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10 mM glucosamine prevents activation of proADAMTS5 (aggrecanase-2) in transfected cells by interference with post-translational modification of furin

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Summary

Objective: Glucosamine has been previously shown to suppress cartilage aggrecan catabolism in explant cultures. We determined the effect of glucosamine on ADAMTS5 (a disintegrin-like and metalloprotease domain (reprolysin type) with thrombospondin type-1 motifs 5), a major aggrecanase in osteoarthritis, and investigated a potential mechanism underlying the observed effects.

Design: HEK293F and CHO-K1 cells transiently transfected with ADAMTS5 cDNA were treated with glucosamine or the related hexosamine mannosamine. Glucosamine effects on *FURIN* transcription were determined by quantitative RT-PCR. Effects on furin-mediated processing of ADAMTS5 zymogen, and aggrecan processing by glucosamine-treated cells, were determined by western blotting. Post-translational modification of furin and N-glycan deficient furin mutants generated by site-directed mutagenesis was analyzed by western blotting, and the mutants were evaluated for their ADAMTS5 processing ability in furin-deficient CHO-RPE.40 cells.

Results: Ten mM glucosamine and 5–10 mM mannosamine reduced excision of the ADAMTS5 propeptide, indicating interference with the propeptide excision mechanism, although mannosamine compromised cell viability at these doses. Although glucosamine had no effect on furin mRNA levels, western blot of furin from glucosamine-treated cells suggested altered post-translational modification. Glucosamine treatment led to decreased glycosylation of cellular furin, with reduced furin autoactivation as the consequence. Recombinant furin treated with peptide N-glycanase F had reduced activity against a synthetic peptide substrate. Indeed, site-directed mutagenesis of two furin N-glycosylation sites, Asn^{387} and Asn^{440}, abrogated furin activation and this mutant was unable to rescue ADAMTS5 processing in furin-deficient cells.

Conclusions: Ten mM glucosamine reduces excision of the ADAMTS5 propeptide *via* interference with post-translational modification of furin and leads to reduced aggrecanase activity of ADAMTS5.

Key words: Aggrecanase, Glucosamine, ADAMTS, Furin, Catabolism.

Introduction

Osteoarthritis (OA) is a common disorder characterized by loss of articular cartilage as a result of degenerative changes in the joint. An early phenomenon in cartilage degradation is proteolytic loss of aggrecan, which forms giant aggregates with hyaluronan in the extracellular matrix (ECM)[1]. These aggregates bind water, and are constrained by a collagen network, which gives cartilage its compressibility. Aggrecan proteolysis diminishes cartilage compressibility and exposes collagen and other cartilage ECM proteins to degradation, thereby setting in motion a vicious cycle of cartilage breakdown[2]. Two secreted metalloproteases, ADAMTS4 and ADAMTS5 (a disintegrin-like and metalloprotease domain (reprolysin type) with thrombospondin type-1 motifs), referred to as aggrecanases, cleave aggrecan efficiently and are implicated as key mediators of OA[3,4]. Therefore, inhibition of these proteases, such as by endogenous inhibitors, medicinal compounds, or biosynthetic interference, is of significant therapeutic interest[5]. ADAMTS5, also known as aggrecanase-2, has attracted considerable interest as a target in OA[5,6] since mice with a targeted deletion of *Adamts5* are resistant to both immune and mechanically induced arthritis[7,8].

ADAMTS5 is synthesized as a zymogen (proADAMTS5) which undergoes proteolytic excision of its propeptide by proprotein convertases (PCs) such as furin and PACE4[9,10]. ADAMTS4 and ADAMTS5 each require propeptide excision for proteolytic activity, but they are activated somewhat differently. ADAMTS4 is processed intracellularly[11], whereas ADAMTS5 is processed extracellularly by furin and/or other PCs. ADAMTS4 and ADAMTS5 zymogens may also be deposited in cartilage ECM, where they are activated by secreted PCs such as PACE4[10]. Both furin and PACE4, which activate ADAMTS5 efficiently, cleave the consensus cleavage site, $RRRR^{261}\downarrow$, which is present at the junction of the ADAMTS5 propeptide and catalytic domain[9,10,12].

The hexosamines glucosamine and mannosamine were previously shown to suppress aggrecan catabolism in cartilage explant cultures[13–19]. Both hexosamines can interfere with N-glycosylation[20,21], and mannosamine is a recognized inhibitor of glycosylphospatidyl-inositol (GPI) anchor formation[14–19]. The effects of hexosamines on ADAMTS4

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biosynthesis, but not on ADAMTS5, were previously investigated at the molecular level. C-terminal processing of furin-activated ADAMTS4 by a GPI-anchored metalloprotease located at the cell surface was inhibited by mannosamine[15,22]. Treatment of cells with hexosamines also led to a prevalence of unprocessed ADAMTS4 zymogen[15]. ADAMTS4 lacks N-linked glycans[4], so the observed effects could not be attributed to inhibition of its N-glycosylation by hexosamines. These published observations led us to examine the effects of glucosamine on ADAMTS5 activity, and to elucidate the molecular mechanisms of the observed effects. Unlike ADAMTS4, ADAMTS5 is N-glycosylated and does not bind to the cell surface, but has been shown to reside in the ECM[23,24]. In cultured cells, which make little ECM, such as HEK293F cells, ADAMTS5 is present in the conditioned medium (CM)[9]. Here, we show that hexosamine treatment of cells interferes with the activation of ADAMTS5 through an indirect mechanism involving the loss of furin activity. These findings provide one possible explanation for previously observed suppressive effects of glucosamine on cartilage catabolism and are thus relevant to OA biochemistry.

Materials and methods

CELL CULTURE AND TREATMENTS

HEK293F cells and CHO-K1 cells (ATCC, Manassus, VA) were cultured in Dulbecco's Modified Eagles Medium (DMEM, high glucose) supplemented with 10% FBS and antibiotics. Furin-deficient CHO-RPE.40 cells[25] were cultured in Ham's F12 medium (high glucose) supplemented with 10% FBS and antibiotics.

