Heparin is essential for the storage of specific granule proteases in mast cells

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All mammals produce heparin, a negatively charged glycosaminoglycan that is a major constituent of the secretory granules of mast cells which are found in the peritoneal cavity and most connective tissues. Although heparin is one of the most studied molecules in the body, its physiological function has yet to be determined. Here we describe transgenic mice, generated by disrupting the *N*-deacetylase/*N*-sulphotransferase-2 gene[1,2], that cannot express fully sulphated heparin. The mast cells in the skeletal muscle that normally contain heparin lacked metachromatic granules and failed to store appreciable amounts of mouse mast-cell protease (mMCP)-4, mMCP-5 and carboxypeptidase A (mMC-CPA), even though they contained substantial amounts of mMCP-7. We developed mast cells from the bone marrow of the transgenic mice. Although these cultured cells contained high levels of various protease transcripts and had substantial amounts of mMCP-6 protein in their granules, they also failed to express mMCP-5 and mMC-CPA. Our data show that heparin controls, through a post-translational mechanism, the levels of specific cassettes of positively charged proteases inside mast cells.

Heparin is generated by a series of enzymatic modifications of a non-sulphated precursor[3]. Thus, we speculated that it might be possible to deduce the physiological function of heparin by creating mice that cannot express this mast-cell specific glycosaminoglycan (GAG) by preventing expression of one of the sulphotransferases used early in its biosynthesis. Two mouse and three human *N*-deacetylase/*N*-sulphotransferases (NDSTs) have been cloned[1,2,4,5]. Because mouse bone-marrow-derived mast cells (mBMMCs) contain a very high level of the NDST-2 transcript relative to the NDST-1 transcript (data not shown), we concluded that mast cells probably require NDST-2 to synthesize their serglycin-bound heparin chains. We therefore used homologous recombination to disrupt the NDST-2 gene in mice. To this end, we isolated the relevant gene from a 129/Sv mouse genomic library and created the targeting construct shown in Fig. 1a. After transfection and positive selection, an embryonic stem-cell clone was obtained that possessed a disrupted NDST-2 allele. This clone was injected into mouse blastocysts to generate chimaeric mice and then NDST-2-null mice (Fig. 1b).

As assessed by chloroacetate-esterase cytochemistry, mast-cell committed progenitors could home to all analysed tissues of the NDST-2-null mouse (Figs 2, 3). However, the fact that the mast cells in most connective tissues were poorly stained by toluidine blue (Fig. 2b) indicates that disruption of the NDST-2 gene markedly affects heparin expression *in vivo*. The amounts of histamine in the exudates of <4-month-old NDST-2-null mice were 31 ± 6% (mean ± range, $n = 2$) of those of wild-type mice. Although the mast cells in the peritoneal cavities of similarly aged NDST-2-null mice contained only small amounts of chloroacetate-esterase activity, the number of chloroacetate-esterase-positive cells at this site was 49 ± 11% of that in wild-type mice. These data indicate that the amount of histamine in a recognizable peritoneal mast cell in a young wild-type mouse is only ~50% higher than that in a

