Chapter 3
Heparanase: Historical Aspects and Future Perspectives

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3.1 Introduction

In this Chapter, a historical overview of our understanding of the functional properties of heparanase (also known as heparanase-1) is provided followed by a general discussion of unanswered questions and future areas of heparanase research. To assist the reader, a Table is provided (Table 3.1) that chronologically lists important advances in our understanding of heparanase. It is also important to note that although heparanase was initially thought to be an endoglycosidase that cleaves HS chains, there are a number of studies showing that heparanase can also perform non-enzymatic functions. Thus, this review will, after an initial historical overview of the general properties and substrate specificity of heparanase, consider the multiple enzymatic functions mediated by heparanase and then, in a separate section, review non-enzymatic processes performed by heparanase.

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Table 3.1 Chronology of major advances in heparanase research

Date	[a]Major advance	References
1975–1977	First evidence that endoglycosidase (heparanase) exists that can degrade HS/heparin	[1–7]
1982–1983	Demonstration that metastatic tumors express heparanase, the level of the enzyme correlating with metastatic potential	[8–10]
1982-present	Development of heparanase assays	Reviewed in [48]
1984	Demonstration that T cells, following activation, upregulate heparanase	[76]
1986–1987	First heparanase inhibitors developed	[106, 107, 109]
1989	First evidence that heparanase can release pro-angiogenic growth factors sequestered by HS in ECMs	[38]
1995	First demonstration that heparanase has non-enzymatic functions, i.e., can act as cell adhesion molecule	[130]
1999	Mammalian heparanase cloned	[12, 13]
1999	Heparanase shown to exist as a pro-enzyme that is protease processed to form an enzymatically active heterodimer	[30]
1999	Discovery that there is only one heparanase enzyme in the mammalian genome	[12, 13]
2000	Discovery of heparanase-2 gene	[35, 36]
2000	Heparanase shown to be a family 79 glycoside hydrolase, with $(\alpha/\beta)_8$ TIM-barrel fold, Glu^{225} and Glu^{343} in active site	[35]
2000-present	Heparanase upregulated in all cancer types, correlated with poor prognosis and can be derived from tumour microenvironment	Reviewed in [73]
2000-present	Heparanase shown to be a key player in inflammatory responses	Reviewed in [71, 85, 89]
2004	First evidence that heparanase can activate signaling pathways	[134]
2004	First reports that heparanase can translocate to the nucleus	[144, 146]
2008	Cathepsin L identified as proheparanase processing enzyme	[32]
2010	First evidence that heparanase-2 can inhibit heparanase function	[155]
2012	Demonstration that heparanase controls transcription by regulating the methylation of histone H3 tails	[148]
2015	Crystal structure of heparanase solved	[42]
2015	Discovery that heparanase can promote autophagy	[56, 57]
2016	Heparanase is required for the activation and function of macrophages	[140]
2015–2019	Heparanase shown to facilitate the spread of HS-binding viruses	[103–105]

[a]Advances in italics represent functions independent of heparanase enzymatic activity

3.2 Historical Overview and General Properties of Heparanase

Heparin and HS degrading activity was first reported in mammalian cells during the mid- to late-1970s, with rat liver lysosomes [1, 2], skin fibroblasts [3, 4], a mouse mastocytoma [5] and platelets [6, 7] being shown to contain such activity, although the functional relevance of HS degradation was unclear (Table 3.1). However, interest in heparanases increased dramatically in the early 1980s when studies by Nicolson and colleagues revealed that metastatic B16 melanoma cells contain a HS-specific endoglycosidase that releases HS fragments from the ECM of cultured vascular endothelial cells [8, 9]. Of particular interest was the observation that the heparanase activity of different B16 melanoma sublines positively correlated with the metastatic potential of the sublines [10]. Similarly, Vlodavsky and colleagues reported that a highly metastatic variant of a T lymphoma cell line very efficiently released HS fragments from a subendothelial ECM whereas the poorly metastatic parent line did not possess this activity [11]. These findings led to the proposal that heparanase(s) may aid tumor metastasis by degrading HS chains in the walls of blood vessels.

Unfortunately, further progress in understanding the biological relevance of heparanase was severely hampered by the inability to purify and clone the enzyme. In fact, it took another 16 years before the enzyme was finally cloned and characterized [12, 13]. This extraordinary delay was mainly due to the lack of a rapid, simple and reliable heparanase assay, most studies measuring by gel filtration the release of radiolabelled HS fragments from radiolabelled ECM [14–16]. This assay approach is semi-quantitative and consequently did not allow accurate estimates of heparanase recovery and purity. Solid phase heparanase assays using radiolabelled HS attached to solid support were also developed, the release of radiolabelled HS from the solid support being a measure of heparanase activity [17–19]. However, these assays suffered from difficulties in coupling HS to the solid support and potentially poor accessibility of the HS substrate to the heparanase enzyme. The assay problem was further compounded by the fact that most tissues contain very low levels of heparanase, only human placenta and platelets eventually being identified as containing sufficient quantities of heparanase for enzyme purification and characterization. The situation was made worse by HS-binding proteins in tissue homogenates binding to HS substrates and blocking HS cleavage. In fact, in the absence of reliable heparanase assays, vastly different molecular masses for heparanase were reported, ranging from 8–137 kDa [18, 20, 21], and claims were also made that heparanase had sequence homology, in one case, to heat shock proteins [22, 23] and in another report to the chemokine β-thromboglobulin [20]. A further complication was the proposal that at least three mammalian heparanases exist, based on the ability of different heparanase preparations to degrade a mast cell heparin precursor, heparin and/or HS [20, 24, 25].