EXPRESSION PLASMIDS AND SITE-DIRECTED MUTAGENESIS

Constructs for expression of full-length human ADAMTS5 and the propeptide and catalytic domain of human ADAMTS5 (ADAMTS5 Pro-Cat) were previously described[9] (Fig. 1). Asn residues within two of three consensus N-linked glycosylation sites in furin (GenBank accession no. NP_002560) were mutated singly (Asn^{387}, Asn^{440}), or in combination ($Asn^{387+440}$), by replacement with Gln using site-directed mutagenesis (Quikchange site-directed mutagenesis kit, Stratagene, La Jolla, CA). The mutations were introduced into full-length untagged human furin and a furin construct with an N-terminal FLAG tag introduced immediately downstream of the furin autoactivation site[26].

Expression plasmids were transiently transfected into HEK293F, CHO-K1 and CHO-RPE.40 cells using FUGENE6 (Roche Diagnostics, Indianapolis, IN). HEK293F and CHO-K1 cells were treated with glucosamine−HCl (neutralized to pH 7.4 before use) or mannosamine (Sigma−Aldrich, St. Louis, MO). Before treatment, cells were transferred to serum-free DMEM containing 5 mM glucose for 3−5 h[13]. Fresh medium containing each compound in a range of concentrations was added to cells. CM was collected, and cells were harvested in phosphate buffered saline after 24 h or 48 h followed by lysis in 1% Triton X-100, 10 mM Tris.HCl, pH 7.6 containing complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). In some experiments, HEK293F and CHO-RPE.40 cells were transfected (or co-transfected) with furin (either wildtype or Asn → Gln mutants) and/or ADAMTS5 Pro-Cat plasmid. As a control, empty vector used for furin cloning (pCI-Neo, Promega, Madison, WI) was co-transfected with ADAMTS5 Pro-Cat in CHO-RPE.40 cells. Serum-free media and cell lysates were collected after 48 h and processed as described above.

QUANTITATIVE RT-PCR

HEK293F cells were treated with glucosamine and harvested after 48 h as described above. Total RNA was extracted using QIAGEN complete RNA isolation kit (QIAGEN, Valencia, CA). RNA concentration and purity were determined with a NanoDrop (ND-100) spectrophotometer (Nanodrop Technologies, Wilmington, DE). All RNA samples used had an A260/280 ratio of >1.8. Two μg of total RNA per condition was reverse transcribed to cDNA in a total volume of 20 μl using the Superscript III kit (Invitrogen, Carlsbad, CA). For quantitative PCR, the *FURIN* primers 5′-CAGCGGTGGCCAACAGTGTG-3′ (forward) 5′-GCGGGCGGTGAGGCGACA-3′ (reverse) and 18s ribosomal RNA primers, 5′-GGGAGGTAGTGACGAAAAATAACAAT-3′ (forward), 5′-TTGCCCTCCAATGGATCCT-3′ (reverse) were used. SYBR-Green (Applied Biosystems, Foster City, CA) incorporation was detected using an iCycler (Bio-Rad. Hercules, CA) and relative levels of *FURIN* mRNA were quantitated using the ΔΔct method as per manufacturer's protocol.

WESTERN BLOTTING, ENZYMATIC DEGLYCOSYLATION OF FURIN, AND DETERMINATION OF AGGRECANASE ACTIVITY

For western blots, CM and cell lysate were electrophoresed by reducing SDS-PAGE in 6% or 7.5% gels prior to electroblotting to polyvinylidene fluoride membrane (Millipore, Billerica, MA). Peptide N-glycosidase F (PNGaseF, New England Biolabs, Ipswich, MA) treatment was carried out as per manufacturer's instructions following reduction of the sample. ADAMTS5 was detected using rabbit polyclonal antibody RP2 (recognizing the propeptide) (Triple Point Biologics, Forest Grove, OR)[9] or anti-myc mouse monoclonal antibody 9E10 (Sigma−Aldrich, St. Louis, MO). Anti-GAPDH mouse monoclonal antibody (Chemicon, Temecula, CA) was used to detect intracellular and extracellular GAPDH to ensure equivalent protein loading. Anti-furin mouse monoclonal

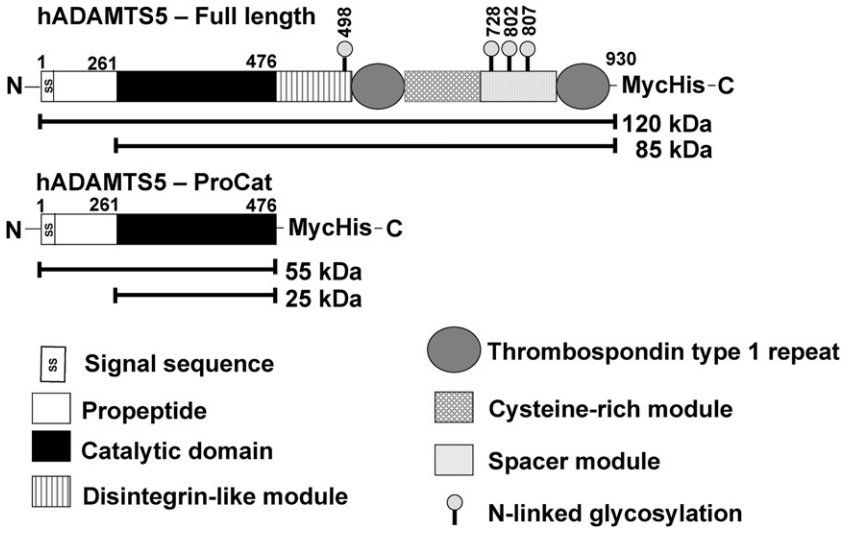


Fig. 1. ADAMTS5 constructs used in the analysis of glucosamine effects. Domain organization of ADAMTS5 and ADAMTS5 Pro-Cat, predicted molecular weights of the relevant protein species, N-linked glycosylation site and furin processing site are shown. The key to the various modules is at the bottom of the figure.