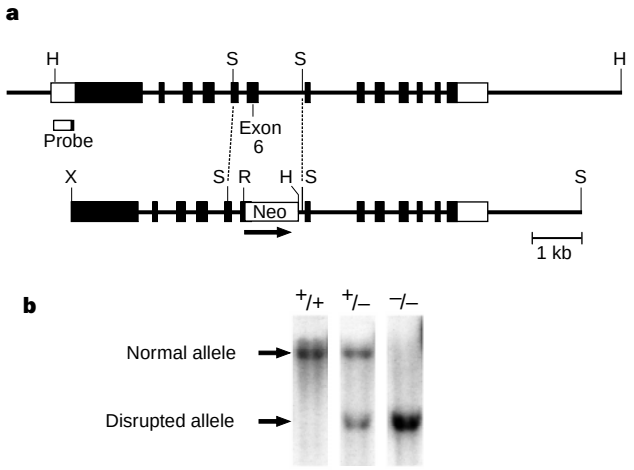

Figure 1 Generation of NDST-2-null mice. **a**, A clone that encodes the entire 13-exon coding region of the NDST-2 gene was isolated from a mouse genomic library to create the indicated targeting vector. H, S, X and R refer to *Hin*dIII-, *Sac*I-, *Xho*I-, and *Eco*RI-susceptible sites, respectively. The 5′ and 3′ untranslated portions of the gene are indicated in open boxes; the translated portions are indicated in solid boxes. **b**, Blot analysis of *Eco*RI-digested genomic DNA was used to genotype +/+, +/– and –/– mice. The probe used in the genotyping is shown in **a**. Because it resides outside the targeting construct, it can be used to test whether homologous recombination took place.

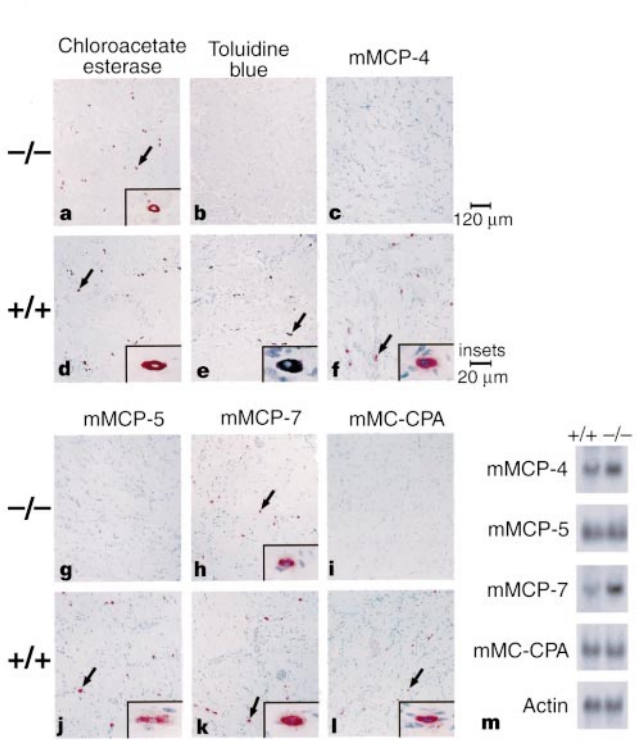

Figure 2 Analysis of mast cells in skeletal muscle and ear. **a**–**l**, Sections of tongue from NDST-2-null (**a**–**c**, **g**–**i**) and wild-type (**d**–**f**, **j**–**l**) mice were stained with toluidine blue (**b**, **e**) or with antibodies specific for mMCP-4 (**c**, **f**), mMCP-5 (**g**, **j**), mMCP-7 (**h**, **k**) and mMC-CPA (**i**, **l**). Alternately, tissue sections were evaluated cytochemically for the presence of chloroacetate-esterase-positive cells (**a**, **d**). Arrows indicate mast cells. Insets: higher magnifications of reactive mast cells (**a**, **d**–**f**, **h**, **j**–**l**). **m**, Blots containing total RNA from the ear of NDST-2-null mice (right lanes) and their wild-type littermates (left lanes) were analysed with mMCP-4, mMCP-5, mMCP-7, mMC-CPA and β-actin gene-specific probes.

NDST-2-null mouse. However, owing to the difficulty in recognizing poorly granulated mast cells in NDST-2-null mice and to age-dependent variations in the levels of chondroitin sulphate in a peritoneal mast cell, the possibility cannot be ruled out that many of the mast cells found at this site in older mice have substantially less histamine. Essentially no Evans blue dye extravasated into the control ears of wild-type and NDST-2-null mice given antigen alone. In contrast, +++ and ++ passive cutaneous anaphylaxis (PCA) reactions occurred in the ears of wild-type ($n = 4$) and NDST-2-null ($n = 4$) mice, respectively, given both IgE and antigen. It has been thought that the PCA reaction is histamine dependent. Thus, these PCA findings are in agreement with the findings obtained with cultured mast cells (see below) that show that fully sulphated heparin is not essential for histamine expression in every mast cell population.

