[6,7], Paul Proost[9] and Ghislain Opdenakker[1]

1 Laboratory of Immunobiology, Rega Institute for Medical Research, KU Leuven, Belgium
2 Ludwig Institute for Cancer Research, Brussels, Belgium
3 Institut de Duve, UCLouvain, Brussels, Belgium
4 Institute of Molecular Physiology, Johannes Gutenberg University, Mainz, Germany
5 Biochemical Institute, University of Kiel, Germany
6 Department of Pediatrics, University Hospitals Leuven, KU Leuven, Belgium
7 Department of Development and Regeneration, KU Leuven, Belgium
8 Department of Clinical Genomics, Mayo Clinic, Rochester, ON, USA
9 Laboratory of Molecular Immunology, Rega Institute for Medical Research, KU Leuven, Belgium

Keywords
galectin-3; matrix metalloproteinase-9; N-linked glycosylation; propeptide; proteolytic activation

Correspondence
G. Opdenakker, Laboratory of Immunobiology, Department of Microbiology and Immunology, Rega Institute for Medical Research, KU Leuven, Herestraat 49 1044, 3000 Leuven, Belgium
Fax: +32 16 33 30 26
Tel: +32 16 37 90 20
E-mail: ghislain.opdenakker@kuleuven.be

(Received 5 July 2018, revised 21 September 2018, accepted 9 November 2018)

doi:10.1111/febs.14698

Matrix metalloproteinases (MMPs) are secreted as proenzymes, containing propeptides that interact with the catalytic zinc, thereby controlling MMP activation. The MMP-9 propeptide is unique in the MMP family because of its post-translational modification with an N-linked oligosaccharide. ProMMP-9 activation by MMP-3 occurs stepwise by cleavage of the propeptide in an aminoterminal (pro-AT) and carboxyterminal (pro-CT) peptide. We chemically synthesized aglycosyl pro-AT and pro-CT and purified recombinant glycosylated pro-AT^{Sf-9}. First, we report new cleavage sites in the MMP-9 propeptide by MMP-3 and neutrophil elastase. Additionally, we demonstrated with the use of western blot analysis a higher resistance of glycosylated versus aglycosyl pro-AT against proteolysis by MMP-3, MMP-9, meprin α, neutrophil elastase and by protease-rich synovial fluids from rheumatoid arthritis patients. Moreover, we investigated the effect of glycosylation on proteolytic activation of human proMMP-9 with the use of zymography and dye-quenched gelatin cleavage analysis. Compared to recombinant Sf-9 proMMP-9 glycoforms, larger oligosaccharides of human neutrophil proMMP-9 increased resistance against proteolytic activation. Additionally, proMMP-9 from Congenital Disorder of Glycosylation patients, compared to healthy controls, showed a higher activation rate by MMP-3. Finally, we demonstrated that glycan-galectin-3 interactions reduced proMMP-9 activation. In conclusion, modification of MMP-9 propeptide glycosylation is a fine-tuning mechanism and co-determines the specific activity of MMP-9 in physiology and pathology.

Enzymes
MMP-9 EC 3.4.24.35, MMP-3 EC 3.4.24.17, meprin α EC 3.4.24.18, neutrophil elastase EC 3.4.21.37, trypsin EC 3.4.21.4 and PNGase F EC 3.5.1.52.

Abbreviations
actMMP-9, activated form of MMP-9; CDG, congenital disorder of glycosylation; cdMMP-3, catalytic domain of MMP-3; DQ-gelatin, Dye-Quenched gelatin; MMP, matrix metalloproteinase; NE, neutrophil elastase; PMM2, phosphomannomutase 2; PNGase F, protein N-glycosidase F; pro-AT^{Sf-9}, recombinant Sf-9-derived aminoterminal peptide of MMP-9 propeptide; pro-AT^{synth}, synthetic aminoterminal peptide of MMP-9 propeptide; pro-CT, synthetic carboxyterminal peptide of MMP-9 propeptide; TIMP-1, tissue inhibitor of metalloproteinases-1; tKK, tissue kallikrein; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

Introduction

ProMMP-9 is presently one of the most studied enzymes in biology and medicine [1]. It plays a fundamental role in physiological processes, such as reproduction [2,3], tissue reorganization [4] and angiogenesis [5,6]. In addition, it is viewed as a marker and possible target in pathological conditions, including cancer [7,8], acute and chronic inflammation and vascular diseases [9,10]. Detection methods with sensitivities in the picogram range [11] and probes for *in situ* demonstration of gelatinase activity have been developed for preclinical mouse models and clinical applications in humans [12,13].

Molecular modelling studies based on sedimentation [14] and small-angle X-ray scattering data [15] have led to a reasonable understanding of the structure of proMMP-9 and insights about the functions of its structural domains. For instance, the hemopexin domain provides the molecule with various non-catalytic activities [16,17], whereas the fibronectin repeats enhance gelatin affinity [18]. The propeptide is the least studied domain of proMMP-9 in terms of its modifications and functions. The human propeptide consists of 87 amino acids and is glycosylated at Asn_{38} (all amino acid numberings in this report include the signal peptide) [1,14]. The propeptide keeps proMMP-9 in a latent state by coordinating the catalytic zinc ion with a sulfhydryl group, the so-called cysteine switch mechanism [19]. Several proteases have been reported to activate proMMP-9 *in vitro*, including MMPs (MMP-1, -2, -3, -7, -10, -13 and -26) [20–25], meprin α and meprin β [26], neutrophil elastase (NE) [27], trypsin [28,29], tissue kallikrein (tKK) [30] and α-chymase [31]. For additional information and graphics we also refer to Fig. 10. Additionally, proMMP-9 itself shows autocatalytic activation particularly in the presence of ligands of the hemopexin domain, such as hemin [32]. Furthermore, plasmin, urokinase type plasminogen activator (uPA) [33] and tissue type plasminogen activator (tPA) are implicated as important physiological activators of proMMP-9 and this protease cascade has been discussed in several diseases [34,35]. Early studies on the enzymatic activation of proMMP-9 by MMP-3 indicated a two-step proteolytic processing [21,25], by which first the aminoterminal part of the propeptide (pro-AT) is cleaved off, followed by the fragment containing the switching cysteine (pro-CT). As a new approach of MMP-9 inhibition with possible therapeutic applications, zymogen activation can be blocked with a compound binding in proximity to this secondary cleavage site [36].

A recent molecular dynamics simulation study based on the crystal structure of an N-terminal proMMP-9 segment by Elkins and colleagues [37] suggests that the glycosylation of the propeptide may regulate this order of stepwise cleavage [38]. However, molecular biology data do not yet exist.

Glycans attached to proMMP-9 generate several glycoforms, increasing structural complexity and generating cell- and tissue-specificities. Recombinant expression in Sf-9 insect cells yields simple oligomannose structures on proMMP-9 [14], while glycans on human neutrophil proMMP-9 are complex [39]. Cancer cells produce proMMP-9 glycoforms that differ from those of normal cells, suggesting a potential role for specific glycosylation of proMMP-9 in cancer. For example, proMMP-9 from MCF-7 breast cancer cells or THP-1 myeloid leukaemia cells contains glycans that show significantly reduced binding to galectin-3, compared to those from neutrophil proMMP-9 [40]. Glycoforms of human neutrophil, MCF-7, THP-1 and recombinant (Sf-9) proMMP-9 are compared in Fig. 1. In general, insights into the biological roles of sugars attached to MMPs are limited [41]. One study refers to the role of the propeptide glycan in secretion of proMMP-9 [42]. However, oligosaccharides attached to glycoproteins also protect against proteolysis, as has been demonstrated for ribonuclease B [43], CD59 [44] and suggested for β-interferon [45].

The role of glycosylation stretches further than the post-translational modification itself. Glycans also create an interface for molecular interactions with lectins, including galectins. Galectins are a protein family with binding specificity for β-galactoside sugars (such as GalβGlcNAc), which can be found on both N-linked and O-linked glycans [46]. Unlike the majority of lectins, galectins are not membrane-bound, but soluble proteins with both intra- and extracellular functions. Galectin-3 is unique among other galectins because of its chimeric structure, having a carbohydrate recognition domain and a long non-lectin domain, through which it can associate into a pentameric form [47]. We discovered that both the glycosylation of the propeptide and the galectin-3 binding to proMMP-9 inhibit the processing of the propeptide, thereby fine-tuning proMMP-9 activation.

Results

Production and detection of the MMP-9 propeptide

Pro-AT and pro-CT, as cleaved from proMMP-9 by MMP-3 during the activation process [25], were

	N-linked glycosylation of proMMP-9							
	Human neutrophil glycans		Human MCF-7 glycans		Human THP-1 glycans		Insect Sf-9 glycans	
FORMULA	Structure	% Glycan pool	Structure	% Glycan pool	Structure	% Glycan pool	Structure	% Glycan pool
	A2G2	-	FcA2G2	19.3	FcA2G2	2.7	Fc	100
	FcA2G1		FcA2G2F(2)	23.6	FcA2G2S(3)	6.3		
	FcA2G2		FcA2G2S(3)		FcA2G2F(3)	16.9		
	FcA2G2F(3)		FcA2G2F(3)	23.6	FcA2G2S(6)	15.1		
	FcA2G2F2(3,3)		FcA2G2F2(2,3)	13.8	FcA2G2F(3)S(3)	11.0		
	FcA3G3		FcA2G2S2(3,3)		FcA2G2S2(3,6)	8.1		
	FcA3G3F(3)		FcA2G2F2(3,3)	15.2	FcA2G2F(3)S(6)	39.9		
	FcA3G3F2(3,3)		FcA2G2F3(2,3,3)	4.5	FcA2G2F2(3,3)			
Reference	Rudd *et al.* 1999 Biochemistry Vol. 38 No. 42		Fry *et al.* 2006 Biochemistry Vol. 45 No. 51				Van den Steen *et al.* 2006 The Journal of Biological Chemistry Vol. 281 No. 27	

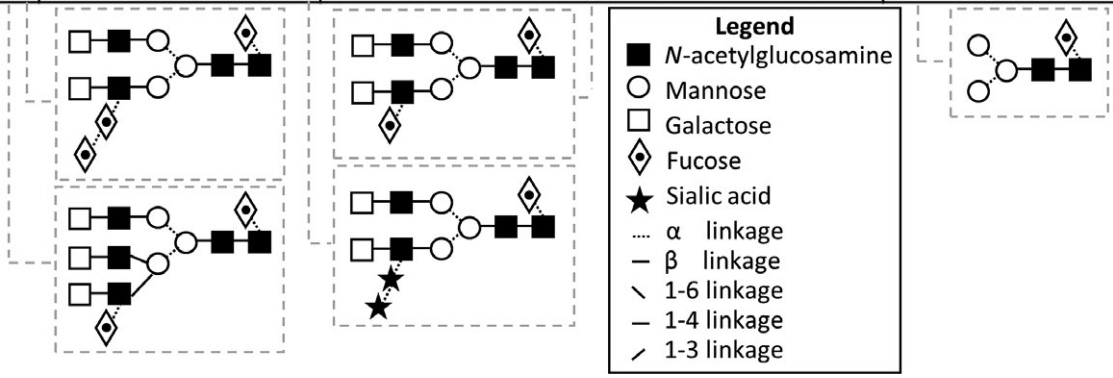

Fig. 1. Different glycoforms of proMMP-9. Sugars are abbreviated in the table as follows. All N-linked glycans have two core N-acetylglucosamines: Fc, fucose linked to inner core; Ax, number x of antennae on trimannosyl core; Gx, number x of galactoses on antennae; Fx, number x of fucoses and Sx, number x of sialic acids on antennae; linkages of outer arm fucose (α1-2 or α1-3) and sialic acid (α2-3 or α2-6) are shown in parentheses. Structures are illustrated as indicated in the legend and are derived from references 14, 39 and 40.

chemically synthesized or produced from natural sources. The synthetic peptides were used to immunize rabbits for the production of polyclonal antibodies for detection by western blot analysis (Fig. 2A,B).