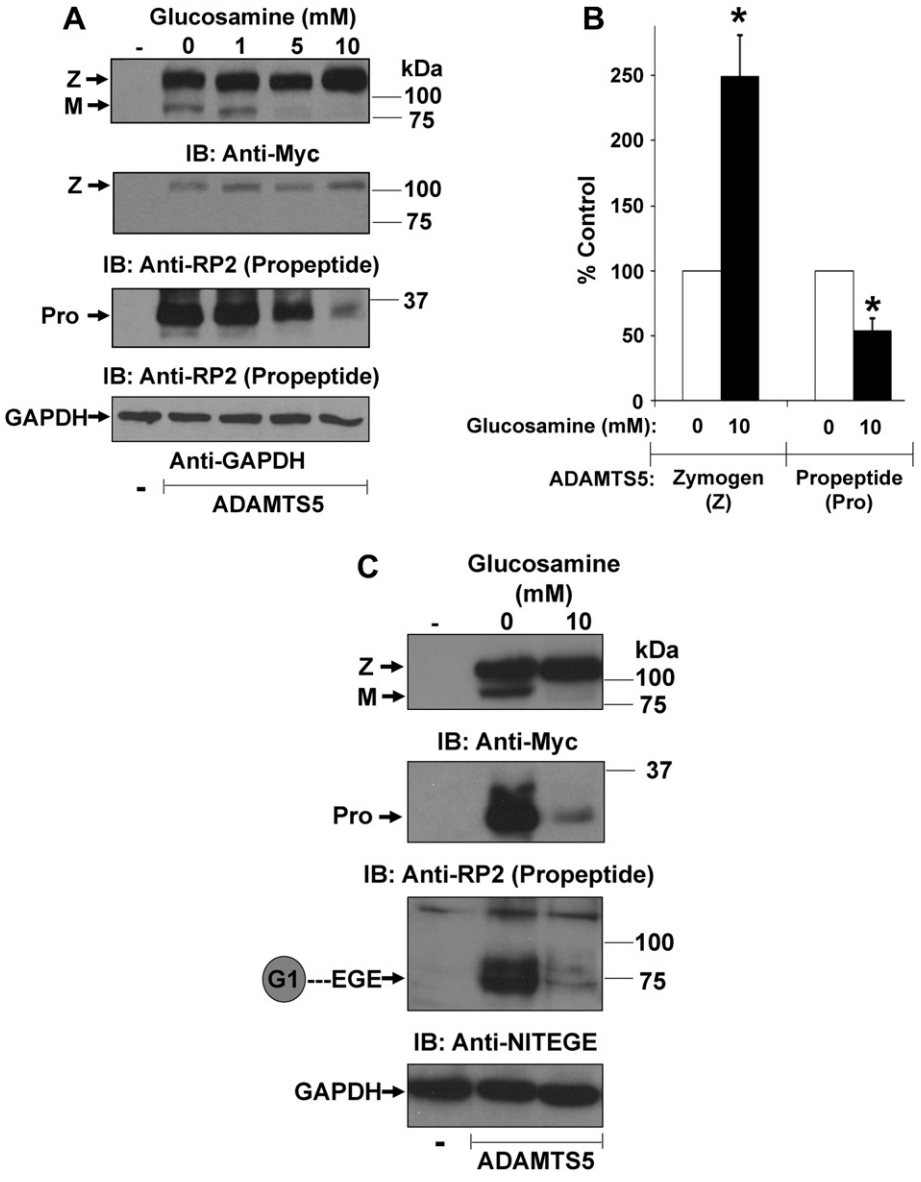


Fig. 2. ADAMTS5 activation is inhibited by glucosamine treatment. A. Treatment of HEK293F cells transiently transfected with ADAMTS5 using 5 mM or 10 mM glucosamine results in increased uncleaved zymogen (top two panels) and decreased processed propeptide (Pro, second panel from the bottom). Western blotting with anti-GAPDH (bottom panel) was used to indicate sample loading. B. Quantitation of the effects of 10 mM glucosamine on the levels of ADAMTS5 zymogen and cleaved propeptide from three independent experiments shows a statistically significant effect of glucosamine on zymogen cleavage (*$P < 0.05$; Student's *t* test, error bars represent S.E.M.). C. Treatment with 10 mM glucosamine results in loss of aggrecanase activity in the medium of ADAMTS5-transfected HEK293F cells. The top two panels illustrate reduced zymogen processing, whereas the anti-NITEGE^{373} immunoblot (second panel from the bottom) shows reduced aggrecanase activity in medium of glucosamine-treated cells.

antibody MON-152 recognizing an epitope in the cysteine-rich domain (Alexis Biochemicals, San Diego, CA) was used for detection of endogenous and untagged furin, whereas mouse anti-FLAG M1 or M2 monoclonal antibody and rabbit anti-FLAG polyclonal antibody were used for detection of FLAG-tagged furin (FLAG antibodies from Sigma–Aldrich, St. Louis, MO). Anti-FLAG M2 antibody recognizes the FLAG tag at any position within the protein, whereas anti-FLAG M1 preferentially recognizes a FLAG tag at the free N-terminus. Antibody binding was detected by enhanced chemiluminescence (ECL, GE Healthcare, Piscataway, NJ). For detection of endogenous furin, ECL-Plus (GE Healthcare, Piscataway, NJ) was utilized. Intensity of relevant bands was quantitated using IMAGE J software (NIH, Bethesda, MD). A type 1 (paired), 2-tailed Student's *t* test was used to determine whether the normalized data obtained from the IMAGE J software was statistically significant ($P \leq 0.05$) between the control and test group, Gaussian distribution was assumed.

ADAMTS5 cleavage of aggrecan at the Glu^{373}–Ala^{374} peptide bond was determined using a modification of a previously described experimental procedure[9]. HEK293F cells were transfected with full-length ADAMTS5 followed by the addition of serum-free medium with or without 10 mM glucosamine. CM was collected after 48 h. Fifty μl of CM from control (untransfected) or from each respective ADAMTS5 transfection was incubated with 4 μg bovine aggrecan (Sigma–Aldrich) for 16 h at 37°C in the presence of an EDTA-free protease inhibitor cocktail (Roche). Each reaction was heat-inactivated (5 min, 100°C), cooled on ice, and aggrecan was deglycosylated with protease-free chondroitinase ABC, keratanase I and keratanase II (Seikagaku, Tokyo, Japan) at 37°C for 2 h. Western blotting was performed using the anti-NITEGE aggrecan neo-epitope antibody that specifically detects cleavage at the Glu^{373}–Ala^{374} peptide bond (kindly provided by Dr. John Sandy, Rush University Medical Center). ADAMTS5 zymogen, mature ADAMTS5 and ADAMTS5 propeptide in the CM were detected by western blot. Anti-GAPDH was used as a control to demonstrate comparable cell numbers.