The mast cells that increase in number in the jejunal mucosal epithelium during helminth infection preferentially express serglycin proteoglycans that contain highly sulphated chondroitins[6]. Therefore, we investigated the consequences of disruption of the NDST-2 gene on this expandable population of mast cells. Not only were mast cells detected in the jejunal mucosa of helminth-infected NDST-2-null mice, but their numbers were ~50% greater than those of their wild-type littermates. As assessed histochemically and cytochemically, the mast cells in the jejunal epithelium of the helminth-infected NDST-2-null mice possessed mature granules (Fig. 3). The one region of the jejunum that lacked metachromatic mast cells was the minor heparin-positive population found in the muscle/submucosa of this tissue in normal mice (Fig. 3, arrows).

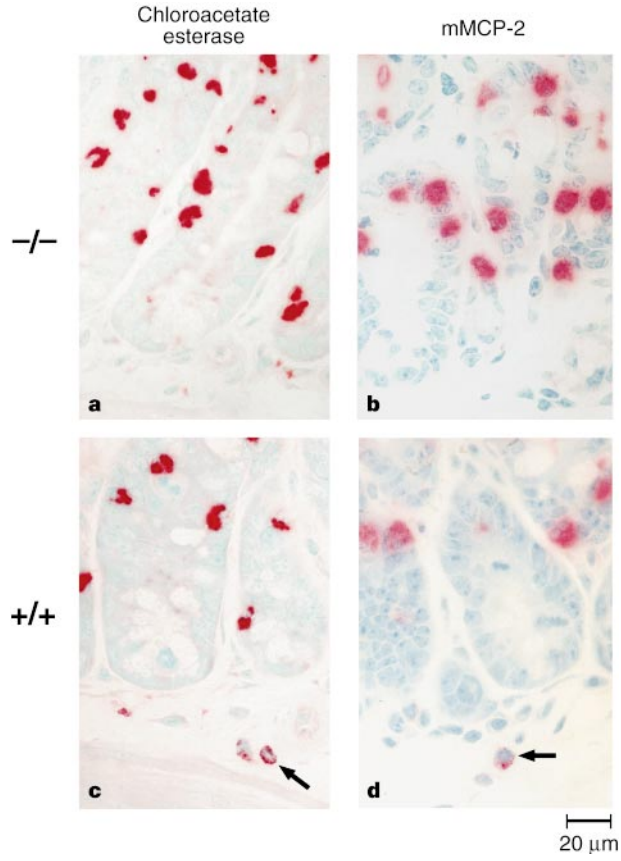

Figure 3 Analysis of mast cells in the jejunum of helminth-infected mice. Sections of jejunum from *T. spiralis*-infected NDST-2-null (**a**, **b**) and wild-type (**c**, **d**) mice were evaluated cytochemically for the presence of chloroacetate-esterase-positive cells (**a**, **c**), or immunohistochemically for the presence of mMCP-2-positive mast cells (**b**, **d**). Arrows (**c**, **d**) point to muscle/submucosa mast cells that are detectable in wild-type mice but not in NDST-2-null mice.

As well as a variety of negatively charged serglycin proteoglycans, mouse mast cells contain substantial amounts of different combinations of the exopeptidase mMC-CPA and at least 13 distinct serine proteases[7–14]. Whereas the mast cells that increase in number in the jejunal epithelium at the height of *Trichinella spiralis* infection of the BALB/c mouse preferentially express mMCP-2 (refs 8, 9), the constitutive mast cells that are found in normal skin preferentially express mMCP-4, mMCP-5, mMCP-6, mMCP-7 and mMC-CPA[10–13,15]. Immunohistochemical analysis revealed that the mast cells in the jejunal epithelium of helminth-infected NDST-2-null mice contain large amounts of mMCP-2 (Fig. 3b). The constitutive mast cells in the tongue, skin and ear (Fig. 2m) were also phenotypically mature in terms of their protease gene expression. The mast cells in the tongue contained substantial amounts of the tryptase mMCP-7 (Fig. 2h) and less mMCP-6 (data not shown). However, their levels of mMCP-4, mMCP-5 and mMC-CPA protein were below detection (Fig. 2c, g, i).