To investigate the function of the oligosaccharide attached to Asn_{38}, glycosylated pro-AT was purified from recombinant proMMP-9, produced in Sf-9 insect cells, after incubation with the catalytic domain of MMP-3 (cdMMP-3) and further referred to as pro-AT^{Sf-9} (Fig. 2C,D). As demonstrated with the use of protein staining, the glycosylated pro-AT^{Sf-9} displays slower electrophoretic mobility, compared to the synthetic aglycosyl pro-AT (Fig. 2D), further referred to as pro-AT^{synth}. Treatment of pro-AT^{Sf-9} with protein N-glycosidase F (PNGase F) to remove N-linked sugars from proteins, resulted in a similar electrophoretic mobility as pro-AT^{synth}, confirming N-linked glycosylation as a post-translational modification (Fig. 2E). Several types of validation experiments were performed to exclude cross-reactivity of the generated antibodies. First, the reactivities of pro-AT and pro-CT were assessed by ELISAs (Fig. 3A) and no cross-reactivity was observed. Secondly, we used western blot analysis to exclude cross-reactivity between pro-AT and pro-CT (Fig. 3B) and to prove that the polyclonal antiserum against pro-AT recognized both the synthetic pro-AT^{synth} and the recombinant human propeptide (pro-AT^{Sf-9}) with similar sensitivities (Fig. 3C,D).

Cleavage of the MMP-9 propeptide by MMP-3 and NE

Stepwise cleavage of proMMP-9 behind Glu_{59} and Arg_{106} in the propeptide by MMP-3 is a well-described

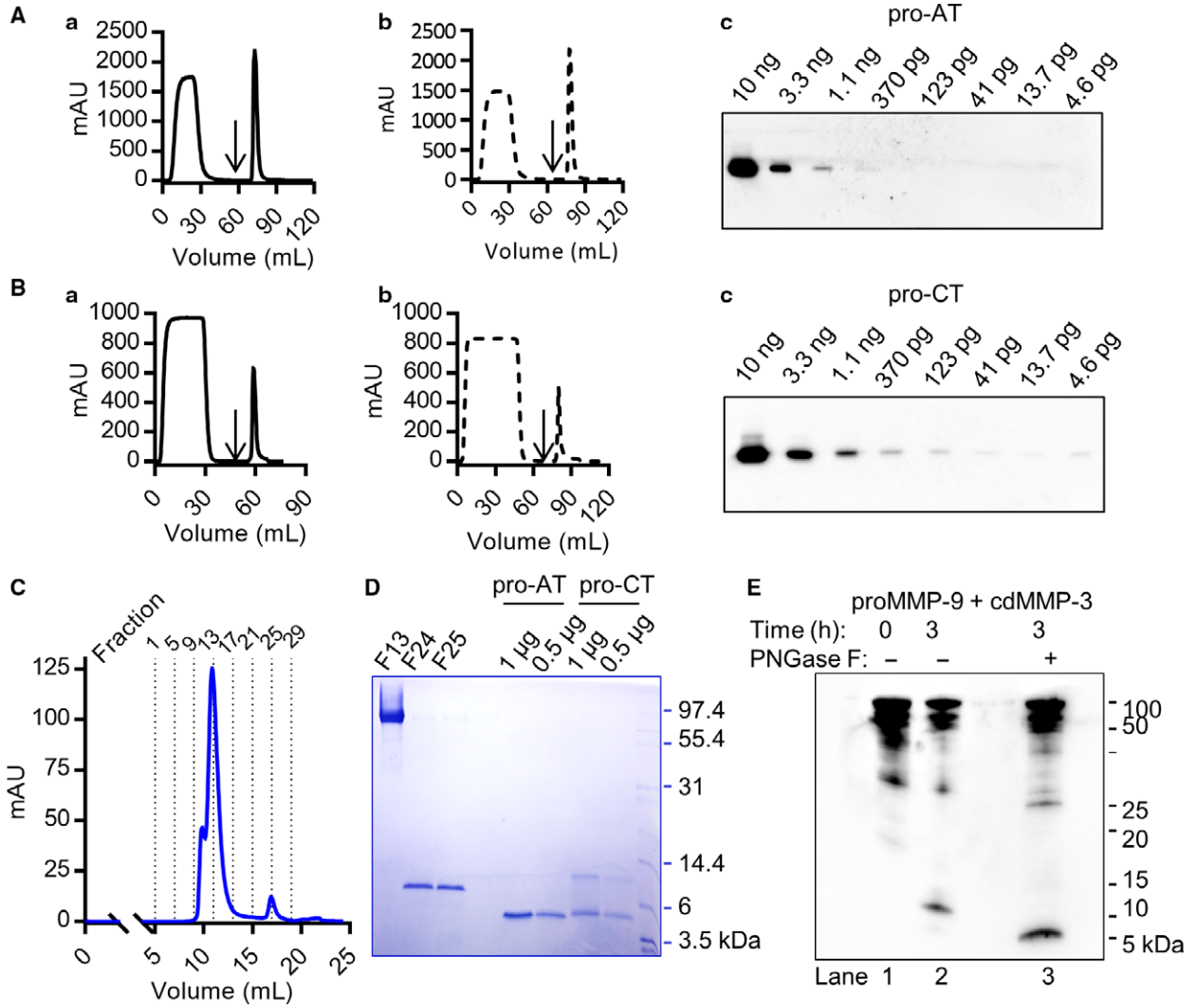

Fig. 2. Detection of pro-AT and pro-CT and purification of pro-AT^{Sf-9} from human MMP-9. (A) Synthetic MMP-9 pro-AT was used as antigen to produce rabbit polyclonal antibodies. UV Absorption (in mAU) for rabbit serum. (a) After immunization with pro-AT, the serum was diluted, filtered and loaded on a recombinant protein A-Sepharose fast flow column (GE Healthcare). Rabbit IgGs were eluted in 0.1 M Glycine pH 2.8 as indicated (vertical arrow). (b) Affinity purification of antibodies on pro-AT-coupled NHS Sepharose (2 mg peptide/mL Sepharose, GE Healthcare) of pooled rabbit IgG fractions (from graph a). (c) Immunoblot detection of pro-AT with the affinity-purified polyclonal antisera. (B) Similar experimental set-up for detection with affinity-purified polyclonal antisera of pro-CT. (C) Purification of pro-AT from recombinant human Sf-9 proMMP-9, further referred to as pro-AT^{Sf-9}, after incubation with the cdMMP-3 (E/S = 1/100). Pro-AT^{Sf-9} was separated from the activated form of MMP-9 by performing gel filtration chromatography in 6 M ureum. UV Absorption (in mAU) is shown for each fraction of 0.5 mL. (D) Coomassie blue staining of proteins in fractions 13 (F13), F24 and F25 separated in a 16% SDS-polyacrylamide gel. F13 and F25 contain, respectively, actMMP-9 and the glycosylated pro-AT^{Sf-9} and are shown next to synthetic pro-AT and pro-CT. (E) Detection of the pro-AT by immunoblotting after incubation with the cdMMP-3 at time 0 (lane 1) and after 3 h (lanes 2–3) incubated at 37 °C, with additional PNGase F treatment for 1 h at 37 °C (lane 3).

in vitro activation process [25]. We investigated the cleavage of the synthetic propeptide parts by cdMMP-3 and discovered two additional cleavage sites, one in pro-AT and one in pro-CT (Fig. 4A). The exact cleavage sites in the propeptide by cdMMP-3 were determined by N-terminal Edman sequencing to be located behind Asp_{34} and Leu_{71} in pro-AT and pro-CT, respectively. So far, the only two described cleavage sites in the MMP-9 propeptide by NE are behind Val_{57} and Ala_{58} [27]. However, we discovered additional cleavage sites, more specifically behind Val_{29} in pro-AT and at several sites in pro-CT (Fig. 4B). All

known and newly discovered cleavage sites in the MMP-9 propeptide by MMP-9 and NE are indicated in Fig. 4C.

Glycosylation of pro-AT reduces cleavage by proMMP-9-activating enzymes

Known and newly discovered cleavage sites in pro-AT are in close proximity to the glycosylated asparagine. Therefore, we tested the hypothesis that glycosylation might affect propeptide cleavage. Comparing the cleavage of pro-AT^{synth} (lacking N-linked glycosylation), with the cleavage of pro-AT^{Sf-9} (with oligosaccharide) by cdMMP-3, we showed a significantly faster decrease in intact peptide levels of pro-AT^{synth} (Fig. 5A). This higher cleavage velocity of the synthetic versus the recombinant pro-AT by cdMMP-3 was confirmed with different substrate concentrations (Fig. 5B,C). These results indicated that glycosylation of the propeptide reduces proteolysis by cdMMP-3. Additionally, cleavage of pro-AT^{synth} by active MMP-9 and meprin α was significantly faster than cleavage of pro-AT^{Sf-9}, confirming our hypothesis that glycosylation of the propeptide decreases proteolysis kinetics (Fig. 6A,B, respectively). Furthermore, while NE cleaves close to the glycosylation site, trypsin cleavage is at a more distant position. In line with our expectations, cleavage of pro-AT^{Sf-9} by NE was slower, compared to cleavage of non-glycosylated pro-AT^{synth} (Fig. 6C), whereas no difference was observed after cleavage by trypsin (Fig. 6D).