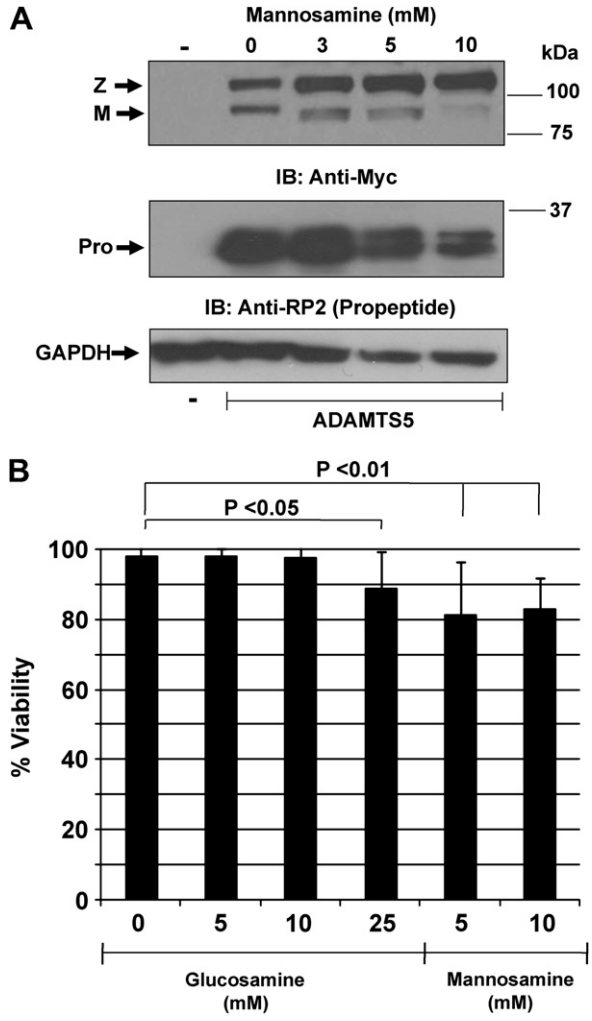


Fig. 3. Effect of mannosamine on ADAMTS5 propeptide processing, and cell viability after glucosamine and mannosamine treatment with various doses. A. Treatment of ADAMTS5-transfected HEK293F cells with 5 mM, and 10 mM mannosamine resulted in a dose-dependent inhibition of ADAMTS5 zymogen processing. GAPDH western blot suggests decreased cell numbers with 5–10 mM mannosamine (bottom panel). B. Trypan blue exclusion assay shows that 5–10 mM mannosamine and 25 mM glucosamine, but not 10 mM glucosamine had a significant decrease in cell viability (the data are presented as the mean ± S.D. of 10 cell counts from duplicate and control hexosamine-treated cultures).

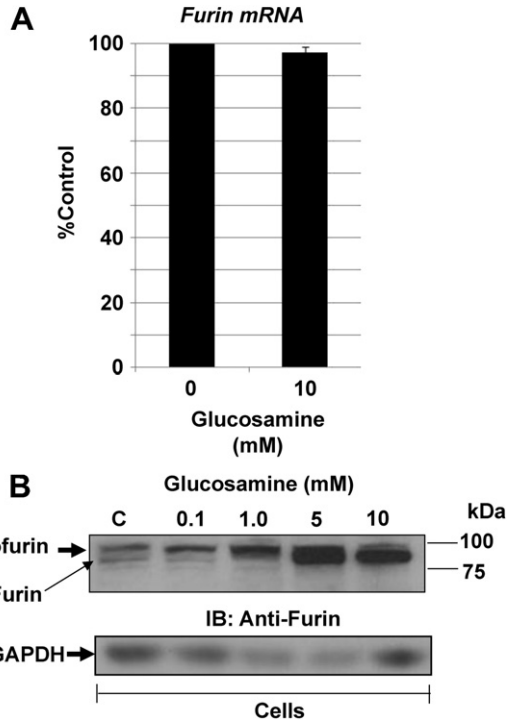


Fig. 4. Glucosamine affects post-translational modification of furin. A. Furin mRNA levels in HEK293F cells treated with 10 mM glucosamine were similar to those of untreated cells. 18s ribosomal RNA levels were used for normalization of furin mRNA. The data are presented as the mean ± S.D. from three independent experiments. B. Treatment with 5 mM and 10 mM glucosamine alters electrophoretic migration of furin in HEK293F cells. The major furin species seen with 5 mM and 10 mM glucosamine treatments corresponds in size to active furin.

FLUOROGENIC SUBSTRATE ASSAY OF FURIN ACTIVITY

Recombinant human furin (rhFurin, purity >95%) was purchased from R&D systems (Minneapolis, MN). Fifty or 100 ng of rhFurin per reaction was treated with PNGaseF (New England Biolabs) or with heat-inactivated PNGaseF (100°C, 5 min) as a control in HEPES buffer (100 mM HEPES, 0.5% Triton X-100, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, pH 7.5) for 2 h at 37°C. Substrate cleavage was measured by the liberation of amino-4-methylcoumarin (AMC) from Boc-RVRR-AMC (final concentration 100 μM, Biomol, Plymouth Meeting, PA) in 100 μl HEPES buffer, pH 7.5. Fluorescence was measured every minute (total 90 min) on a Victor 2 1420 Multilabel Counter fluorescent plate reader (Perkin Elmer, Waltham, MA) with excitation/emission wavelength of 355/460 nm respectively. Each assay was run in triplicate in two separate assays in 96-well plates. The extinction co-efficient (ε) of AMC was determined to be 17.3 $\mu m^{-1} cm^{-1}$ using an evenly spaced standard curve (range 40–400 μm). Specific activity was determined for each individual reaction using the following formula: μm/min = ([Rate (OD/min)/ε] × 1^{e-4}]). Statistical analysis was performed using a Student *t* test on the raw data generated for control (glycosylated) and test (deglycosylated) furin activity. The data are presented as the mean specific activity of three individual rates per assay ± standard deviation.