Thanks to an outstanding effort, Craig Freeman in our laboratory developed a rapid and highly quantitative heparanase assay that was instrumental in us purifying human platelet heparanase to homogeneity [21, 26]. The assay took advantage of our finding that the HS/heparin-binding plasma protein, histidine-rich glycoprotein

(HRG), binds across heparanase cleavage sites in HS. Thus, when HRG was immobilized on beads radiolabelled HS bound strongly to the beads whereas if HS chains had been cleaved by heparanase they failed to bind to the HRG coated beads. One major advantage of the assay was that its ability to detect heparanase activity was unaffected by the presence of large quantities of irrelevant proteins, as occurs with tissue homogenates.

Of course, another reason for the long delay in cloning heparanase was that the cloning procedures employed in the 1980s and 1990s were tedious and technically difficult. They involved initially purifying the protein of interest to homogeneity, obtaining some amino acid sequence of the protein and then designing PCR primers, based on the available amino acid sequence, to eventually deduce the nucleotide sequence of the protein. We eventually obtained the N-terminal amino acid sequence as well as the sequence of 10 heparanase tryptic peptides but were still struggling to clone the enzyme. The breakthrough came with the emergence of expressed sequence tags (ESTs) as part of the human genome project, ESTs being short mRNA sequences generated by a single sequencing reaction from randomly selected clones from cDNA libraries [27]. Initially, the public EST database was incomplete and so many transcripts, including that of heparanase, were not represented. However, the database was being continually updated, and eventually, an EST appeared, derived from a human placenta cDNA clone, that contained the predicted nucleotide sequence of five of the heparanase peptides we had identified and encoded the 3′ end of the gene. Once this information was available, we were then able to use standard techniques to rapidly deduce the complete nucleotide sequence of human heparanase and, subsequently, rat and mouse heparanase.

In August 1998, one of the authors of this Chapter, Chris Parish, attended the Xth International Vascular Biology Meeting in Cairns, Australia. We were well aware of the major contributions Israel Vlodavsky and his colleagues were making to the heparanase field and so were excited to hear that he would be attending the conference. When Chris and Israel presented their heparanase research to the meeting, it was obvious that both groups had successfully cloned heparanase and were close to submitting their findings for publication. Also, following their conference presentations Chris and Israel compared their heparanase sequences, without giving away too much information, and it became abundantly clear that both laboratories had cloned the same protein. The question then arose how to proceed. Both Chris and Israel were not comfortable with a ‘race to the summit’ scenario and decided to submit their findings as back-to-back papers in *Nature Medicine*. In due course, the two manuscripts were submitted simultaneously and there followed an anxious wait. When the decision was received from the *Nature Medicine* Editor it was short and sweet – accept without change and a single sentence from the reviewers stating that ‘this work must be published’!

The two *Nature Medicine* articles [12, 13] reported that human heparanase consists of a polypeptide of 543 amino acids and has a molecular mass of 61.2 kDa, a finding confirmed by four other groups soon afterward [28–31]. However, the N-terminus of the enzymatically active enzyme was found to be 157 amino acids downstream of the initiation codon, implying that heparanase initially exists as a pro-enzyme that is proteolytically cleaved to yield the active enzyme. Indeed, it was

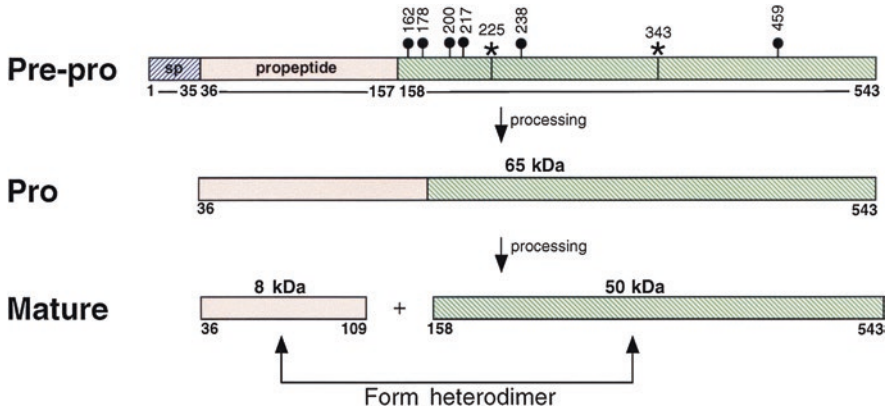

Fig. 3.1 Predicted structure and processing of human heparanase (*circa 2001)*. A schematic representation of the domain structure of the heparanase protein and the proposed processing steps to produce the active form of the enzyme are shown. The enzyme is predicted to be synthesized in a pre-pro-form, which is processed to an inactive pro-form upon removal of the putative signal peptide. The pre-pro form is then processed into the active mature enzyme by removal of amino acids 110–157 to give two polypeptides of 8 kDa (residues 36–109) and 50 kDa (158–543), which form a heterodimer. The locations of the six putative N-linked glycosylation sites (N162, 178, 200, 217, 238 and 459) are indicated by the solid circles, and the putative catalytic proton donor (Glu225) and nucleophile (Glu343) residues by asterisks. The domain boundaries are numbered. (Figure reproduced from Parish, et.al [41]. with permission)