Next, we evaluated if glycosylation also plays a role in MMP-9 propeptide cleavage by complex biological fluids, which contain many proteases additionally to those tested *in vitro*. The non-glycosylated pro-AT^{synth} and the glycosylated pro-AT^{Sf-9} (Fig. 6E) were incubated with serum from healthy control subjects. Upon immunoblotting, almost no cleavage of the peptides was detected. However, incubation of the pro-AT peptides with synovial fluids of patients with rheumatoid arthritis showed a trend towards faster degradation of pro-AT^{synth} compared to pro-AT^{Sf-9}, with a significant difference after 24 h (Fig. 6F). In conclusion,

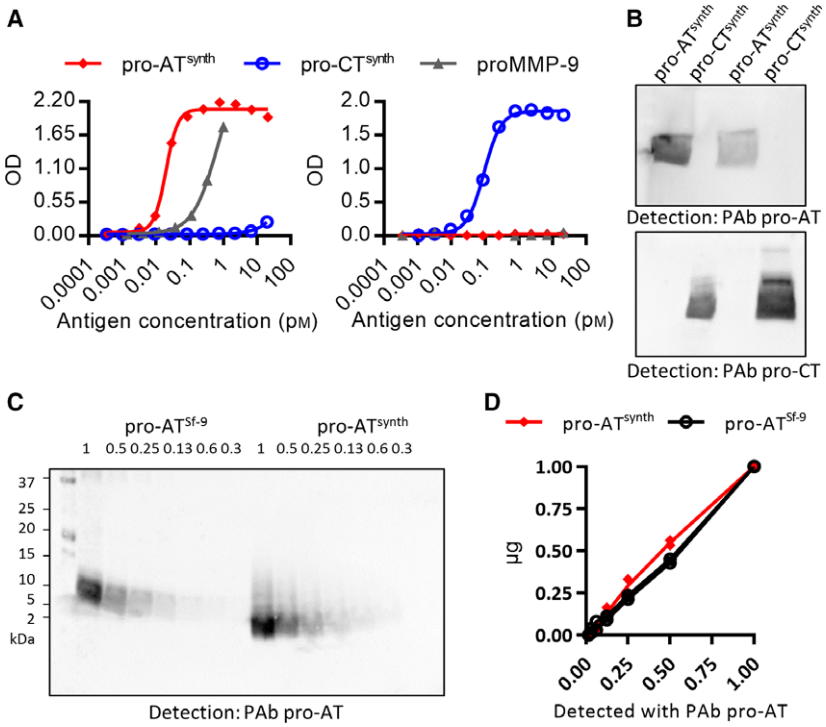

Fig. 3. Polyclonal antibodies detecting pro-AT and pro-CT show no cross-reactivity for pro-CT and pro-AT, respectively. PAb for pro-AT detect both pro-AT^{Sf-9} and pro-AT^{synth} in a similar way. (A) Specificity of the produced polyclonal antibodies was assessed by ELISAs with the antisera as coating and detection antibodies. Recognition of the synthetic pro-AT^{synth} (red) and pro-CT^{synth} (blue) as well as recombinant proMMP-9 (grey) was analysed. Left: optical density (OD) of antigen concentrations using polyclonal antibodies against pro-AT. Right: OD of antigen concentrations using polyclonal antibodies against pro-CT. (B) Western blot of pro-AT^{synth} and pro-CT^{synth} detected with PAb pro-AT (top) and after stripping with PAb pro-CT (bottom). (C) Detection of 1/2 dilution series of pro-AT^{Sf-9} and pro-AT^{synth} with anti-pro-AT immunoblotting. (D) Quantification of the detection of pro-AT^{Sf-9} and pro-AT^{synth} with anti-pro-AT immunoblotting, proving similar detection sensitivities. The synthetic pro-AT^{synth} migrates at a relatively lower molecular weight than pro-AT^{Sf-9}.

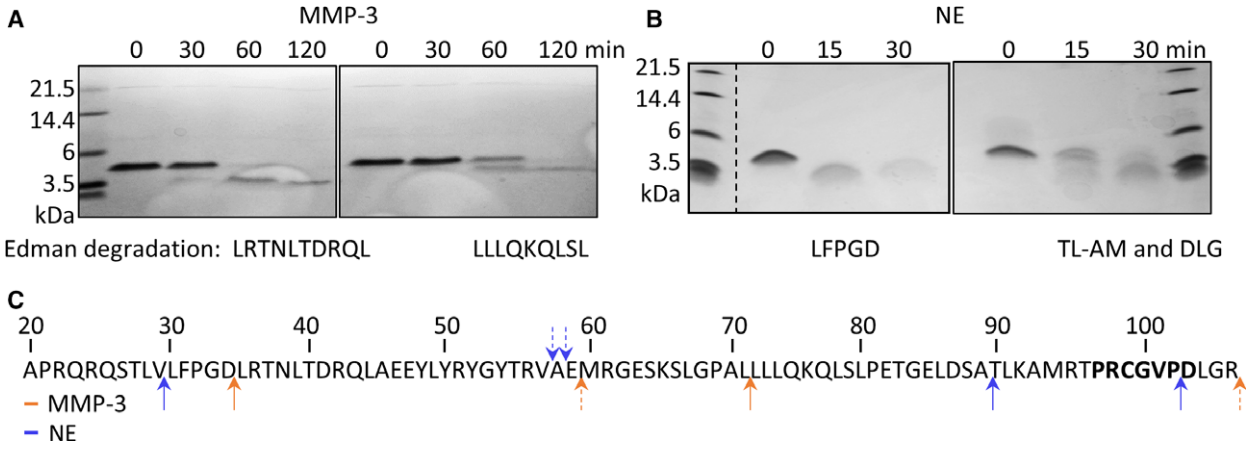

Fig. 4. Cleavage of the MMP-9 propeptide by MMP-3 and NE. (A) Left: Coomassie blue staining of synthetic pro-AT (10 μM) incubated with the cdMMP-3 (E/S = 1/50) for 0, 30, 60 and 120 min reveals a novel cleavage site behind Asp_{34}. Right: Similar experimental set-up with pro-CT reveals an additional cleavage site in this part of the propeptide behind Leu_{71}. Experimentally determined peptide sequences of the cleaved fragments are indicated in one-letter code for amino acids below the respective stained peptides. (B) Left: Coomassie blue staining of synthetic pro-AT (10 μM) incubated with the NE (E/S = 1/100) for 0, 15 and 30 min shows proteolytic cleavage, behind Val_{29}. Right: Similar experimental set-up with pro-CT reveals several cleavage sites by NE. Several peptide sequences were detected with Edman degradation, probable cleavage sites are behind Ala_{89} and Pro_{102}. Dotted black line marks splicing of image, due to an empty lane, which was deleted. (C) Propeptide sequence with dashed arrows indicating known and full arrows indicating newly identified cleavage sites for MMP-3 (red) and NE (blue).

glycosylation of the propeptide decreases proteolysis kinetics of the propeptide.

Neutrophil proMMP-9 is more resistant against stepwise activation by MMP-3 than recombinant proMMP-9

To test if glycosylation of proMMP-9 affects its activation in human, we used zymography (picogram sensitivity [11]) and DQ-gelatin degradation assays, since these are more sensitive methods and require less material than western blot analysis (nanogram sensitivity). We compared the stepwise activation of equimolar amounts of intact human recombinant Sf-9 and neutrophil proMMP-9 by MMP-3 with the use of gelatin zymography analysis. This was done by inclusion of several protein forms of recombinant MMP-9 and by standardization to quantify specific MMP-9 forms: proMMP-9 (Ala_{20} form, arrow 1), intermediate MMP-9 (Met_{60} form, arrow 2) and the activated MMP-9 form (Phe_{107} form, arrow 3) (Fig. 7A–C). ProMMP-9 from neutrophils was more resistant to stepwise processing by cdMMP-3 compared to recombinant proMMP-9. The decrease of proMMP-9 levels (Fig. 7D), in combination with the increase of the activated form of MMP-9 (actMMP-9, Fig. 7E) indicated that activation levels of neutrophil proMMP-9 were lower compared to those of recombinant Sf-9 proMMP-9 with a smaller sized N-glycosylation. Together, these data suggested that higher sugar complexity interfered more with the activation of proMMP-9 by MMP-3 *in vitro*.

ProMMP-9 from CDG patients versus healthy controls has a higher activation rate

Genetic defects in glycosylation have been first described in 1980 [48]. The most common disease is phosphomannomutase 2 deficiency, also named PMM2-Congenital Disorder of Glycosylation (CDG) [49]. We detected more efficient conversion of proMMP-9 into its activated form by MMP-3 in PMM2-CDG patients (n = 5) compared to healthy controls (n = 7), as analysed with zymography (Fig. 8A). Quantification of the percentage of proMMP-9 (Fig. 8B) and its activated form (Fig. 8C) showed a significantly faster activation of proMMP-9 from CDG patients compared to controls.

Galectin-3 interaction with proMMP-9 inhibits its activation by MMP-3

An important function of protein glycosylation is to mediate glycan–lectin interactions. Such interactions may be relevant in cancer biology [40]. We evaluated the role of galectin-3, a galactoside-binding lectin, on the activation of human neutrophil proMMP-9 by MMP-3. Interestingly, we discovered that stepwise activation of neutrophil proMMP-9 by MMP-3 into