Like the *in-vivo*-differentiated mast cells in the peritoneal cavity, the mBMMCs developed from a normal mouse contain mMC-CPA and mMCP-5[12]. The two populations of mast cells differ in the nature of the prominent type of GAG attached to serglycin[16]. The proteoglycans present in the secretory granules of the mast cells that reside in the peritoneal cavity contain small amounts of chondroitin sulphate and large amounts of heparin. In contrast, the proteoglycans in mBMMCs contain small amounts of heparin and large amounts of chondroitin sulphate. If the protease/proteoglycan interactions in a mast cell are strictly ionic, disruption of heparin biosynthesis should not alter the amounts of mMCP-5 and mMC-CPA in the *in vitro*-differentiated mBMMCs. However, if the protease/proteoglycan interactions are heparin-dependent, there should be substantially less mMCP-5 and mMC-CPA in those mBMMCs developed from NDST-2-null mice. Thus, we generated mBMMCs from NDST-2-null mice to resolve these and other issues. As in the wild type, mast cells could be readily generated *in vitro* from the bone marrows of NDST-2-null mice (Fig. 4). This finding indicates that disruption of the NDST-2 gene does not lead to a decrease in the viability of the mast-cell-committed progenitors in the bone marrow or in their ability to respond to interleukin (IL)-3. The amount of [^{35}S]sulphate transferred from

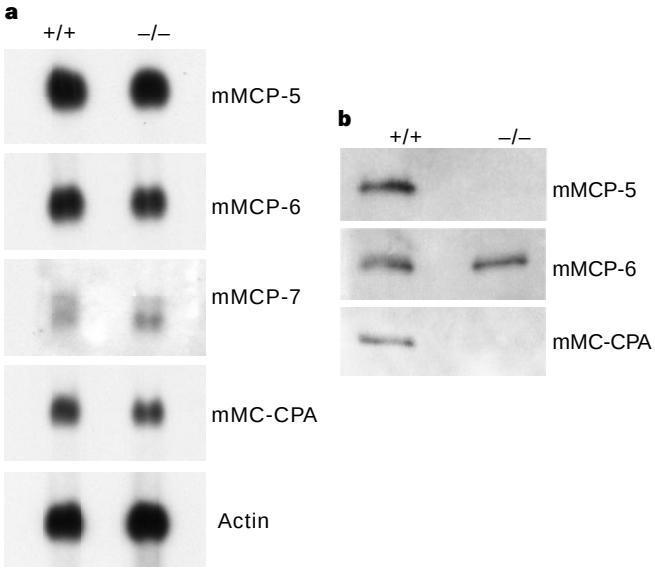

Figure 4 RNA blot and SDS–PAGE/immunoblot analysis of mBMMCs. **a**, IL-3-dependent mBMMCs developed from NDST-2-null mice (right lanes) and their wild-type littermates (left lanes) were analysed for mMCP-5, mMCP-6, mMCP-7, mMC-CPA and β-actin transcripts. **b**, Lysates of these cells were also evaluated for mMCP-5, mMCP-6 and mMC-CPA protein.

[^{35}S]PAPS to *N*-desulphated heparin was reduced by at least 75% in the NDST-2-null mBMMCs relative to wild-type mBMMCs. Concomitantly, the granules in the NDST-2-null mBMMCs were poorly stained by toluidine blue (data not shown). Despite the poor granulation, disruption of the NDST-2 gene did not adversely affect histamine expression in these *in vitro*-differentiated mast cells. The histamine content of the null and wild-type mBMMCs was 330 and 320 ng per 10^6 cells in one experiment and 530 and 440 ng per 10^6 cells in another, respectively.