subsequently reported that the enzyme is a heterodimer composed of a 50 kDa subunit (Lys^{158}-IIe_{543}) associated non-covalently with an 8 kDa peptide (Gln_{36}-Glu_{109}), with an interconnecting 6 kDa peptide (Ser_{110}-Gln_{157}) being excised by proteolysis (Fig. 3.1) [30]. Another 9 years elapsed before cathepsin-L was identified as the key protease that processes pro-heparanase to form the active heparanase enzyme [32].

One of the most intriguing aspects of the cloning of mammalian heparanase is, however, that there appears to be only one heparanase encoding gene in the mammalian genome. This surprising conclusion has remained unchanged for the last 20 years, in humans the gene being located on chromosome 4q21.3 [33], spanning 40 kb and being composed of 12 exons separated by 11 introns [13, 34]. We did discover, however, based on exhaustive sequence homology studies, that heparanase is a clan A glycoside hydrolase (family 79), with secondary structure predictions suggesting that heparanase contains an $(\alpha/\beta)_8$ TIM-barrel fold, which is characteristic of clan A glycoside hydrolases [35]. Soon afterward a human cDNA was identified by McKenzie et al. that encodes a protein, designated **heparanase-2**, that has 40% overall identity and 59% sequence resemblance with heparanase (heparanase-1), and resembles a family 79 glycoside hydrolase [36] although it was subsequently shown to lack enzymatic activity [37]. Recent research indicates, however, that although heparanase-2 lacks endoglycosidase activity it has a higher affinity for HS than heparanase-1 and thus, via competition for HS, inhibits heparanase-1 enzymatic activity [38]. Furthermore, heparanase-2 regulates a range of genes associated with tumor suppression, implying that heparanase-2 acts as a tumor suppressor, a truly remarkable finding (reviewed in [37]).

Additional, molecular modeling studies of heparanase-1 established critical active site residues, Glu^{225} being identified as the proton donor and Glu^{343} as the nucleophile (Fig. 3.1). Site-directed mutagenesis studies with human heparanase confirmed these predictions [35]. Thus, by 2001 we had a reasonably detailed understanding of the secondary structure of heparanase (Fig. 3.1), although at that stage the position of disulfide bonds within the molecule had not been determined, a deficiency that was rectified in 2007 with the report that heparanase has two disulfide bonds, namely Cys127-Cys179 and Cys437-Cys542, with the latter disulfide being essential for enzymatic activity [39]. We also predicted six glycosylation sites in human heparanase (Fig. 3.1) and relatively soon after this prediction was made it was reported that all 6 sites were glycosylated, with glycosylation not being required for enzymatic activity, but is required for heparanase secretion [40].

We then constructed a space-filling model of heparanase based on the crystal structure of the endo-1,4-β-xylanase from *Penicillium simplicissium*, a member of the glycoside hydrolase 79 family, the active site residues of heparanase being shown to be surrounded by patches of basic residues that could potentially bind to negatively charged HS [41]. Following these initial studies of the structure of heparanase, we keenly awaited the determination of the crystal structure of the molecule. Unfortunately, another 14 years elapsed before the 3D structure of heparanase was determined (Table 3.1) [42], although we were pleased to discover that the deduced structure did confirm essentially all of our predictions.

3.3 Overview of Substrate Specificity of Heparanase

Since HS is the substrate of the endoglycosidase heparanase, some structural and functional features of HS should be highlighted prior to discussing the enzymatic functions of heparanase. HS is a linear glycosaminoglycan consisting of repeating disaccharides of D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) (reviewed in [43]). During biosynthesis of HS substantial changes are made to the molecule, sulfate groups being attached to specific hydroxyl groups, GlcNAc residues being N-deacetylated and N-sulfated and GlcA residues undergoing epimerization to become iduronic acids (IdoA). Such changes are not evenly distributed throughout HS molecules but tend to be concentrated in ‘hot spots’ of sulfation and epimerization and result in HS molecules that exhibit great structural diversity. In fact, >300 proteins have been shown to interact with HS, many of these proteins binding specifically to unique structural motifs in HS (reviewed in [43]).

HS is ubiquitously expressed on cell surfaces and in the extracellular matrix (ECM) of most animal species, with some studies suggesting that HS can also accumulate in the nuclei of cells (reviewed in [44]). A major function of HS is to provide a scaffolding with which HS-binding proteins can interact and become stably positioned within 3-dimensional space. A classic example of this process is the establishment of chemokine gradients, HS-binding chemokines interacting with HS and forming such gradients [45]. Similarly, HS molecules can oligomerize HS-binding proteins and act as a scaffold that promotes protein-protein interactions

(reviewed in [43]). These unique structural features of HS should be kept in mind when analyzing the functional consequences of heparanase degradation of HS chains.