Results

GLUCOSAMINE TREATMENT INTERFERES WITH ADAMTS5 ZYMOGEN PROCESSING AND AGGRECANASE ACTIVITY

To investigate a possible effect of glucosamine on ADAMTS5, myc-tagged full-length ADAMTS5 (Fig. 1) was transiently transfected in HEK293F cells and the molecular species present in the CM were evaluated by western blotting with anti-myc-antibody and anti-propeptide antibody. The medium of untreated cells showed the ADAMTS5 zymogen (120 kDa) and mature ADAMTS5 (85 kDa) using anti-myc, whereas the zymogen and cleaved 25 kDa propeptide were recognized by anti-RP2 [Fig. 2(A)]. The relatively weak reactivity of unprocessed ADAMTS5 with anti-RP2, compared to anti-myc, likely reflects the stronger affinity of the myc-antibody. The relatively low amount of the mature ADAMTS5 in medium possibly reflects the previously reported observation that heparin specifically enhances the detection of mature ADAMTS5 in medium of these cells. Thus mature ADAMTS5 may be sequestered in the ECM by ionic interactions. Mature ADAMTS5 detected by anti-myc was decreased in the presence of 5 mM and 10 mM glucosamine with a corresponding increase in the intact zymogen [Fig. 2(A), top panel]. Reduction of ADAMTS5 activation, as represented by a decrease in proteolytically cleaved propeptide [detected by RP2,

Fig. 2(A), second panel from bottom] in CM, was observed upon treatment with 5 and 10 mM glucosamine in both HEK293F cells [Fig. 2(A, B)] and CHO-K1 cells (data not shown).

Medium from ADAMTS5-expressing cells treated with 10 mM glucosamine contained significantly less proteolytic activity when cleavage of the Glu^{373}–Ala^{374} bond of aggrecan core protein was compared to untreated cells, consistent with reduced proADAMTS5 activation [Fig. 2(C)]. This suggested that aggrecan proteolysis by ADAMTS5 was profoundly affected by 10 mM glucosamine due to impaired ADAMTS5 activation. Similarly, we found that constructs of ADAMTS9 and ADAMTS20 expressed in HEK293F cells also had reduced propeptide processing in the presence of 10 mM glucosamine (data not shown). We concluded that reduction of ADAMTS5-dependent aggrecan processing was probably not a direct effect of glucosamine on ADAMTS5, but an indirect effect on the processing mechanism.

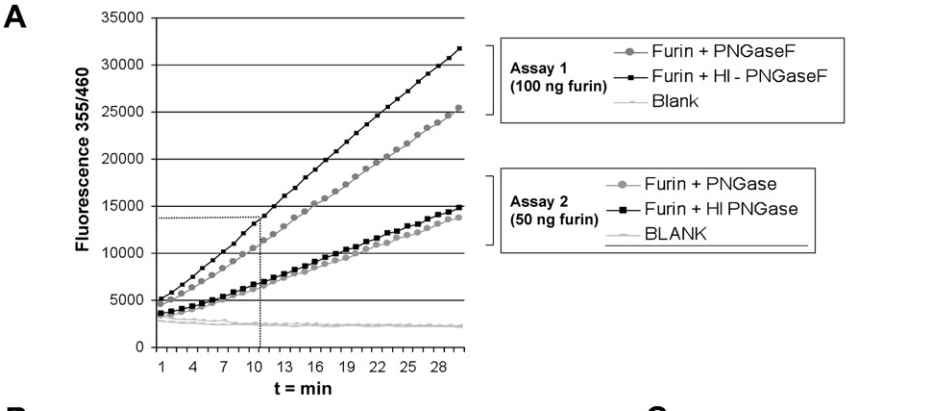
Treatment of transfected HEK293F cells with 5 mM or 10 mM mannosamine also resulted in decreased removal of the ADAMTS5 propeptide [Fig. 3(A)]. Because high levels of hexosamines may have deleterious effects on cells, we analyzed cell viability at the effective glucosamine and mannosamine concentrations tested, as well as 25 mM glucosamine. There was decreased cell viability in the presence of 25 mM glucosamine, as well as 5 mM and 10 mM mannosamine [Fig. 3(B)]. Therefore, we primarily investigated the effects of 10 mM glucosamine, since this did not affect cell viability.

GLUCOSAMINE AFFECTS ADAMTS5 ACTIVATION BY INTERFERENCE WITH FURIN

Previous work has established the role of furin in ADAMTS zymogen processing, including ADAMTS5[9,24,27,28]. Glucosamine is reported to affect transcription of many genes[29,30], prompting us to examine whether it affected furin transcription. Quantitative RT-PCR demonstrated no significant change in furin mRNA in 10 mM glucosamine-treated HEK293F cells [Fig. 4(A)]. Next, we investigated the effect of glucosamine on endogenous cellular furin by western blot analysis. Furin is detected as a doublet of the zymogen (90 kDa) and activated enzyme (85 kDa), in untreated HEK293F cells [Fig. 4(B)]. In the presence of 5 mM and 10 mM glucosamine, there was an increased intensity of a furin species apparently co-migrating with active furin in untreated cells [Fig. 4(B)]. However, since the activity of furin was clearly reduced in the presence of 10 mM glucosamine (Fig. 2), we concluded that this altered electrophoretic migration likely represented reduced furin glycosylation rather than increased profurin conversion to active furin, consistent with a role for glucosamine in inhibition of N-glycosylation[20]. The reduced furin activity in the presence of glucosamine suggested that 85 kDa furin species not only lacked glycosylation, but also failed to be processed in the presence of 10 mM glucosamine. The enhanced furin immunoreactivity seen [Fig. 4(B)] could also result from blockade of furin shedding from the cell surface but could not be resolved by analysis of the CM of cells owing to the relatively low levels present in medium. These data strongly suggested that glucosamine impaired furin function *via* an effect on post-translational modification of furin, such as altered glycosylation, decreased conversion to furin, decreased shedding or a combination of these.