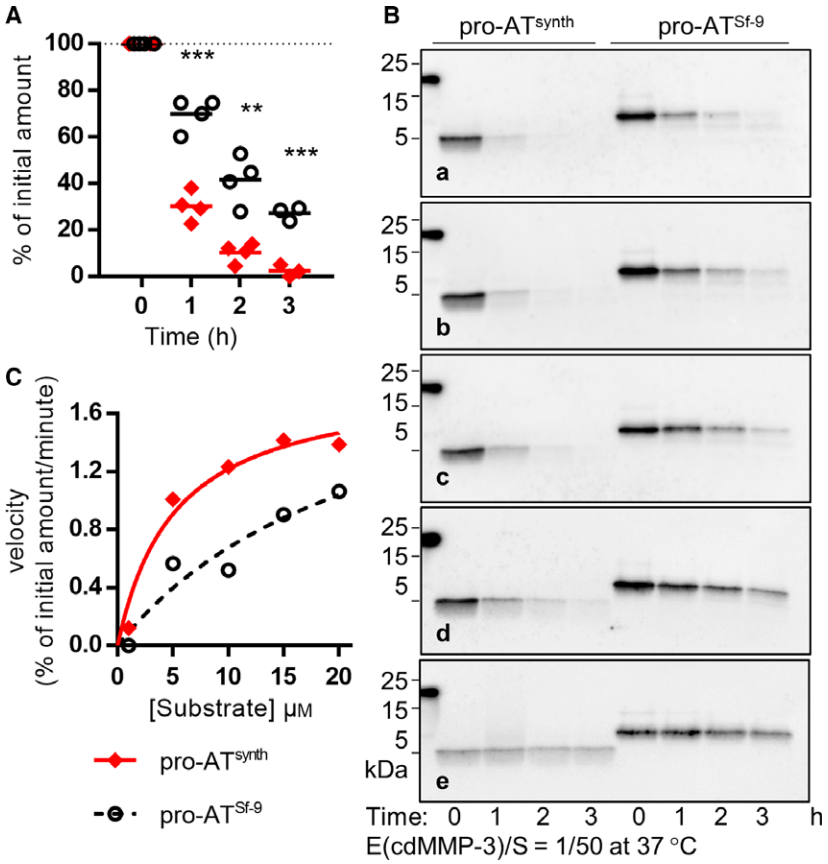

Fig. 5. Differential proteolysis of pro-AT^{synth} and pro-AT^{Sf-9} by cdMMP-3 suggesting protection by glycosylation. (A) Quantification of detection of pro-AT^{synth} (red, $n = 4$) and pro-AT^{Sf-9} (black, $n = 4$) by immunoblotting after incubation with the cdMMP-3 for 0, 1, 2 and 3 h at 37 °C. Significantly different proteolysis, assessed with the unpaired t-test, is indicated with *** ($P < 0.001$) and ** ($P < 0.01$). Horizontal bars indicate means. (B) Example of immunoblot analysis of aglycosyl pro-AT^{synth} (left) and glycosylated pro-AT^{Sf-9} (right) at different peptide concentrations (a = 20 µM, b = 15 µM, c = 10 µM, d = 5 µM and e = 1 µM) after incubation and proteolysis by cdMMP-3. After cleavage, the equivalent amounts of 10 nanogram starting substrate ($t = 0$) and similar equivalents after the indicated time intervals were loaded. The locations of molecular markers, indicated in kilodaltons are included. (C) Velocity of cleavage of the aglycosyl (red) and glycosylated (black) peptides by cdMMP-3 at different substrate concentrations.

its activated form is reduced by co-incubation with galectin-3 (Fig. 9A). Consequently, gelatinolytic activity of MMP-9 after incubation with MMP-3, quantified by the velocity of Dye-Quenched (DQ) gelatin cleavage, was also significantly reduced (Fig. 9C). Overnight incubation of neutrophil proMMP-9 with galectin-3 (1/10) prevented activation by MMP-3, while recombinant proMMP-9 was fully converted into its active form (Fig. 9B). Interestingly, a galectin-3 mutant, defective in the formation of pentameric structures, lacking the non-lectin domain failed to inhibit proMMP-9 activation by MMP-3 (Fig. 9D). Although addition of homodimeric galectin-1 slightly reduced MMP-3-induced activation of proMMP-9 (Fig. 9E), this effect was much less compared to inhibition by galectin-3 and was not statistically significant.

Discussion

The two-step proteolytic activation of proMMP-9 by MMP-3 is known since long [25], but the role of propeptide glycosylation in this process remained enigmatic. Here, we investigated the role of MMP-9 propeptide glycosylation. First, we report new cleavage sites in the MMP-9 propeptide by MMP-3 and NE. All known and newly discovered cleavage sites in the MMP-9 propeptide are indicated in Fig. 10A. Additionally, we discovered a role of the propeptide glycosylation in decreasing its proteolytic cleavage by MMP-3, MMP-9, meprin α, NE and by proteases in synovial fluids of rheumatoid arthritis patients. Interestingly, larger glycan structures, such as those occurring in neutrophil proMMP-9, increased the resistance against proteolysis,

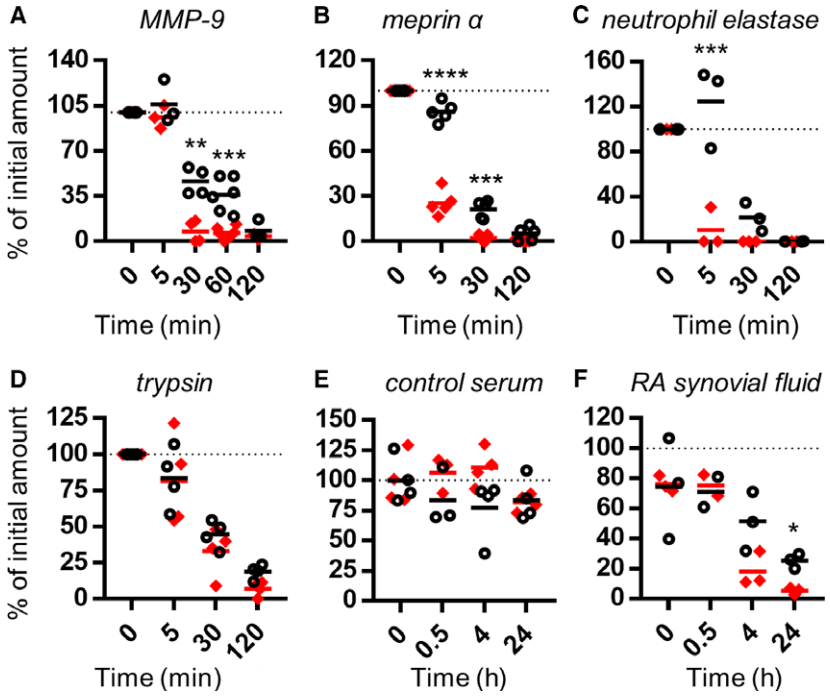

Fig. 6. Differential proteolysis of pro-AT^{synth} and pro-AT^{Sf-9} by active MMP-9, meprin α, NE and with synovial fluids of rheumatoid arthritis (RA) patients. Quantification of immunoblotting of pro-AT^{synth} (red) and pro-AT^{Sf-9} (black) after incubation with (A) actMMP-9 (1/200, $n = 4$), (B) meprin α (1/5000, $n = 5$), (C) NE (1/500, $n = 3$) and (D) trypsin (1/10 000, $n = 4$) for several time points at 37 °C. Proteolysis of the peptides was also assessed after incubation in serum of healthy controls (E) and in the presence of synovial fluid of patients with rheumatoid arthritis (F) for 0, 0.5, 4 and 24 h ($n = 3$). Significantly different proteolysis, assessed with the unpaired t-test, is indicated with *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) and ****($P < 0.0001$). Horizontal bars indicate means.

compared to smaller oligosaccharides of recombinant Sf-9 proMMP-9 and proMMP-9 from CDG patients. Besides this direct effect on altering proteolysis kinetics, glycosylation of proMMP-9 decreased the activation rate and enzyme activity through binding with galectin-3 in an indirect way.

A three-dimensional model of full-length proMMP-9 with the N-linked sugar from Sf-9 cells and the more complex N-linked sugar from neutrophils on the propeptide illustrates the importance of investigating the role of sugars, given their relative sizes in comparison with protein domains (Fig. 10B). Our data document a significant role of glycosylation in protection of the MMP-9 propeptide against proteolysis. This finding is reminiscent of the protective function of oligosaccharides against proteases, as was originally described by Rudd and co-workers for pancreatic ribonuclease B as a protease substrate [43,50].

Noteworthy, the used pro-AT^{Sf-9} contains five additional amino acids in comparison with the 40-amino acid pro-AT^{synth}, namely Asp$_{-5}$Pro$_{-4}$Ser$_{-3}$Ser$_{-2}$Arg$_{-1}$, increasing the molecular weight by about 500 Da [14], whereas the neutrophil-derived natural proMMP-9 has the same amino-terminus as the synthetic peptide. However, these five amino acids were unlikely to induce any difference in proteolysis of the peptide since the cleavage by trypsin, which cleaves in the closest proximity to Arg$_{-1}$, is similar for pro-AT^{synth} and pro-AT^{Sf-9}. Furthermore, in the comparison of recombinant and neutrophil proMMP-9 cleavage, this was not an issue, since we observed more resistance against cleavage of neutrophil proMMP-9, lacking these amino acids.

Glycans of the O-glycosylated domain of proMMP-9 have been suggested to be important for lectin binding, enzyme elongation [14], domain organization [51] and interdomain flexibility of proMMP-9 [52]. Terminal sialylation of sugars is essential for the interaction of MMP-9 with its inhibitor TIMP-1 [53] and, as recently shown, with staphylococcal superantigen-like protein 5 [54]. Out of three potential N-linked glycosylation sites in proMMP-9, only Asn$_{38}$ in the propeptide and Asn$_{120}$ in the active site are glycosylated [14]. Interestingly, while the N-linked glycosylation of the active site of MMP-9 is also present in collagenases MMP-8, -13, -17 and MMP-23, the N-linked glycan in the propeptide is unique for MMP-9 [41]. By site-directed mutagenesis, a role of both N-glycosylation sites for the secretion of