In terms of substrate specificity we now know that heparanase is an endo-β-glucuronidase that cleaves HS chains at a limited number of sites, usually the non-reducing side of highly sulfated regions of HS [46], to yield fragments of ~3–5 kDa in size. Hydrolysis of HS chains proceeds via a double displacement mechanism, with the anomeric configuration of the substrate being retained and, consequently, heparanase being classified as a retaining glycosidase [47]. A number of studies indicate that the HS cleavage site consists of the linkage between GlcA and *N*-sulfoglucosamine that is either 3-*O*- or 6-*O*-sulfated, with the minimum sequence being a trisaccharide (reviewed in [48]). However, the substrate specificity of heparanase is not fully resolved, recent studies suggesting that the specificity of the enzyme is somewhat plastic, being dependent on saccharide structures around the cleavage site [49].

3.4 Functions Dependent on Heparanase Enzymatic Activity

3.4.1 HS Turnover

As discussed earlier, heparanase is the only known *endo*glycosidase in mammals that can cleave heparan sulfate (HS) chains, either free or attached to HS proteoglycans (HSPG) (reviewed in [37, 41, 50]). In contrast, there are at least 9 mammalian *exo*glycosidases present in lysosomes that very specifically and sequentially degrade HS chains from their non-reducing termini. The importance of these exoglycosidases in degrading HS is highlighted by their deficiency resulting in the massive accumulation of HS in lysosomes in various tissues and disease syndromes called 'mucopolysaccharidoses' (reviewed in [51]). It was originally thought that heparanase plays an important role in initiating degradation of HS chains, particularly as heparanase accumulates in lysosomes like the exoglycosidases that degrade HS [52, 53]. However, to date, there have been no reports of a mucopolysaccharidosis in humans resulting from heparanase deficiency [54]. Furthermore, heparanase deficient mice, although producing HS chains of higher molecular mass than their wild type counterparts, exhibit no signs of HS accumulation in their tissues [55]. These data imply that lysosomal heparanase is not required for HS turnover but it has been proposed that lysosomal heparanase promotes **autophagy** and thereby maintains cellular homeostasis in damaged cells [56, 57].

3.4.2 Involvement in Cell Invasion

It has been proposed for many decades that the subendothelial basement membrane represents a major barrier to the passage of cells through the blood vessel wall and entry into tissues. Basement membranes are a specialized type of ECM that separate

different cell types and tissues and are composed predominantly of collagen type 4, laminin and HSPGs [58]. To overcome this barrier it was discovered that invading cells use a battery of degradative enzymes that disassemble the basement membranes and allow the passage of cells [59, 60]. By the early 1980s, a number of proteases had been identified that degrade ECMs/basement membranes and aid tumor metastasis. Thus, when it was first reported in 1983 that heparanase activity correlated with melanoma and lymphoma metastasis [10, 11], the finding was received with much enthusiasm as it implied that heparanase aids **tumor metastasis** by cleaving HS chains, rather than polypeptide chains, in basement membranes. In fact, it was anticipated that proteases and heparanase(s) acted cooperatively to degrade BMs and allow the passage of cells, a view that was supported by a study some years later [61]. Furthermore, additional investigations confirmed that the metastatic potential of tumor cells correlated with their content of heparanase [62–64] and heparanase inhibitors were found to be very effective at inhibiting tumor metastasis (see Sect. 3.4.6 below). However, developments in this area were severely hampered by the heparanase enzyme not being cloned and characterized.

The situation changed dramatically in 1999 with the cloning of heparanase (Table 3.1). It was immediately shown that heparanase mRNA is highly expressed in metastatic rat and human mammary adenocarcinoma cell lines whereas the poorly metastatic parent cell lines contained little or no heparanase mRNA [12, 65]. In situ hybridization and immunohistochemical studies confirmed the mRNA results, with heparanase transcripts and protein expression being upregulated in highly invasive breast and colon cancers when compared to non-cancerous tissue nearby [65, 66]. It was also shown that stable transfection of lymphoma and melanoma cell lines with the heparanase gene increased the metastatic ability of the cell lines [13, 65]. Conversely, knockdown of heparanase transcripts reduced the metastatic potential of highly metastatic tumors [67–69]. These early studies also confirmed that most normal tissues contain very low levels of heparanase, the exceptions being lymphoid organs, peripheral blood leukocytes and the placenta [12, 28]. Thus, the results were consistent with the view that heparanase is mainly expressed by cells that are in an invasive rather than a resting state. In fact, subsequent studies have demonstrated that heparanase expression is enhanced in all major cancer types, namely carcinomas, sarcomas and hematological malignancies [50, 70–74]. Furthermore, numerous clinical studies have shown that upregulation of heparanase expression correlates with increased metastasis and poor prognosis [37, 50, 71, 74, 75]. However, it is increasingly being recognized that some tumors rely on heparanase being provided by components of the tumor microenvironment (e.g., fibroblasts, platelets), the tumor cells in these cases being essentially heparanase deficient (reviewed in [37]).