REDUCED N-GLYCOSYLATION OF FURIN INHIBITS ITS ACTIVITY

To resolve these various possibilities, we first examined whether mature furin was dependent on N-glycosylation for its activity. Recombinant, purified, N-terminally His-tagged human furin was treated with PNGaseF to eliminate



B

Comparison of activity of furin and deglycosylated furin

Furin	Glycosylated	Deglycosylated	% Reduction	ng Furin
Assay 1	55.8+/- 6.96	42.23 +/- 1.7	29**	100
Assay 2	48.58+/- 5.72	43.25 +/- 0.95	12*	50

(nmol/min/ng Furin)

**P<0.01
*P<0.05

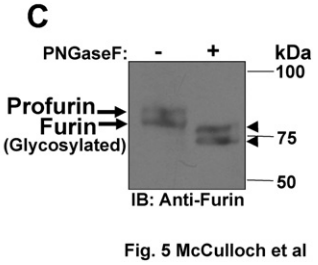


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Fig. 5. Deglycosylation of purified recombinant furin reduces its activity. A. Kinetic data for two independent assays are plotted as a function of fluorescence (AMC liberation) vs time at 25°C. Each curve represents the mean kinetic data from triplicate reactions for each deglycosylated furin preparation (HI = heat-inactivated). B. Activity of glycosylated vs deglycosylated furin towards the fluorogenic substrate Boc-RVRR-AMC was measured at 10 min. The liberation of AMC by furin was significantly decreased by the removal of N-linked sugars with PNGaseF. C. Western blot of recombinant furin following treatment with PNGaseF, showing complete deglycosylation.

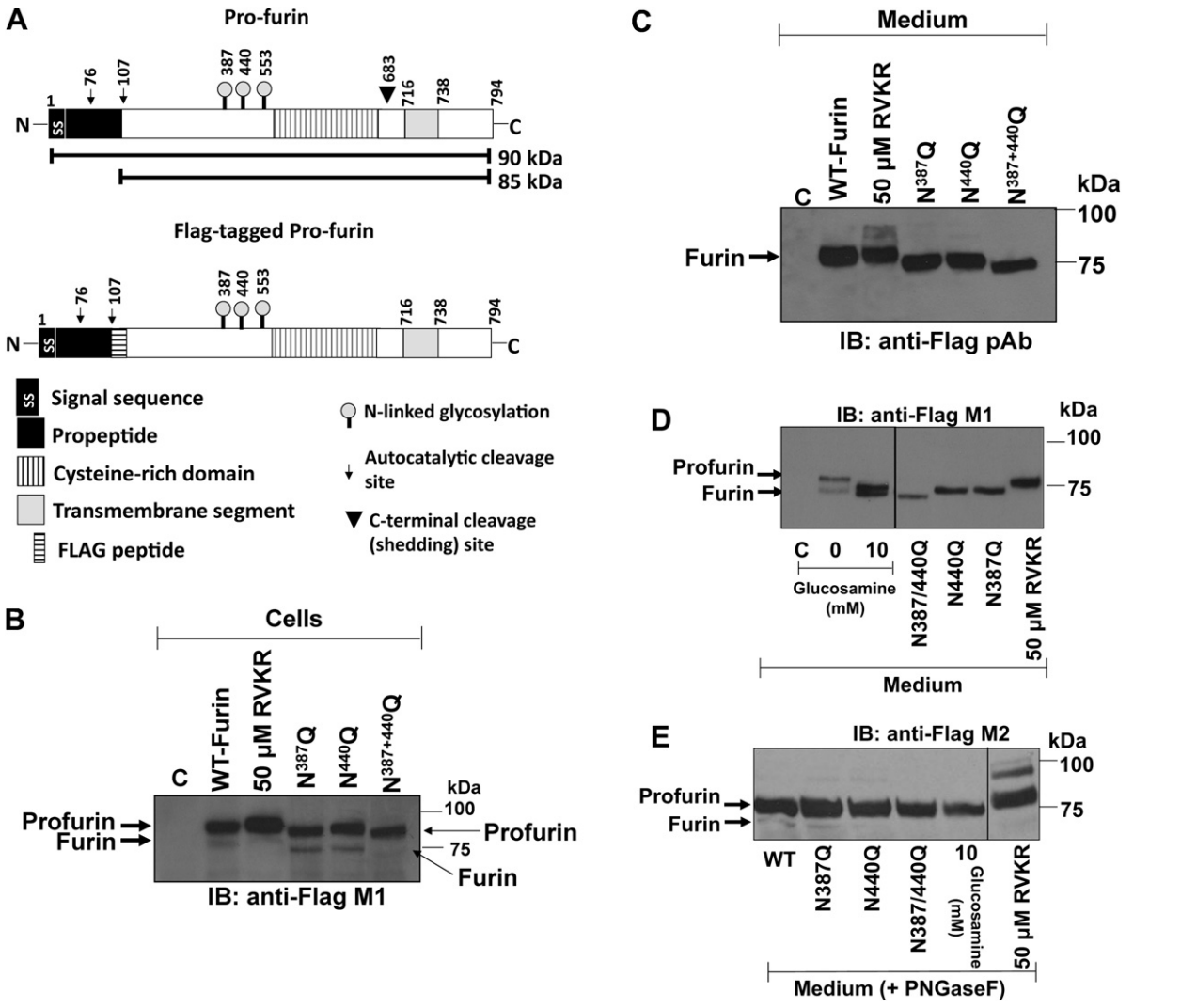

Fig. 6. Mutation of the N-glycosylation sites of furin inhibits its autoactivation. A. Schematic of human furin constructs used in the study depicting their domain structure (key to various domains is at the bottom), predicted molecular weights, autocatalytic and C-terminal cleavage sites and N-linked glycosylation sites. B. Detection of transiently expressed N-terminal FLAG-tagged WT-furin with or without dec-RVKR-CMK (dec-RVKR-chloromethylketone) as well as FLAG-tagged $Asn^{387}Gln$, $Asn^{440}Gln$, $Asn^{387+440}Gln$ furin mutants in HEK293F cells using anti-FLAG M1 antibody. Note enhanced in-gel migration of the mutants, complete loss of activation of $Asn^{387+440}Gln$ furin, and reduced activation of $Asn^{440}Gln$ furin. $Asn^{387+440}Gln$ furin migrates as a species intermediate in size between WT profurin and furin. C. Shed furin was detected in the CM of the cells shown in B using a polyclonal anti-FLAG antibody, where similar relative migration of the various constructs is seen as in cells, albeit with reduction of molecular mass owing to C-terminal processing. The active form of furin is not detected using this antibody. D. Electrophoretic mobility of shed furin from transfected cells treated with or without 10 mM glucosamine was compared alongside the single and double N-glycosylation mutants, suggestive of impaired N-glycosylation of WT furin in the presence of glucosamine. E. Western blot analysis of the samples shown in panel D was done following enzymatic deglycosylation with PNGaseF. Note that the 10 mM glucosamine-treated furin and glycosylation mutant furin co-migrated with deglycosylated WT profurin, as well as with profurin from RVKR treated cells expressing WT furin, indicating retention of the propeptide following 10 mM glucosamine treatment (compare with 6D). The identity of the 90 kDa band seen upon RVKR treatment is not known. In panels B, C, and D, C indicates untransfected control.