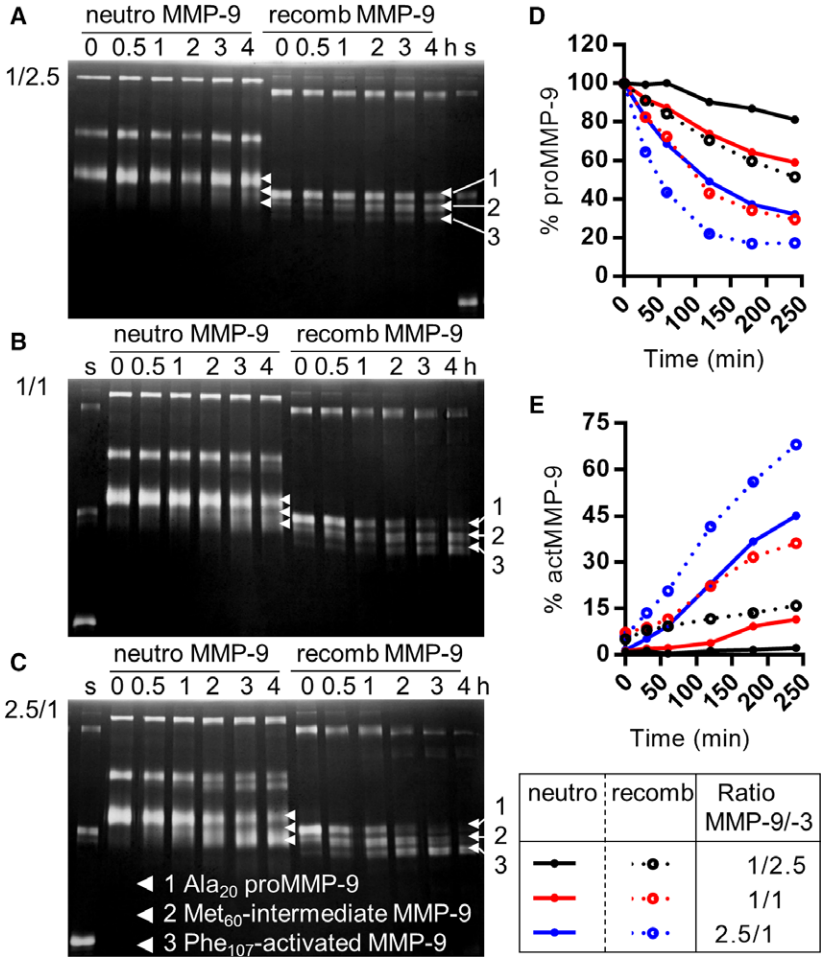

Fig. 7. Differential cleavage of recombinant and neutrophil proMMP-9 by MMP-3. Zymography analysis of the cleavage of proMMP-9 (white arrow 1 = Ala_{20} form) into intermediate (white arrow 2 = Met_{60} form) and activated (white arrow 3 = Phe_{107} form) MMP-9 by cdMMP-3. Neutrophil proMMP-9 (neutro) and recombinant proMMP-9 (recomb) were incubated with cdMMP-3 for 0, 0.5, 1, 2, 3 and 4 h at 37 °C at an enzyme/substrate ratio of (A) 1/2.5, (B) 1/1 and (C) 2.5/1. Recombinant standard (s) is a mixture of MMP-9 multimers (250 kDa) and monomers (92 kDa) and a deletion mutant (46 kDa, lacking the O-glycosylated and hemopexin domains) of proMMP-9 and was loaded as a reference. Quantification of percentage neutrophil (solid lines) and recombinant (dotted lines) (D) proMMP-9 and (E) actMMP-9 at enzyme/substrate ratios of 1/2.5 (black), 1/1 (red) and 2.5/1 (blue).

proMMP-9 has been proven. Both Asn_{38} and Asn_{120} glycosylation-deficient mutants showed retention in the endoplasmic reticulum (ER), through a strong N-glycosylation-independent interaction with ER-resident calreticulin [42]. Additionally, Duellman *et al.* showed a loss of proMMP-9 expression due to the degradation of N-glycosylation-deficient proMMP-9 by the proteasome [55], suggesting protection by N-glycosylation against intracellular proteolysis. Recently, a molecular dynamics simulation study suggested that glycosylation might induce a conformation of the propeptide that facilitates the temporal order of the first and second cleavage events [38]. The authors speculated that glycosylation does not hinder the activation process directly but that glycosylation of proMMP-9 decreases the flexibility of the protein backbone, having a stabilizing effect, as described previously [50]. However, we demonstrated decreased proteolysis due to glycosylation.

While investigating galectin-8 interactions, Nishi *et al.* [56] showed increased proMMP-9 activation by MMP-3 when adding galectin-8. This effect was not observed when adding galectin-1 or -3 [56], which is in agreement with our results proving an inhibitory effect of galectin-3 on proMMP-9 activation. Interestingly, galectin-3 is efficiently cleaved by MMP-9 and MMP-2 in the non-lectin domain [57], which is necessary for multimerization. Our results indicate that a multimerization-deficient mutant of galectin-3 is less capable of inhibiting proMMP-9 activation, suggesting a loop, that once MMP-9 is active, it promotes activation of other proMMP-9 molecules through galectin-3 cleavage.

MMP-9 plays a role in many organ systems, including the endocrine, nervous, reproductive and immune systems [1] and in CDGs several organ systems are affected [49]. The present findings, illustrating diminished fine-tuning of proMMP-9 activation by altered propeptide glycosylation, may be relevant in these contexts.

In conclusion, we document the functional role of glycosylation of the propeptide of MMP-9 in controlling proteolysis kinetics, directly and indirectly by galectin-3 binding, thereby regulating proMMP-9 activation, *in vitro* and *ex vivo*.

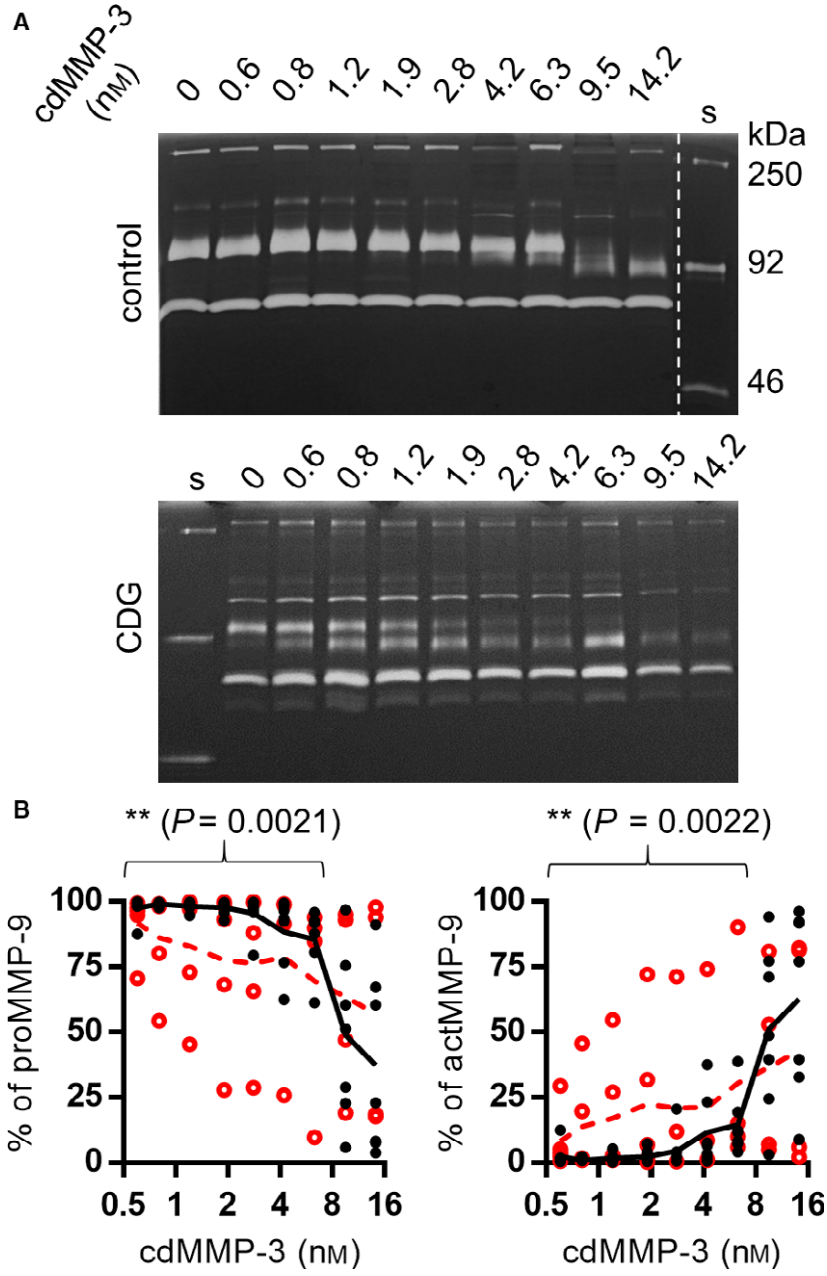

Fig. 8. Congenital disorder of glycosylation patients, having defective N-glycosylation, show higher proMMP-9 activation rate compared to healthy controls. (A) Zymography analysis of gelatin-Sepharose purified serum samples of healthy controls (n = 7) and CDG patients (n = 5) after 3-h incubation with 0-14.2 nM cdMMP-3. One zymography gel is shown for both groups. Recombinant proMMP-9 standard (s) was added. Dotted white line marks splicing of image. Quantification of zymolytic bands of (B) proMMP-9 and (C) actMMP-9 in the serum of healthy controls (black) and CDG patients (red) are shown as a percentage of both forms at different concentrations of cdMMP-3. Lines connect the mean of all data points. Significance, assessed with one-way ANOVA, is indicated with ** (P < 0.01).

Materials and methods

Proteins and antibodies

Synthetic peptides (pro-AT:APRQRQSTLVLFPGDLRTN LTDRQLAEEYLYRYGYTRVAE and pro-CT: MRGES KSLGPALLLLQKQLSLPETGELDSATLKAMRTPRCG VPDLGR) were synthesized by Thermo Scientific (Waltham, MA, USA). Recombinant full-length human proMMP-9 was expressed in Sf-9 insect cells and purified as described previously [14]. The human recombinant catalytic domain of MMP-3 (cdMMP-3) was purchased from Calbiochem (San Diego, CA, USA, cat# 444217). For the purification of recombinant pro-AT^{Sf-9}, human recombinant proMMP-9 was incubated with cdMMP-3 at an enzyme substrate ratio of 1/100 for 3 h at 37 °C in 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$ and 0.01% Tween-20 (Tris buffer). The recombinant peptide, pro-AT^{Sf-9}, was separated from the activated form of MMP-9 by gel filtration chromatography in 6 M ureum (Superdex 200 10/300 GL, GE Healthcare, Chicago, IL, USA; Fig. 2C). Afterwards, pro-AT was concentrated and dialysed using Tris buffer and 3 kDa centrifugal filters (Amicon, Merck Millipore, Burlington, MA, USA).