Initially, the focus of heparanase research was on the ability of the enzyme to facilitate tumor metastasis by degrading and remodeling ECM and basement membranes. Under normal circumstances, however, the function of heparanase is to aid the invasion of non-malignant cells through ECM barriers, with cells of the immune system entering sites of **inflammation** being major participants. In fact, it was demonstrated as early as 1984 that following activation T lymphocytes produce heparanase [76] and, soon after, neutrophils were shown to also release heparanase [77]. In both studies, it was proposed that the released heparanase plays a key role in leucocyte extravasation through subendothelial basement membranes. Support for

this view was the demonstration that heparanase inhibitors exhibit anti-inflammatory activity (see Sect. 3.4.6 below).

Subsequent studies demonstrated that in inflammatory responses heparanase is not only expressed by leucocytes, inflammatory cytokines inducing epithelial and/or endothelial cells to produce the enzyme and aid leucocyte entry into inflammatory sites [78–81]. In fact, in addition to cytokines a range of other stimuli are able to augment heparanase expression, e.g., high glucose, reactive oxygen species [78, 82] and estrogens [83, 84]. Based on these observations it is not surprising that heparanase has been shown to play a key role in the pathogenesis of a range of inflammatory disorders, notably inflammatory lung disease, rheumatoid arthritis and chronic colitis (reviewed in [70, 71, 85, 86]). However, inflammatory reactions are complex, involving multiple cell types and cytokines, and so the precise role of heparanase in inflammation remains to be elucidated. There is also the intriguing observation that inflammation is associated with cancer progression [87, 88] and mounting evidence that heparanase may play a central role in the induction of **inflammation-associated cancers** (reviewed in [71, 85, 89]).

3.4.3 Involvement in Release of ECM Bound Proteins

During both tumor progression and inflammatory responses, heparanase enzymatic activity not only contributes to the breakdown of ECM barriers and cell invasion but also results in the release of ECM bound proteins. Examples of such proteins are HS-binding growth factors, such as basic fibroblast growth factor, hepatocyte growth factor and vascular endothelial growth factor [90]. These factors are sequestered by HS in the ECM and are also protected from degradation by proteases. Following liberation from the ECM by heparanase, however, the growth factors become available to stimulate nearby cells, this process being particularly important for the induction of endothelial cell proliferation and resultant **angiogenesis** as well as wound healing (reviewed in [91–93]) [38, 94]. Released growth factors also remain associated with HS-fragments that can crosslink growth factors to their receptors and thereby enhance signaling by the receptors [95, 96]. Since >300 proteins have been identified that bind heparin/HS [43] and, consequently, can potentially bind to ECM- and cell surface-associated HS chains, the influence of heparanase on the functional behavior of these proteins must be immense and would be expected to underpin many biological processes, not just the release of HS-binding growth factors from the ECM.

3.4.4 Involvement in Depletion of Intracellular Anti-Oxidant Stores of HS

Usually, HS is predominantly expressed outside cells either in the surrounding ECM or on cell surfaces, with relatively little in the cytoplasm and nucleus of cells (see Sect. 3.3). In collaboration with Charmaine Simeonovic, we discovered,

however, that the insulin-producing β-cells in the Islets of Langerhans of the pancreas contain extremely high levels of intracellular HS [97]. We also discovered that this intra-islet HS made the β-cells extremely resistant to reactive oxygen species (ROS) by a mechanism that is being currently elucidated [97]. It is not surprising that β-cells possess such potent anti-oxidant activity as they are one of the most biosynthetically and metabolically active cells in the body. In Type-1 Diabetes (T1D) in mice and humans, however, autoreactive T cells against islet auto-antigens enter the islets and, via depletion of intracellular HS by T cell-dependent heparanase, render the insulin-producing β-cells susceptible to ROS killing [97, 98] (reviewed in [99, 100]). In addition, we found that T1D is particularly dependent on heparanase as auto-reactive T cells are required to not only pass through the subendothelial basement membrane of pancreatic blood vessels but also to traverse a basement membrane surrounding the Islets of Langerhans. This conclusion was further supported by the finding that a heparanase inhibitor (PI-88) markedly reduces diabetes incidence in NOD mice that spontaneously develop T1D (Simeonovic et al., Chap. 24 in this volume). An intriguing question that arises from these findings is whether other tissues/cells use cytoplasmic HS as an anti-oxidant and, as a result, are susceptible to heparanase exposure.