Normal mBMMCs markedly increase their biosynthesis of heparin, as well as their granular content of mMCP-5 and mMC-CPA, when co-cultured with fibroblasts[17]. We therefore co-cultured mBMMCs developed from NDST-2-null mice with fibroblasts to investigate how disruption of the NDST-2 gene adversely affects heparin biosynthesis and packaging of these two granule proteases. Like normal BALB/c mBMMCs[17], the mBMMCs developed from NDST-2-null mice readily adhered to confluent monolayers of fibroblasts, indicating that they have normal adhesion receptors. The co-cultured mBMMCs developed from the wild-type mice contained safranin-positive granules (Fig. 5a). In contrast, no safranin-positive mast cells could be detected in the co-cultured mBMMCs developed from the NDST-2-null mice. The difference in toluidine blue staining was also marked. Wild-type mBMMCs that had been co-cultured with fibroblasts for 2–4 weeks incorporated 1.4- to 2.1-fold (range, $n = 3$) more [^{35}S]sulphate into proteoglycan than the corresponding mBMMCs developed from NDST-2-null mice. Thus, disruption of the NDST-2 gene caused an overall reduction in sulphated GAGs in these mast cells. We found that 67–74% and 41–51% of the total [^{35}S]proteoglycans produced by the co-cultured NDST-2-null mBMMCs and their wild-type littermates, respectively, were susceptible to chondroitinase ABC. In contrast, 19 to 22% and 46 to 56% of the total [^{35}S]proteoglycans produced by the co-cultured NDST-2-null mBMMCs and their wild-type littermates, respectively, were at least partially susceptible to nitrous acid. DEAE chromatography also revealed that the chondroitinase-ABC-resistant [^{35}S]GAGs produced by the NDST-2-null mBMMCs that had been co-cultured with fibroblasts were less sulphated than those produced by wild-type mBMMCs (data not shown). Moreover, fewer of these heparin-like GAGs were able to bind to an anti-thrombin-III affinity column. The cumulative data indicate that the failure of mast cells to express NDST-2 results in defective formation of structural motifs in the heparin chain, such as the 3-*O*-sulphated glucosamine-containing pentasaccharide that specifically recognizes anti-thrombin III[18].

It has been proposed that heparin is essential for the maturation of tryptase zymogens[19]. We could not determine whether the immunoreactive tryptases (Fig. 2h) in the tongue mast cells of NDST-2-null mice were enzymatically active. Thus, the mBMMCs developed from these animals allowed us to ascertain whether or not heparin was critical for the activation of mMCP-6 and/or mMCP-7 zymogens. The steady-state levels of the transcripts that encode the two tryptases in NDST-2-null mBMMCs were comparable to those in the control mBMMCs developed from their wild-type littermates (Fig. 4a). As assessed by SDS–PAGE/immunoblot analysis and immunohistochemistry, IL-3-developed mBMMCs (Fig. 4b) contained normal levels of mMCP-6 protein. Moreover, the observation that these mast cells contained tryptases in their granules that could cleave a chromogenic substrate indicated that at least some of the expressed tryptases were properly folded and enzymatically active. The data presented in Fig. 4 indicate that mMCP-6 can bind to chondroitin sulphate E. During fibroblast co-culture, mBMMCs switch from synthesis of chondroitin sulphate E to heparin[17]. A modest reduction in mMCP-6 content was observed in the co-cultured mBMMCs developed from NDST-2-null mice (Fig. 5b). This probably occurred because there is less total sulphated glycosaminoglycan available to interact with this tryptase.

Protein modelling, crystallographic and site-directed mutagenesis studies have revealed that the GAG-binding domain on the surface of each mast cell granule protease is conformation dependent. Because the granule storage capacity of a mast cell is limited, serglycin proteoglycans appear to be used by this cell to ensure that only properly folded proteases are targeted to the secretory granule. Mast cells must be able to respond quickly to changes in the immune status of their microenvironments[9]. Thus, the proteases must be packaged in granules in their mature forms. A larger number of distinct glycosyltransferases and sulphotransferases are required to synthesize heparin and chondroitin sulphate. Why mast cells place very different types of negatively charged GAGs onto serglycin has not been deduced. Nevertheless, the observation that the post-translational modification of serglycin is conserved in evolution from mice to humans indicates that it is very important to the function of the mast cell. The observation that the mBMMCs developed with IL-3 from NDST-2-null mice can still package tryptases (Fig. 4) that can cleave tosyl-Gly-Pro-Arg-pNA is consistent with the *in vivo* data (Fig. 2) that showed that fully sulphated heparin is not essential for tryptase expression. Because the chondroitin-sulphate-rich mast cells residing in the jejunum contain substantial amounts of mMCP-2 (Fig. 3), heparin is also not essential for expression of this chymase.