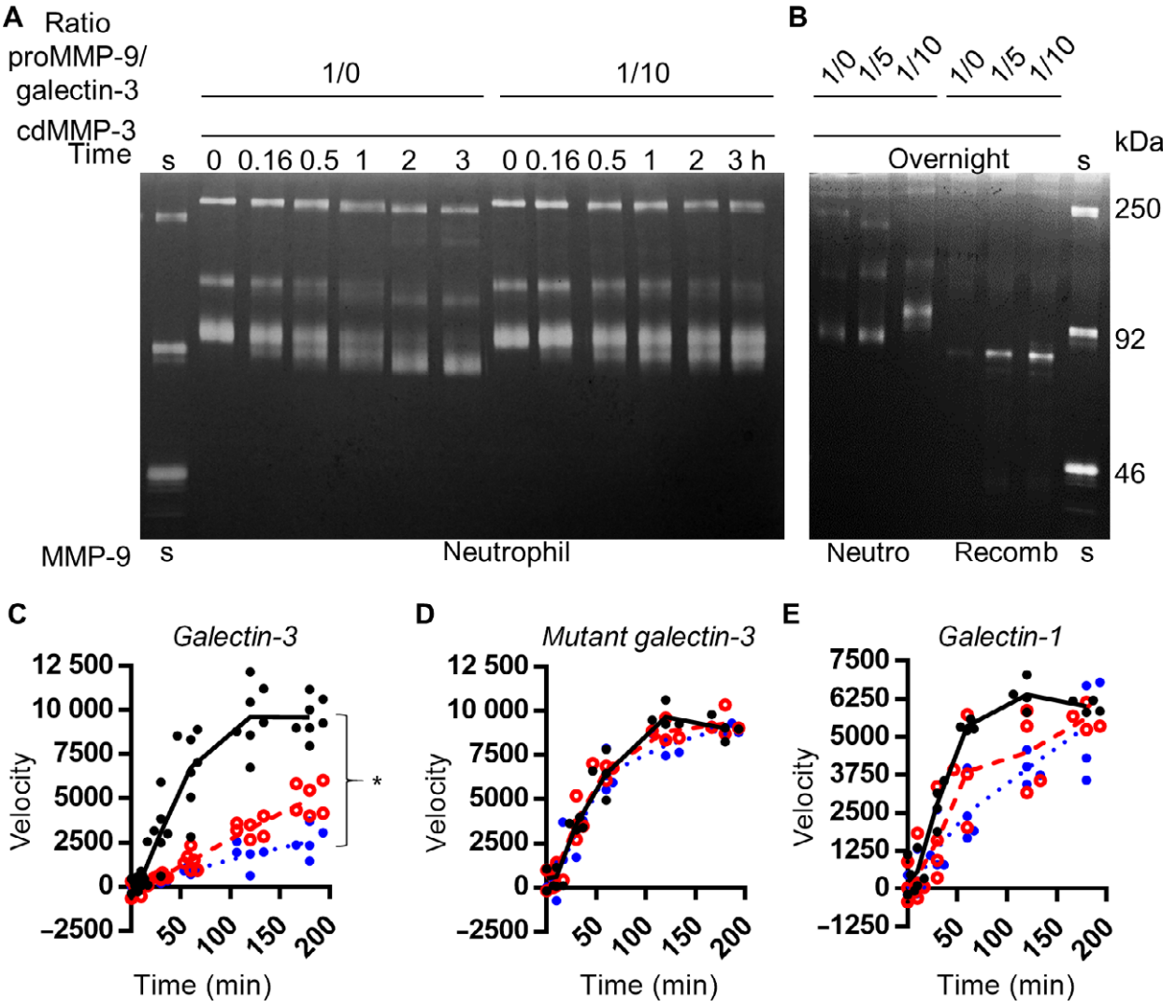

Fig. 9. Galectin-3 binding with proMMP-9 inhibits its activation and gelatinolytic activity. (A) Zymography analysis of neutrophil proMMP-9 cleavage into actMMP-9 by cdMMP-3 (1/20), alone or co-incubated with galectin-3 (with proMMP-9/galectin-3: 1/10) for 0, 0.16, 0.5, 1, 2 and 3 h. Recombinant proMMP-9 standard (s) was loaded as a reference. (B) Overnight incubation of neutrophil (neutro) and recombinant (recomb) proMMP-9 and cdMMP-3, with or without galectin-3 (1/5 and 1/10). Standard (s) is as in panel A. (C) Gelatinolytic activity of neutrophil proMMP-9/galectin-3 (1/0 in black, 1/5 in red and 1/10 in blue) after incubation with cdMMP-3 was assessed by DQ-gelatin cleavage. Significance, assessed with one-way ANOVA, is indicated with *($P < 0.05$). (D) Similar experimental set-up with an oligomerization-deficient mutant of galectin-3 and with (E) galectin-1.

Coomassie Blue-stained proteins within SDS/PAGE gels (of protein peak fractions after gel filtration chromatography) confirmed the separation of activated MMP-9 and the pro-AT^{Sf-9} (Fig. 2D). Recombinant human active meprin α was produced in insect cells and purified and activated as described previously [58,59]. Human recombinant active neutrophil elastase (NE) was from Abcam (Cambridge, UK, ab91099) and trypsin was obtained from Sigma-Aldrich (Saint-Louis, MO, USA, T1426). cDNAs coding for the human galectin-1 and galectin-3 were generated by PCR with reverse transcription on total RNA extracted from a melanoma cell line. Galectin-3 cDNA was used as template to amplify by PCR the sequence coding for galectin-3 (amino acids Met_1-Ile_{250}) fused to the C-terminal tag: SG(H)6SC. The galectin-3 mutant, lacking the non-lectin domain corresponds to Gly_{112}-Ile_{250}. The PCR products were cloned into a derivative of plasmid pET9 (Novagen, Merck Millipore, Burlington, MA, USA). The soluble proteins were expressed in *E. coli* BL21-AI (Invitrogen, Thermo Fisher Scientific) and purified by affinity chromatography on α-lactose agarose (Sigma-Aldrich, St. Louis, MO, USA). Peroxidase-conjugated affinity-purified donkey anti-rabbit IgG was purchased from Jackson

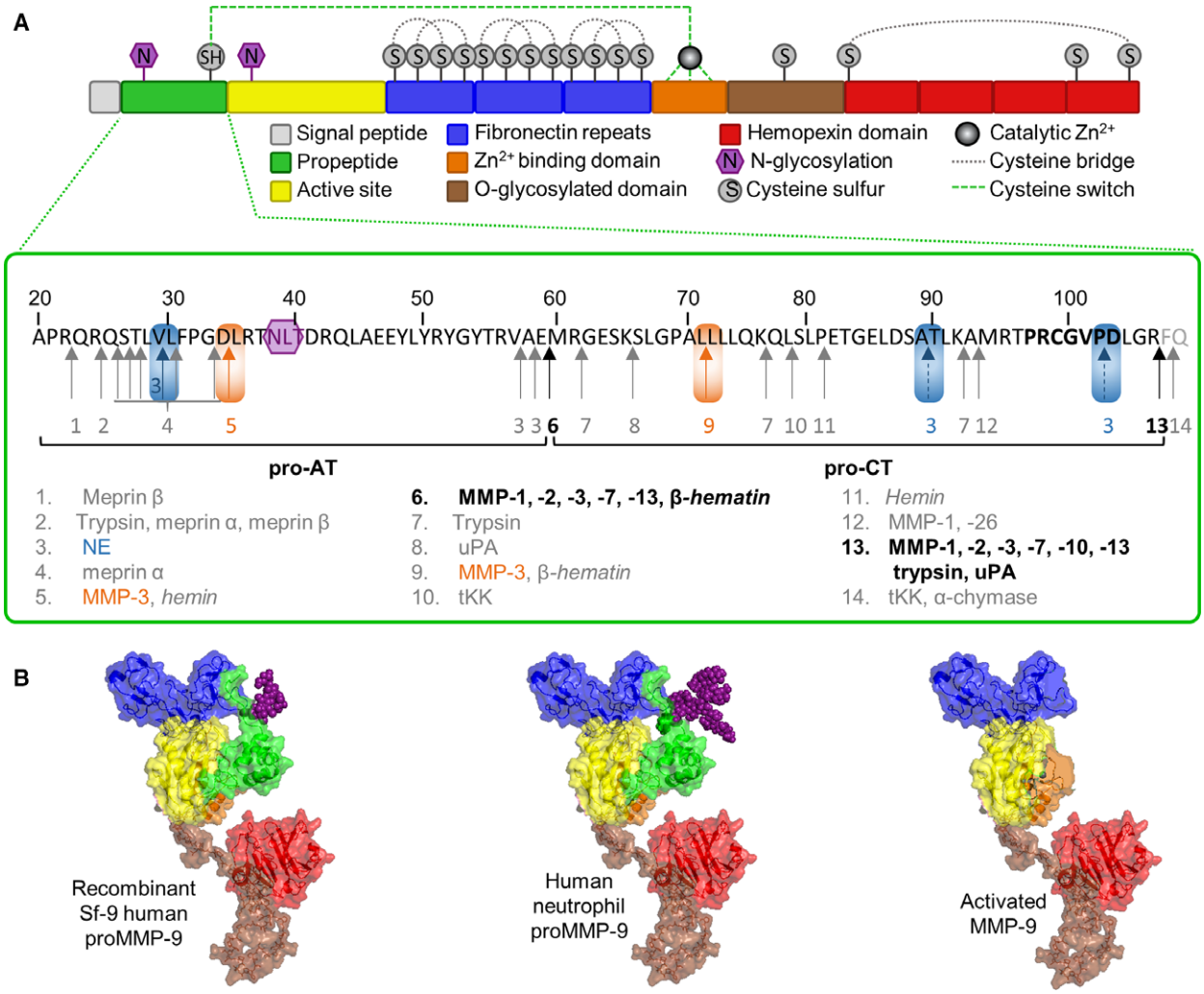

Fig. 10. Domain organization, post-translational modifications and cleavage sites in the MMP-9 propeptide. Human proMMP-9 is composed of an aminoterminal signal peptide (grey), a propeptide (green), an active site (yellow), three fibronectin repeats (blue), a Zn^{2+} binding domain (orange), an O-glycosylated domain (brown) and a carboxyterminal hemopexin domain (red). (A) The propeptide (Ala_{20}-Arg_{106}) contains the cysteine switch consensus sequence **PRCXXPD** (bold) of which Cys_{99} interacts with the Zn^{2+} ion coordinated in the catalytic site. Numbered arrows indicate cleavage sites in the propeptide by meprin α, α-chymase, meprin β, β-*hematin*, *hemin (italic=interaction induces autocatalytic activation of proMMP-9)*, MMP-1, -2, -3, -7, -10, -13 and -26, neutrophil elastase (NE), tissue kallikrein (tKK), trypsin and urokinase type plasminogen activator (uPA). For MMP-3 and NE, newly discovered cleavage sites are indicated in orange and blue respectively. At Asn_{38} (in the NLT sequon, indicated in purple), the propeptide contains an N-linked oligosaccharide. (B) Three-dimensional model of proMMP-9 and the N-linked oligosaccharide (purple) at Asn_{38} on human recombinant Sf-9 proMMP-9 (left), which is smaller compared to oligosaccharides on human neutrophil proMMP-9 (middle, glycoform A3G3F2) and a model of activated MMP-9 (right) (model based on references 14, 15, 37 and 61) and figure adapted from (1). The N-linked oligosaccharides at Asn_{120} are not included.