3.4.5 Facilitator of Spread of HS-Binding Viruses

Many viruses bind HS and use it as a co-receptor for infecting cells (reviewed in [101]). This observation provides an intriguing paradox, namely, if viruses bind HS how do they escape from a primary site of infection and spread to other organs, particularly as HS is ubiquitously expressed on cell surfaces and the ECM. A similar situation also occurs with the influenza virus, in this case, the hemagglutinin of the virus binding sialic acid, an interaction that facilitates influenza virus infection of cells but also impedes the spread of progeny virus. Influenza overcomes this problem by expressing neuraminidase, a glycosidase that removes sialic acid from neighboring molecules and allows virus spread (Fig. 3.2a) (reviewed in [102]). Thus, an attractive hypothesis based on these data is that HS-binding viruses recruit

Fig. 3.2 Schematic representation of the role of neuraminidase in influenza virus spread and the participation of heparanase in HSV-1 and VACV dissemination. (**a**) Neuraminidase, a major protein expressed on the surface of influenza virus, cleaves sialic acid from neighboring molecules, which prevents the influenza hemagglutinin from interacting with sialic acid and, consequently, promotes virus release. In contrast, heparan sulfate (HS) binding viruses like HSV-1 and VACV rely on heparanase (HPSE) mediated cleavage of HS in order to spread. However, unlike influenza, HSV-1 and VACV do not encode for such enzymatic activity. (**b**) In fact, HSV-1 has been shown to induce the expression of HPSE in infected cells, release of which results in degradation of extracellular matrix (ECM) and cell surface HS and allows localized spread of HSV-1. (**c**) In contrast, VACV being highly cytopathic attracts platelets to sites of infection, which in turn release pre-formed stored HPSE to promote inflammation in response to vascular injury. VACV released from the infection site ECM by platelet HPSE would also be expected to carry HS fragments, which could potentially block the virus from further interactions with ECM HS. This would allow an easier long-range spread of VACV (Figure reproduced from Khanna et al. [105] with permission)

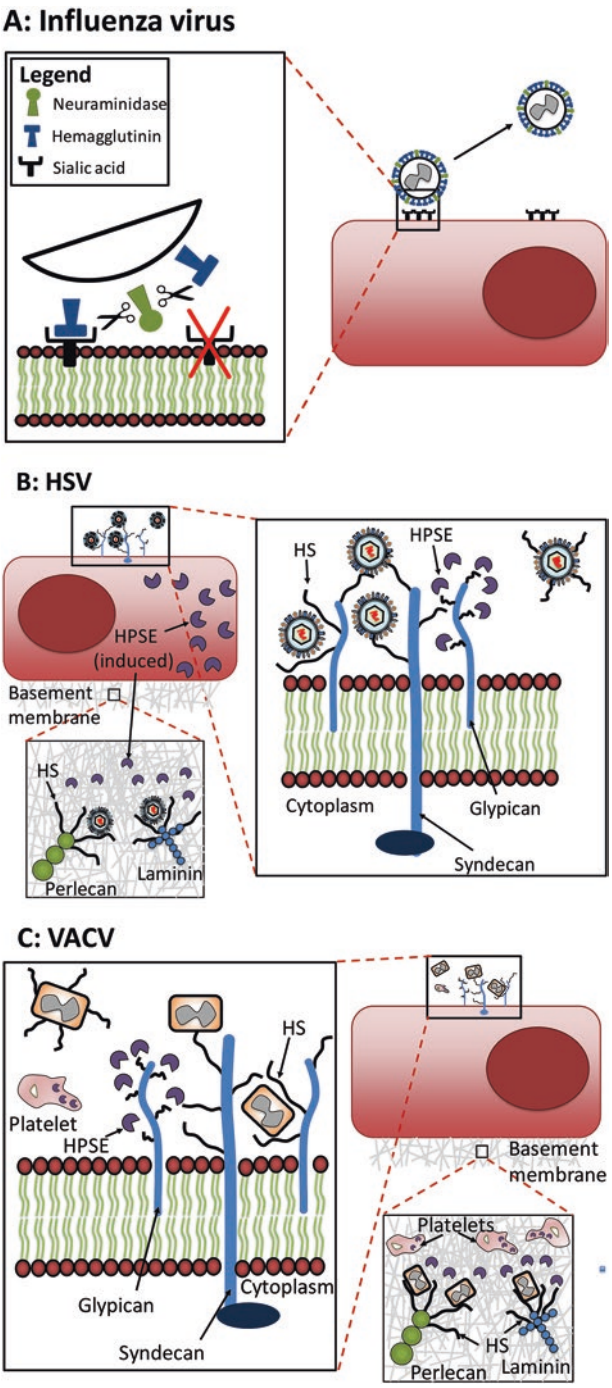

Fig. 3.2 (continued)

heparanase to degrade HS in the vicinity of the virus, although in this case, the heparanase must be host rather than virus derived.

In fact, herpes simplex virus-1 (HSV-1), an HS-binding virus, has been shown recently to induce the expression of heparanase in HSV-1 infected cells, the enzyme then allowing the localized spread of the virus (Fig. 3.2b) [103, 104]. We have undertaken similar studies with vaccinia virus (VACV), an HS-binding virus that very effectively spreads to distant organs. Using heparanase deficient mice we discovered that spread of the virus was substantially reduced and in some cases, depending on the inoculation site, delayed by up to 3 days (Fig. 3.2c) [105]. In contrast, heparanase deficiency had no effect on virus replication at the inoculation sites. Additional *in vitro* experiments showed that heparanase treatment released VACV from the ECM of infected HS-expressing cells but not from infected HS-deficient cells. We were also unable to find any evidence of VACV inducing heparanase expression in infected cells as was seen with HSV-1. In this case, we suggest that since VACV is highly cytopathic it attracts platelets to sites of infection, platelets being a rich source of pre-formed heparanase (Fig. 3.2c) [105]. It is also highly likely that VACV liberated from the ECM by heparanase carries HS-fragments that block any further interaction of the virus with the ECM. These findings suggest that heparanase inhibitors may interfere with the spread of HS-binding viruses, particularly if administered in the first few days after virus infection (Agelidis and Shukla, Chap. 32 in this volume).