N-linked sugars. Furin activity towards the fluorogenic substrate Boc-RVRR-AMC was significantly impaired after treatment with PNGaseF in two independent assays [Fig. 5(A, B)]. Western blotting confirmed complete deglycosylation of the PNGaseF treated furin used in these assays [Fig. 5(C)]. However, since the activity of mature furin was only partially abolished by enzymatic deglycosylation, we investigated the effect of glucosamine on cellular furin.

To better resolve the effect of glucosamine on profurin conversion and shedding, we used plasmids for expression of wild-type (WT) profurin or a profurin construct containing a FLAG tag downstream of the autocatalytic processing site in HEK293F cells [Fig. 6(A)]. Indeed, studies examining the fate of furin have typically utilized such constructs since endogenous furin is poorly detected[31,32]. Since profurin conversion to the active form is an intrinsic, autocatalytic process[33], over-expression of these constructs by transient transfection is valid for extrapolation of any observed changes in activation status to endogenous furin. To better visualize the active form of furin, we used the anti-FLAG M1 antibody, which is reported to have increased reactivity to free N-terminal FLAG epitopes, since upon autoactivation, the tagged construct will have a free N-terminal FLAG tag [Fig. 6(A)]. N-terminal FLAG-tagged furin, or respective

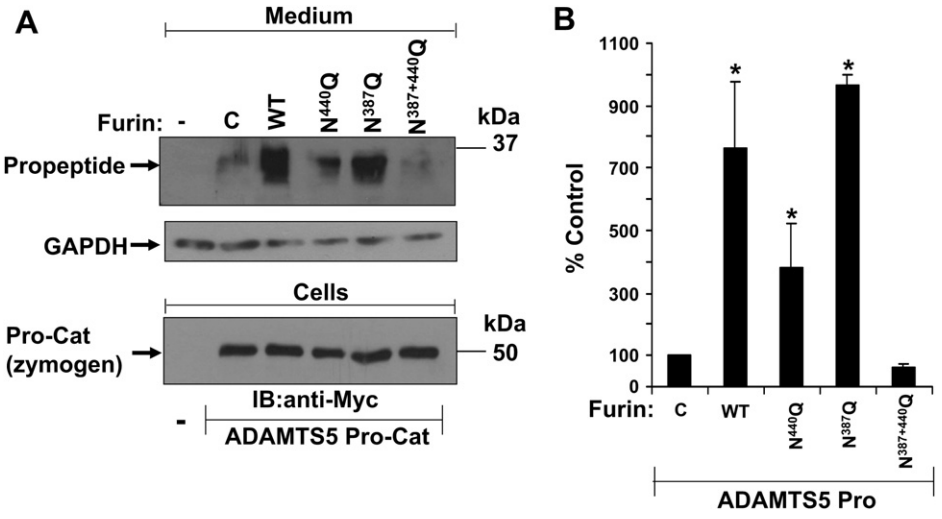

Fig. 7. Glycosylation-deficient furin mutants cannot process ADAMTS5 zymogen in CHO.RPE40 cells. A. WT furin and Asn^{387}Gln-furin increased ADAMTS5 propeptide processing above background, whereas Asn^{440}Gln furin and Asn$^{387+440}$Gln furin did not rescue processing (upper panel). GAPDH was used as a loading control (center panel). Intracellular levels of unprocessed ADAMTS5 Pro-Cat zymogen were similar in each transfection (lower panel). B. Quantitation of ADAMTS5 propeptide in the CM (normalized to GAPDH) from the respective transfections in A. These data were derived from three independent experiments and show statistically significant reduction of activity of Asn^{440}Gln furin and Asn$^{387+440}$Gln furin (*$P < 0.05$ Student's t test; error bars represent S.E.M).

mutants of the N-linked carbohydrate attachment sites (Asn^{387}Gln, Asn^{440}Gln and Asn$^{387+440}$Gln) were transfected into HEK293F cells. Cells transfected with FLAG-tagged furin were also treated with or without 50 μM dec-RVKR-CMK, a furin inhibitor, to inhibit autocatalytic activation. Anti-FLAG M1 antibody detected furin species at ~90 kDa (profurin) and ~85 kDa [zymogen and mature furin respectively, Fig. 6(B)] in untreated cells. Cells treated with dec-RVKR-CMK contained only furin zymogen [Fig. 6(B)]. Asn^{387}Gln and Asn^{440}Gln mutants showed an expected increase in electrophorectic mobility of both furin species, but the relative position of the zymogen with respect to the active form in each of these mutants was similar to the WT furin [Fig. 6(B)]. Active furin was undetectable in the Asn$^{387+440}$Gln mutant [Fig. 6(B)], suggesting that lack of glycosylation at these two sites impaired furin autoactivation. The migration of Asn$^{387+440}$Gln profurin at a size intermediate between WT profurin and active furin therefore appears to reflect the net effect of reduced glycosylation and impaired autoactivation, suggesting that the 85 kDa band noted in cells treated with 10 mM glucosamine [Fig. 4(B)] could be profurin deficient in N-glycosylation, and having reduced autoactivation. All transfected furin constructs were shed from cells, and their reduced molecular mass relative to that observed for cellular furin [Fig. 6(C)] was consistent with C-terminal processing of the membrane-anchored enzyme[26].