ImmunoResearch (Cambridgeshire, UK, cat# 711-035-152). Synthetic pro-AT and pro-CT were used to immunize New Zealand White rabbits for the production of polyclonal antibodies, in the presence of complete Freund's adjuvant. A boost injection of the antigen with incomplete Freund's adjuvant was given monthly and 14 days prior to serum collection. Hyperimmune rabbit serum was diluted (1/5 with 20 mM NaH_2PO_4 pH 7.5, 0.2 M NaCl) and filtered before loading on a recombinant protein A-Sepharose fast flow column (GE Healthcare). Rabbit IgGs were eluted with 0.1 M Glycine pH 2.8 followed by a neutralization step with the use of 1 M Tris pH 8.0. Affinity purification of pooled rabbit IgG fractions was performed on N-hydroxysuccinimide (NHS)-Sepharose (GE Healthcare), coupled with either pro-AT or pro-CT (2 $mg{\cdot}mL^{-1}$) according to the manufacturer's protocol, with similar buffers to load, elute and neutralize, as indicated above. These affinity-

purified rabbit polyclonal antibodies were used to optimize immunoblot analysis and ELISA for the detection of pro-AT and pro-CT (Figs 2Ac,Bc and 3A–D).

Immunoblot analysis

Peptides were separated in 16% acrylamide (Biorad, Hercules, CA, USA) gels, transferred to polyvinylidene difluoride (PVDF) membranes (Biorad) and probed with indicated polyclonal rabbit antibodies (0.5 $\mu g \cdot mL^{-1}$), recognizing either pro-AT or pro-CT of human proMMP-9. As secondary reactant, we used peroxidase-conjugated polyclonal donkey anti-rabbit antibodies. Detection was performed after addition of chemiluminescent substrate (Super Signal West Pico #34080 and Femto #34095, ThermoFisher, Waltham, MA, USA).

Patient samples

Blood of healthy controls and synovial fluid samples from rheumatoid arthritis patients were collected in accordance with the Declaration of Helsinki and rules of the local Ethics Committee (KU Leuven, Belgium). Serum samples of PMM2-CDG patients and healthy controls were obtained at the University Hospitals of Leuven under the supervision of J. Jaeken. Written informed consent from healthy controls and patients was obtained.

DQ-gelatin or fluorigenic peptide degradation assay

A dye-quenched (DQ) gelatin degradation assay was performed as previously described [60] with fluorescence detection (Clariostar, MBG Labtech, ex 485 nm/em 530 nm). All data were corrected by subtraction of negative control values.

Proteolytic processing of propeptide

The synthetic pro-AT and pro-CT (10 μM) were incubated with cdMMP-3 (enzyme substrate ratio of 1/50) in Tris buffer or with NE (1/100) in 200 mM Tris/HCl pH 8.8 at 37 °C to determine cleavage sites. Pro-AT^{synth} and pro-AT^{Sf-9} were incubated at different concentrations (20 μM, 15 μM, 10 μM, 5 μM and 1 μM) with the cdMMP-3 (1/50) at 37 °C in Tris buffer. ProMMP-9 was activated with cdMMP-3 (1/100) for 3 h at 37 °C in Tris buffer. Active MMP-9 (1/200), active meprin α (1/5000) were used to cleave pro-AT^{synth} and pro-AT^{Sf-9} (10 μM) at 37 °C in Tris buffer. Active NE (1/500) was used to cleave pro-AT^{synth} and pro-AT^{Sf-9} (10 μM) at 37 °C in 200 mM Tris/HCl pH 8.8. Trypsin (1/10 000) was used to cleave pro-AT^{synth} and pro-AT^{Sf-9} (10 μM) at 37 °C in Tris buffer (without $CaCl_2$). Finally, pro-AT^{synth} and pro-AT^{Sf-9} (5 μM) were incubated in the presence of serum of healthy controls or synovial fluid of patients with rheumatoid arthritis (1/5 diluted in PBS) for several time points.

Peptide sequencing to identify cleavage sites

Peptide fragments of pro-AT or pro-CT were transferred onto a PVDF membrane of a ProSorb cartridge (Applied Biosystems, Foster City, CA, USA) followed by Edman degradation (Procise 491cLC, Applied Biosystems).

Gelatin zymography analysis

Samples of MMP-9 were analysed on 7.5% polyacrylamide gels containing 0.1% gelatin, as previously described [11]. Multimeric and monomeric proMMP-9 and a specific domain deletion mutant of human proMMP-9 (lacking the O-glycosylated and the hemopexin domains) were included as molecular size marker and standardization of the gelatin zymography method (Figs 7–9).

Gelatin-Sepharose bound MMP-9 activation

Five microlitre of serum was incubated for 30 min with gelatin-Sepharose (GE Healthcare) in 50 mM Tris/HCl pH 7.4, 10 mM $CaCl_2$ and 0.01% $Tween_{20}$ under agitation. After washing, the gel was incubated with cdMMP-3 (from 0 to 14.2 nM) for 3 h at 37 °C while shaking and the residual gelatin-bound MMPs were quantified by zymography.

Statistical analysis

Statistical analyses were performed using GRAPHPAD PRISM 6 software (La Jolla, CA, USA). Data points were assessed for normality with the use of the Shapiro–Wilk test. Depending on the distribution of the data points, unpaired t-test (for normal distribution) or Mann–Whitney test (for non-normal distribution) were used to calculate P values. All P values <0.05 were considered significant.

Molecular modelling

For molecular modelling of proMMP-9 with the glycan attached to Asn_{38}, we used a previous model of proMMP-9 created by Rosenblum *et al.* [15], which is based on SAXS data and crystal structures obtained by Elkins *et al.* [37] and Cha *et al.* [61]. N-linked glycans, Fc and FcA3G3F2 (Fig. 1) for recombinant Sf-9 and natural neutrophil proMMP-9, respectively, were build using the Glycam webserver (http://www.glycam.org) and then attached to Asn_{38} in PyMOL (Fig. 10).

Acknowledgements

The present study was funded by the Geconcerteerde OnderzoeksActies (GOA 2013/015) and C1 funding of KU Leuven (16/17/010), the Charcot Foundation of Belgium and the Research Foundation of Flanders

(FWO-Vlaanderen), the Rega Foundation and the Deutsche Forschungsgemeinschaft SFB 877 (project A9).

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

LB and GO designed research. LB, EU-B, EM, JV, VR and PP performed research. DC, MG-A, PB, WS, CB-P provided recombinant proteins. JJ, EM and PW provided CDG patient samples and LB and GO wrote the paper. All authors critically improved the manuscript and agreed with the final version.

References

1 Vandooren J, Van den Steen PE & Opdenakker G (2013) Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9): the next decade. *Crit Rev Biochem Mol Biol* **48**, 222–272.
2 Dubois B, Arnold B & Opdenakker G (2000) Gelatinase B deficiency impairs reproduction. *J Clin Invest* **106**, 627–628.
3 Plaks V, Rinkenberger J, Dai J, Flannery M, Sund M, Kanasaki K, Ni W, Kalluri R & Werb Z (2013) Matrix metalloproteinase-9 deficiency phenocopies features of preeclampsia and intrauterine growth restriction. *Proc Natl Acad Sci USA* **110**, 11109–11114.
4 Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM & Werb Z (1998) MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* **93**, 411–422.
5 Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, Carbone DP & Lin PC (2004) Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* **6**, 409–421.
6 Ardi VC, Kupriyanova TA, Deryugina EI & Quigley JP (2007) Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. *Proc Natl Acad Sci USA* **104**, 20262–20267.
7 Overall CM & Kleifeld O (2006) Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer* **6**, 227–239.
8 Kessenbrock K, Plaks V & Werb Z (2010) Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* **141**, 52–67.
9 Hu J, Van den Steen PE, Sang QX & Opdenakker G (2007) Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat Rev Drug Discov* **6**, 480–498.
10 Vandenbroucke RE & Libert C (2014) Is there new hope for therapeutic matrix metalloproteinase inhibition? *Nat Rev Drug Discov* **13**, 904–927.
11 Vandooren J, Geurts N, Martens E, Van den Steen PE & Opdenakker G (2013) Zymography methods for visualizing hydrolytic enzymes. *Nat Methods* **10**, 211–220.
12 Gerwien H, Hermann S, Zhang X, Korpos E, Song J, Kopka K, Faust A, Wenning C, Gross CC, Honold L *et al.* (2016) Imaging matrix metalloproteinase activity in multiple sclerosis as a specific marker of leukocyte penetration of the blood-brain barrier. *Sci Transl Med* **8**, 364ra152.
13 Hingorani DV, Lemieux AJ, Acevedo JR, Glasgow HL, Kedarisetty S, Whitney MA, Molinolo AA, Tsien RY & Nguyen QT (2017) Early detection of squamous cell carcinoma in carcinogen induced oral cancer rodent model by ratiometric activatable cell penetrating peptides. *Oral Oncol* **71**, 156–162.
14 Van den Steen PE, Van Aelst I, Hvidberg V, Piccard H, Fiten P, Jacobsen C, Moestrup SK, Fry S, Royle L, Wormald MR *et al.* (2006) The hemopexin and O-glycosylated domains tune gelatinase B/MMP-9 bioavailability via inhibition and binding to cargo receptors. *J Biol Chem* **281**, 18626–18637.
15 Rosenblum G, Van den Steen PE, Cohen SR, Grossmann JG, Frenkel J, Sertchook R, Slack N, Strange RW, Opdenakker G & Sagi I (2007) Insights into the structure and domain flexibility of full-length pro-matrix metalloproteinase-9/gelatinase B. *Structure* **15**, 1227–1236.
16 Piccard H, Van den Steen PE & Opdenakker G (2007) Hemopexin domains as multifunctional liganding modules in matrix metalloproteinases and other proteins. *J Leukoc Biol* **81**, 870–892.
17 Ugarte-Berzal E, Bailon E, Amigo-Jimenez I, Vituri CL, del Cerro MH, Terol MJ, Albar JP, Rivas G, Garcia-Marco JA & Garcia-Pardo A (2012) A 17-residue sequence from the matrix metalloproteinase-9 (MMP-9) hemopexin domain binds alpha4beta1 integrin and inhibits MMP-9-induced functions in chronic lymphocytic leukemia B cells. *J Biol Chem* **287**, 27601–27613.
18 Collier IE, Krasnov PA, Strongin AY, Birkedal-Hansen H & Goldberg GI (1992) Alanine scanning mutagenesis and functional analysis of the fibronectin-like collagen-binding domain from human 92-kDa type IV collagenase. *J Biol Chem* **267**, 6776–6781.
19 Van Wart HE & Birkedal-Hansen H (1990) The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to