3.4.6 Inhibitors of Heparanase Enzymatic Activity

Soon after heparanase had been implicated in tumor metastasis the search for heparanase inhibitors began, an endeavor that became more attractive following the discovery that there is only one heparanase gene in the mammalian genome. At the outset it was already known that heparanase enzymatic activity could be inhibited by heparin [10, 11], so initial inhibitors were derived from heparin and involved the production of heparin preparations with reduced anticoagulant activity, this being achieved by chemical modification [106, 107]. Further development of heparin-based inhibitors lacking anticoagulant activity has ensued since these early studies, although structural heterogeneity of heparin has made quality control difficult [50]. Sulfated polysaccharides were also identified that inhibit tumor metastasis via heparanase inhibition [108, 109], these studies leading to the production of sulfated oligosaccharides or HS mimetics with substantial heparanase inhibitory activity but much better safety profiles than sulfated polysaccharides [110]. Totally synthetic HS mimetics were also developed, such as sulfated linked cyclitols [111] and variants of suramin, polysulfated naphthyl urea, that have a better safety profile than the parent compound [112].

PI-88 (Muparfostat) resulted from the sulfated oligosaccharide approach and is the first heparanase inhibitor that has reached Phase III clinical trials (reviewed in [113, 114]). It contains a mixture of sulfated mannose-based oligosaccharides (Fig. 3.3) [110]. It was designed to simultaneously inhibit heparanase and a number of HS-binding pro-angiogenic growth factors, in the case of growth factors the drug blocking the cross-linking of growth factor/growth factor receptors by HS. PI-88

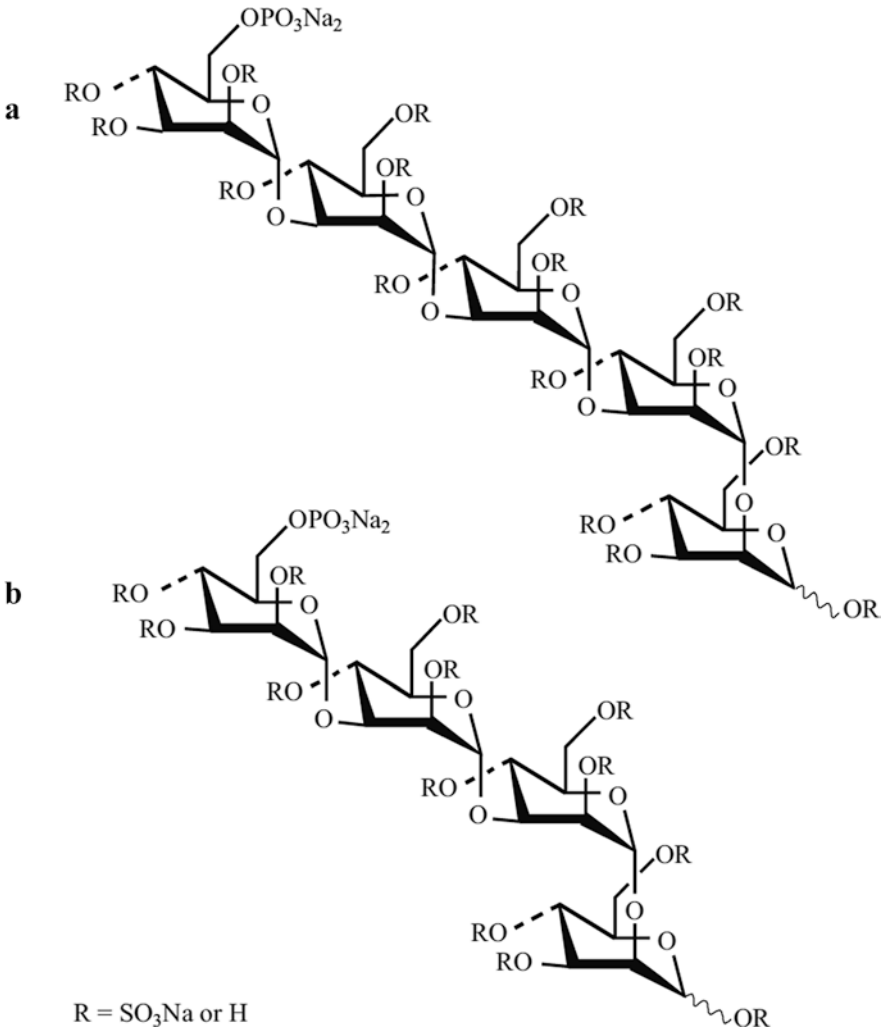

Fig. 3.3 The chemical structure of PI-88, the first heparanase inhibitor to reach a Phase III clinical trial. PI-88 is composed predominantly (~90%) of **(a)** phosphomannopentaose and **(b)** phosphomannotetraose sulfates, with the ratio between the pentasaccharide and tetrasaccharide ranging from approximately 2:1–3:2. This structural heterogeneity resulted in a drug that simultaneously inhibits heparanase and the pro-angiogenic activity of a number of HS-binding growth factors) (Figure reproduced from Khachigian and Parish [113] with permission)