One of the mysteries in immunology is how mast cells can store large amounts of enzymatically active proteases in their granules in such a way that these enzymes do not undergo rapid autolysis. Based on data from numerous *in vitro* studies it has been speculated that heparin is an essential co-factor in mast cells for the dipeptidyl-peptidase-I-mediated conversion of zymogens into active proteases[20]. Whether NDST-2-null mBMMCs were cultured in the absence (Fig. 4) or presence (Fig. 5) of fibroblasts, the resulting cells could not store mMCP-5 and mMC-CPA in their granules, even though they contained high levels of the chymase and exopeptidase transcripts. mMCP-5 and mMC-CPA have relative molecular masses of 30,000 and 36,000 ($M_r = 30$K and 36K), respectively. Consistent with the SDS–PAGE/immunoblot data, the levels of the 30K and 36K proteins in wild-type mBMMCs was greater than that in NDST-2-null mBMMCs (Fig. 5b). These data indicate that fully sulphated heparin is essential for the expression of two mast-cell chymases and an exopeptidase. The mast cell uses transcriptional and post-transcriptional mechanisms to alter the levels of its individual protease transcripts. This is the first example where the expression of one granule constituent markedly affects the expression of another at the post-translational level. Thus, the mast cell uses multiple mechanisms to regulate the levels of its various granule proteases. □

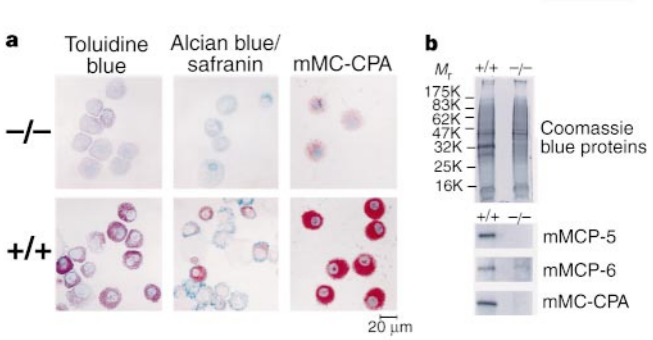

Figure 5 Histochemistry, immunohistochemistry and SDS–PAGE/immunoblot analysis of fibroblast/mBMMC co-cultures. **a**, Co-cultured mBMMCs, developed from NDST-2-null mice and their wild-type littermates, were evaluated histochemically for their proteoglycan content and immunohistochemically for their mMC-CPA content. **b**, SDS–PAGE/immunoblot analysis was used to evaluate the relative levels of Coomassie blue stainable proteins, as well as mMCP-5, mMCP-6 and mMC-CPA proteins in the two populations of co-cultured mast cells.

Methods

Generation of NDST-2-null mice. We screened a 129/Sv mouse genomic library with a probe corresponding to residues 1488–2128 in the transcript[2] to isolate the coding portion of the NDST-2 gene. We prepared a targeting construct in which amino-acids 539 to 580 of the mouse NDST-2 gene were replaced by the Neomycin resistance (Neo^r) gene. The carboxy-terminal halves of the varied NDSTs exhibit considerable homology with other sulphotransferases and previous functional studies carried out on recombinant NDSTs have revealed that the *N*-sulphotransferase activity of this family of enzymes is entirely contained within residues 479–880. Thus, to ensure a nonfunctional enzyme, we also placed a premature stop codon after residue 538. Embryonic stem cells were transfected with the targeting construct. After positive selection, a clone that possessed a disrupted NDST-2 allele was injected into blastocysts to eventually obtain NDST-2-null mice.