the entire matrix metalloproteinase gene family. *Proc Natl Acad Sci USA* **87**, 5578–5582.
20 Fridman R, Toth M, Pena D & Mobashery S (1995) Activation of progelatinase B (MMP-9) by gelatinase A (MMP-2). *Cancer Res* **55**, 2548–2555.
21 Sang QX, Birkedal-Hansen H & Van Wart HE (1995) Proteolytic and non-proteolytic activation of human neutrophil progelatinase B. *Biochim Biophys Acta* **1251**, 99–108.
22 Nakamura H, Fujii Y, Ohuchi E, Yamamoto E & Okada Y (1998) Activation of the precursor of human stromelysin 2 and its interactions with other matrix metalloproteinases. *Eur J Biochem* **253**, 67–75.
23 Knäuper V, Smith B, Lopez-Otin C & Murphy G (1997) Activation of progelatinase B (proMMP-9) by active collagenase-3 (MMP-13). *Eur J Biochem* **248**, 369–373.
24 Uria JA & Lopez-Otin C (2000) Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. *Cancer Res* **60**, 4745–4751.
25 Ogata Y, Enghild JJ & Nagase H (1992) Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *J Biol Chem* **267**, 3581–3584.
26 Geurts N, Becker-Pauly C, Martens E, Proost P, Van den Steen PE, Stöcker W & Opdenakker G (2012) Meprins process matrix metalloproteinase-9 (MMP-9)/gelatinase B and enhance the activation kinetics by MMP-3. *FEBS Lett* **586**, 4264–4269.
27 Jackson PL, Xu X, Wilson L, Weathington NM, Clancy JP, Blalock JE & Gaggar A (2010) Human neutrophil elastase-mediated cleavage sites of MMP-9 and TIMP-1: implications to cystic fibrosis proteolytic dysfunction. *Mol Med* **16**, 159–166.
28 Duncan ME, Richardson JP, Murray GI, Melvin WT & Fothergill JE (1998) Human matrix metalloproteinase-9: activation by limited trypsin treatment and generation of monoclonal antibodies specific for the activated form. *Eur J Biochem* **258**, 37–43.
29 Okada Y, Gonoji Y, Naka K, Tomita K, Nakanishi I, Iwata K, Yamashita K & Hayakawa T (1992) Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT 1080 human fibrosarcoma cells. Purification and activation of the precursor and enzymic properties. *J Biol Chem* **267**, 21712–21719.
30 Desrivieres S, Lu H, Peyri N, Soria C, Legrand Y & Menashi S (1993) Activation of the 92 kDa type IV collagenase by tissue kallikrein. *J Cell Physiol* **157**, 587–593.
31 Fang KC, Raymond WW, Blount JL & Caughey GH (1997) Dog mast cell alpha-chymase activates progelatinase B by cleaving the Phe88-Gln89 and Phe91-Glu92 bonds of the catalytic domain. *J Biol Chem* **272**, 25628–25635.
32 Geurts N, Martens E, Van Aelst I, Proost P, Opdenakker G & Van den Steen PE (2008) Beta-hematin interaction with the hemopexin domain of gelatinase B/MMP-9 provokes autocatalytic processing of the propeptide, thereby priming activation by MMP-3. *Biochemistry* **47**, 2689–2699.
33 Zhao Y, Lyons CE Jr, Xiao A, Templeton DJ, Sang QA, Brew K & Hussaini IM (2008) Urokinase directly activates matrix metalloproteinases-9: a potential role in glioblastoma invasion. *Biochem Biophys Res Commun* **369**, 1215–1220.
34 Cuzner ML & Opdenakker G (1999) Plasminogen activators and matrix metalloproteases, mediators of extracellular proteolysis in inflammatory demyelination of the central nervous system. *J Neuroimmunol* **94**, 1–14.
35 Lijnen HR (2001) Plasmin and matrix metalloproteinases in vascular remodeling. *Thromb Haemost* **86**, 324–333.
36 Scannevin RH, Alexander R, Haarlander TM, Burke SL, Singer M, Huo C, Zhang YM, Maguire D, Spurlino J, Deckman I *et al.* (2017) Discovery of a highly selective chemical inhibitor of matrix metalloproteinase-9 (MMP-9) that allosterically inhibits zymogen activation. *J Biol Chem* **292**, 17963–17974.
37 Elkins PA, Ho YS, Smith WW, Janson CA, D'Alessio KJ, McQueney MS, Cummings MD & Romanic AM (2002) Structure of the C-terminally truncated human ProMMP9, a gelatin-binding matrix metalloproteinase. *Acta Crystallogr D Biol Crystallogr* **58**, 1182–1192.
38 Kumar S & Cieplak P (2018) Role of N-glycosylation in activation of proMMP-9. A molecular dynamics simulations study. *PLoS ONE* **13**, e0191157.
39 Rudd PM, Mattu TS, Masure S, Bratt T, Van den Steen PE, Wormald MR, Kuster B, Harvey DJ, Borregaard N, Van Damme J *et al.* (1999) Glycosylation of natural human neutrophil gelatinase B and neutrophil gelatinase B-associated lipocalin. *Biochemistry* **38**, 13937–13950.
40 Fry SA, Van den Steen PE, Royle L, Wormald MR, Leathem AJ, Opdenakker G, McDonnell JM, Dwek RA & Rudd PM (2006) Cancer-associated glycoforms of gelatinase B exhibit a decreased level of binding to galectin-3. *Biochemistry* **45**, 15249–15258.
41 Boon L, Ugarte-Berzal E, Vandooren J & Opdenakker G (2016) Glycosylation of matrix metalloproteases and tissue inhibitors: present state, challenges and opportunities. *Biochem J* **473**, 1471–1482.
42 Duellman T, Burnett J & Yang J (2015) Functional roles of N-linked glycosylation of human matrix metalloproteinase 9. *Traffic* **16**, 1108–1126.
43 Rudd PM, Joao HC, Coghill E, Fiten P, Saunders MR, Opdenakker G & Dwek RA (1994) Glycoforms modify

the dynamic stability and functional activity of an enzyme. *Biochemistry* **33**, 17–22.
44 Rudd PM, Morgan BP, Wormald MR, Harvey DJ, van den Berg CW, Davis SJ, Ferguson MA & Dwek RA (1997) Roles for glycosylation in the anti-inflammatory molecule CD59. *Biochem Soc Trans* **25**, 1177–1184.
45 Nelissen I, Martens E, Van den Steen PE, Proost P, Ronsse I & Opdenakker G (2003) Gelatinase B/matrix metalloproteinase-9 cleaves interferon-beta and is a target for immunotherapy. *Brain* **126**, 1371–1381.
46 Barondes SH, Castronovo V, Cooper DN, Cummings RD, Drickamer K, Feizi T, Gitt MA, Hirabayashi J, Hughes C, Kasai K *et al.* (1994) Galectins: a family of animal beta-galactoside-binding lectins. *Cell* **76**, 597–598.
47 Ahmad N, Gabius HJ, Andre S, Kaltner H, Sabesan S, Roy R, Liu B, Macaluso F & Brewer CF (2004) Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes. *J Biol Chem* **279**, 10841–10847.
48 Jaeken J, Vanderschueren-Lodeweyckx M, Casaer P, Snoeck L, Corbeel L, Eggermont E & Eeckels R (1980) Familial psychomotor retardation with markedly fluctuating serum proteins, FSH and GH levels, partial TBG-deficiency, increased serum arylsulphatase A and increased CSF protein: a new syndrome? *Pediatr Res* **14**, 179.
49 Jaeken J & Peanne R (2017) What is new in CDG? *J Inherit Metab Dis* **40**, 569–586.
50 Rudd PM & Dwek RA (1997) Glycosylation: heterogeneity and the 3D structure of proteins. *Crit Rev Biochem Mol Biol* **32**, 1–100.
51 Mattu TS, Royle L, Langridge J, Wormald MR, Van den Steen PE, Van Damme J, Opdenakker G, Harvey DJ, Dwek RA & Rudd PM (2000) O-glycan analysis of natural human neutrophil gelatinase B using a combination of normal phase-HPLC and online tandem mass spectrometry: implications for the domain organization of the enzyme. *Biochemistry* **39**, 15695–15704.
52 Rosenblum G, Van den Steen PE, Cohen SR, Bitler A, Brand DD, Opdenakker G & Sagi I (2010) Direct visualization of protease action on collagen triple helical structure. *PLoS ONE* **5**, e11043.
53 Van den Steen PE, Opdenakker G, Wormald MR, Dwek RA & Rudd PM (2001) Matrix remodelling enzymes, the protease cascade and glycosylation. *Biochim Biophys Acta* **1528**, 61–73.
54 Kurisaka C, Oku T, Itoh S & Tsuji T (2018) A role of sialic acid-containing glycans of matrix metalloproteinase-9 (MMP-9) in the interaction between MMP-9 and staphylococcal superantigen-like protein 5 (SSL5). *Microbiol Immunol* **62**, 168–175.
55 Duellman T, Warren C & Yang J (2014) Single nucleotide polymorphism-specific regulation of matrix metalloproteinase-9 by multiple miRNAs targeting the coding exon. *Nucleic Acids Res* **42**, 5518–5531.
56 Nishi N, Shoji H, Seki M, Itoh A, Miyanaka H, Yuube K, Hirashima M & Nakamura T (2003) Galectin-8 modulates neutrophil function via interaction with integrin alphaM. *Glycobiology* **13**, 755–763.
57 Ochieng J, Fridman R, Nangia-Makker P, Kleiner DE, Liotta LA, Stetler-Stevenson WG & Raz A (1994) Galectin-3 is a novel substrate for human matrix metalloproteinases-2 and -9. *Biochemistry* **33**, 14109–14114.
58 Becker-Pauly C, Kruse MN, Slotty KA, Kohler D, Harris JR, Rosmann S, Sterchi EE & Stöcker W (2003) Differences in the activation mechanism between the alpha and beta subunits of human meprin. *Biol Chem* **384**, 825–831.
59 Becker-Pauly C, Howel M, Walker T, Vlad A, Aufenvenne K, Oji V, Lottaz D, Sterchi EE, Debela M, Magdolen V *et al.* (2007) The alpha and beta subunits of the metalloprotease meprin are expressed in separate layers of human epidermis, revealing different functions in keratinocyte proliferation and differentiation. *J Invest Dermatol* **127**, 1115–1125.
60 Vandooren J, Geurts N, Martens E, Van den Steen PE, Jonghe SD, Herdewijn P & Opdenakker G (2011) Gelatin degradation assay reveals MMP-9 inhibitors and function of O-glycosylated domain. *World J Biol Chem* **2**, 14–24.
61 Cha H, Kopetzki E, Huber R, Lanzendorfer M & Brandstetter H (2002) Structural basis of the adaptive molecular recognition by MMP9. *J Mol Biol* **320**, 1065–1079.