Since ablation of N-linkage sites and 10 mM glucosamine each led to enhanced electrophoretic mobility of furin, we considered whether glucosamine may hinder furin autoactivation by interference with its glycosylation. Accordingly, we compared the effect of glucosamine on FLAG-tagged furin directly alongside the N-glycosylation mutants. Anti-FLAG M1 antibody showed enhanced mobility of furin species originating from the FLAG-tagged furin in the medium of cells treated with 10 mM glucosamine, where two bands were detected, the lower band migrated similarly to the Asn$^{387+440}$Gln furin mutant [Fig. 6(D)]. The two molecular species seen in the medium of 10 mM glucosamine-treated cells were spaced more closely together than the two species present in medium of untreated cells [Fig. 6(D)]. This is suggestive of both species originating from the zymogen with impaired activation and impaired glycosylation. To resolve this, we enzymatically deglycosylated the CM obtained from cells transfected with WT furin, furin mutants, and 10 mM glucosamine-treated cells expressing WT furin. Following enzymatic deglycosylation, furin from the medium of 10 mM glucosamine-treated cells co-migrated with the deglycosylated WT profurin and mutant profurin [Fig. 6(E)]. This result establishes that treatment with 10 mM glucosamine severely compromises furin glycosylation. Since N-glycosylation is a co-translational event and precedes furin autoactivation in the *trans*-Golgi, this would argue that the observed lack of furin activation upon 10 mM glucosamine treatment could be secondary to impaired glycosylation of furin.

To determine whether elimination of one or more furin N-linked glycosylation sites did indeed reduce furin activity, CHO-RPE.40 cells that are deficient in furin activity were co-transfected with WT-furin (to eliminate potential functional interference by a FLAG-tag), Asn^{387}Gln-furin, Asn^{440}Gln-furin, or Asn$^{387+440}$Gln furin mutants, together with ADAMTS5 Pro-Cat. A low level of baseline processing of ADAMTS5 Pro-Cat was seen in CHO-RPE.40 cells (presumably mediated by other convertases), but was markedly increased by co-expression of WT furin [Fig. 7(A)]. In contrast, Asn^{440}Gln furin and Asn$^{387+440}$Gln furin, but not the Asn^{387}Gln-furin mutant, showed statistically significant loss of ADAMTS5 Pro-Cat processing [Fig. 7(A, B)]. The complete loss of activity of Asn$^{387+440}$Gln furin in regard to propeptide excision [Fig. 7(A, B)] was consistent with the observed lack of autocatalytic activation in this mutant [Fig. 6(B)].

Discussion

Glucosamine is widely utilized as a presumed anabolic agent, based on it being a precursor of cartilage glycosaminoglycans[34]. Although patients often report symptomatic benefit from glucosamine or a combination of glucosamine

and chondroitin-sulfate, prospective randomized clinical trials have not consistently supported a beneficial effect[35]. On the other hand, *in vitro* studies demonstrated suppression of the catabolic response of cartilage explants and chondrocytes to retinoic acid and interleukin-1 in the presence of glucosamine at similar concentrations used here[13,16,18,19]. Inhibition of aggrecan catabolism in cartilage explants in the presence of 5–10 mM glucosamine was reversible after removal of glucosamine[16]. Glucosamine lacks a direct inhibitory effect on soluble aggrecanase activity, and was only anti-catabolic in explant cultures undergoing a catabolic stimulus[16]. Taken together, the previously published work strongly suggested an effect of glucosamine on post-translational modification of aggrecanases. Indeed, molecular analysis of ADAMTS5 in this study demonstrates that 10 mM glucosamine impairs its activation by furin. Glucosamine is an inhibitor of N-linked glycosylation[20], yet no consistent effect was seen on electrophoretic mobility of ADAMTS5. We found that glucosamine led to altered furin migration on gels, as well as reduced activity upon elimination of N-linked carbohydrate in mature furin. Altered in-gel furin migration and concomitant loss of processing activity upon treatment of cells with 10 mM glucosamine leads us to propose that impairment of furin glycosylation compromises its conversion to active furin, which secondarily leads to failed activation of ADAMTS zymogen.

The mechanisms uncovered in this study may provide insights on several previously published observations regarding the effect of glucosamine and mannosamine on aggrecan catabolism in cartilage[13,16,18,19]. Suppression of aggrecan catabolism by glucosamine, which we confirmed here, could be explained by the suppression of activation of both major aggrecanases in cartilage explants by glucosamine, since removal of the ADAMTS4 and ADAMTS5 propeptides is essential for their catalytic activity[9,10]. The lack of N-glycosylation sites in ADAMTS4 argues that decreased ADAMTS4[15] or ADAMTS5 activation in the presence of glucosamine is not a consequence of altered glycosylation of these ADAMTS proteases, and indeed, there was no clear effect of glucosamine on migration of ADAMTS5, which is N-glycosylated. It was previously demonstrated that pretreatment of cartilage explants with glucosamine did not confer a protective effect on the subsequent exposure to IL-1, i.e., that the suppression of catabolic activity in the presence of glucosamine was lost after removal of glucosamine from the medium[16]. Since the intracellular pool of glucosamine is rapidly depleted following its removal from the medium of cultured cells[21] and because furin is constitutively and continuously synthesized and recycled by cells[31], *de novo* furin production after removal of glucosamine would allow ADAMTS processing to occur. In addition, furin and/or other PCs secreted or shed from cells after removal of glucosamine could activate ADAMTS zymogens that were secreted unprocessed in the presence of glucosamine and deposited in the ECM[10].

The present studies contribute to understanding the previously documented protective effect of glucosamine on cartilage catabolism *in vitro* in conjunction with other reported effects of glucosamine such as increased transcription of aggrecan, collagen II, and the aggrecanase inhibitor TIMP3[29,36]. Indeed, Chan *et al.*[37], described suppression of *ADAMTS5* mRNA in bovine cartilage explants in response to glucosamine, which we did not observe in HEK293F cells. Thus glucosamine has the potential to suppress cartilage catabolism in several ways, albeit at doses that currently appear to be too high to be clinically relevant. The effect described here is of broad biological significance, since furin is responsible for molecular maturation of numerous proproteins relevant to cartilage and OA, including prohormones, growth factors, receptors, ECM proteins, and proteases[38]. Treatment with glucosamine may be useful for reversible reduction or suppression of maturation of such substrates in cell culture, since we found there were no changes in cell morphology or viability using 10 mM glucosamine.

Glucosamine is well-tolerated and is not toxic orally, but the serum and tissue concentrations achieved using the typical dose of 1500 mg/day in short-term studies are far lower than the levels we found to be required for blocking ADAMTS5 activation[34,39]. Indeed the clinical trials testing long-term glucosamine administration do not support a slowing of arthritis progression[40,41]. Nevertheless, the effect we report supports inhibition of aggrecanase zymogen activation as a potential therapeutic measure.

Conflict of interest

None of the authors have any conflict of interest relating to the submitted manuscript.

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