Helminth infection and IgE-dependent passive anaphylaxis. To evaluate the consequence of disrupted heparin expression on the chondroitin sulphate population of mast cells in the jejunum of helminth-infected mice, NDST-2-null mice and their wild-type littermates were each infected orally with 400 *T. spiralis* larvae, as described[9]. We examined the jejunal mast cells at the height of the mastocytosis at day 14. For the IgE-dependent PCA reactions, PBS (20 μl) with or without 20 ng of anti-dinitrophenyl IgE (Sigma) was injected into separate ears of each NDST-2-null and wild-type mouse. Twenty-four hours later, a 100-μl solution of 0.5% Evans blue containing 100 μg dinitrophenyl albumin was injected into the tail vein of each animal; the uptake of the dye into the two ears was then compared ~30 min later.

Histochemistry, enzyme cytochemistry and immunohistochemistry. mBMMCs were obtained as described[16] by culturing isolated progenitors from NDST-2-null mice and their wild-type littermates for 3 weeks in IL-3-supplemented medium. Some of these mBMMCs were then co-cultured with 3T3 fibroblasts[17]. For histological examination, we stained cytocentrifuge preparations of mBMMCs and 1.5-μm-thick glycolmethacrylate sections of various tissues with a 0.5% solution of toluidine blue in 0.6 M HCl. mBMMCs were also stained with 0.5% alcian blue in 0.3% acetic acid and then counterstained with 0.1% safranin O in 1% acetic acid. Those mast cells that contained esterase activities were identified using a modification[9] of the method of ref. 21. We incubated tissue sections and cultured mast cells with antibodies specific for mMCP-2 (ref. 22), mMCP-4 (R.L.S., unpublished data), mMCP-5 (ref. 23), mMCP-6 (ref. 24), mMCP-7 (ref. 24) and mMC-CPA[25]. To confirm the immunocytochemical data, lysates of pooled mBMMCs developed from NDST-2-null mice and their wild-type littermates were electrophoresed in 12% SDS–PAGE gels. Protein blots prepared from the separated proteins were probed with anti-mMCP-5, anti-mMCP-6 or anti-mMCP-CPA antibodies.

RNA blot analysis. We isolated total RNA[15] from the ear and tongue of NDST-2-null mice and their wild-type littermates, as well as the mBMMCs developed from these mice. RNA blots were prepared and analysed with NDST-2 (the 641-base-pair fragment that corresponds to residues 1488–2128 in the transcript[2]), NDST-1 (ref. 4), mMCP-5 (ref. 12), mMCP-6 (ref. 11), mMCP-7 (ref. 13), mMC-CPA or β-actin[26] radiolabelled cDNAs.

***N*-Sulphotransferase, proteoglycan, tryptase and histamine biochemical assays.** We evaluated mBMMCs for their *N*-sulphotransferase activities as described[27]. To evaluate the consequences of disrupting the NDST-2 gene on proteoglycan expression in mast cells, mBMMCs from NDST-2-null mice and their wild-type littermates were co-cultured with fibroblasts for an additional 2–4 weeks. The adherent cells in the resulting co-cultures were radiolabelled in medium containing 150 μCi [^{35}S]sodium sulphate[16]. After this step, each culture was washed and then cultured for an additional 2 h in normal medium. The mast cell/fibroblast co-cultures were then exposed to a solution containing 0.25% trypsin and 1 mM EDTA for 5 min to remove the remaining fibroblast-derived [^{35}S]proteoglycans. After the detached fibroblasts and mast cells were centrifuged, the cell pellets were disrupted and the liberated [^{35}S]proteolgycans were subjected to CsCl density-gradient centrifugation[28]. The purified proteoglycans were then treated with either chondroitinase ABC[29] to degrade their chondroitin-sulphate chains or nitrous acid[28] to degrade their heparin-like chains. The intracellular, heparin-like population of GAGs produced by the NDST-2-null and wild-type mBMMCs were subjected to DEAE-cellulose column chromatography to evaluate their overall net charges. In one experiment, samples also were subjected to anti-thrombin-III affinity chromatography to evaluate the presence of a specific structural motif[18] within the chain.

To determine whether or not the immunoreactive tryptases present in the mBMMCs developed from NDST-2-null mice were enzymatically active, we tested the ability of cell lysates to cleave tosyl-Gly-Pro-Arg-pNA[30]. Finally, an ELISA kit (ICN Pharmaceuticals) was used to measure the levels of histamine in sonicates of mBMMCs and peritoneal cavity exudates.

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