has also been shown to block the enzymatic activity of endoglucosamine 6-sulfatases, these enzymes having pro-angiogenic activity [115]. The structural heterogeneity of PI-88 potentially increases the number of HS-binding proteins it interacts with and, thereby, reduces the chances of treatment escape variants arising in cancer patients. The most impressive preclinical data was obtained in the RIP-Tag2 tumor model, a mouse model of multistage pancreatic islet carcinogenesis. It was found that PI-88 acted at several stages of carcinogenesis from the formation of early progenitors to

invasive carcinomas, the drug inhibiting tumor cell proliferation, increasing tumor cell apoptosis, impairing angiogenesis and, ultimately, reducing the number of invasive carcinomas [94]. Based on this excellent preclinical data and acceptable Phase I clinical trial safety, the drug entered a randomized Phase II clinical trial in hepatocellular carcinoma patients, the drug exhibiting preliminary efficacy as adjuvant therapy following tumor resection [116]. A subsequent Phase III clinical trial, however, failed to significantly improve disease-free survival (DFS) in the overall treatment group but did significantly prolong DFS in the microvascular invasion group that constituted 40% of the trial population and includes patients with the poorest prognosis [117, 118]. The drug was also shown to have a good safety profile. These data indicate that Muparfostat has potential as a mono-therapy but, as an anti-metastatic and anti-angiogenic drug, is much more likely to exhibit efficacy when combined with other anti-cancer agents, such as checkpoint inhibitors. A second generation PI-88, PG545, has been developed that is a cholesterol-conjugated maltotetraose sulfate that has a much longer half-life *in vivo* than PI-88 and is structurally more homogeneous [119, 120]. PG545 has shown efficacy in acute kidney injury as a nephroprotective agent [121], inhibits colon cancer initiation and growth [122] and is a potent anti-lymphoma drug [123]. For more information see Chapters by Chhabra and Ferro; Hammond and Dredge; and Abassi and Goligorsky, Chaps. 19, 22 and 28 in this volume.

More recently a plethora of heparanase inhibitors have been identified based on high throughput screening of small molecule libraries and of natural products (reviewed in [124]. In addition, there have been successful approaches using biologicals, such as peptides [125] and oligonucleotide-based inhibitors [126], neutralizing antibodies [127], RNA interference [128] and immunotherapy [129] (Fig. 3.4).

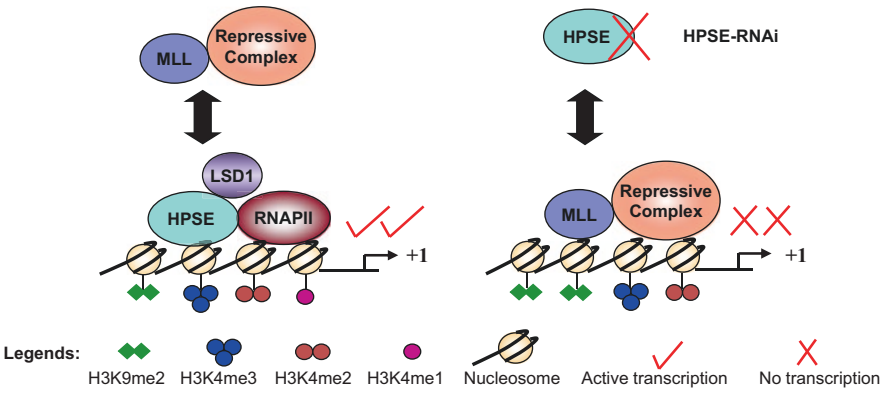

Fig. 3.4 Proposed model of the interplay of nuclear heparanase (HPSE) with LSD1, RNAP II, MLL, and histone methylation marks. In the left schematic, heparanase displaces the MLL-Repressive Complex and recruits the demethylase LSD 1 and RNAP II to the promoter of genes and imposes histone methylation marks that result in transcription. In contrast (right schematic), in the absence of heparanase (induced by heparanase-specific RNAi in this case), the methyltransferase MLL-Repressive Complex binds to gene promoters and changes the histone methylation marks such that transcription is halted

LSD1: lysine-specific histone demethylase 1; MLL: mixed lineage leukemia methyltransferase; RNAP II: RNA polymerase II (Figure reproduced from He et al. [148] with permission)

Despite all this activity, however, PI-88 (Muparfostat) appears to be the only heparanase inhibitor that has reached Phase III clinical trials, a drug that was developed over 20 years ago and is now off patent, the original PI-88 patent being filed in 1995 (Chhabra and Ferro, Chap. 19).

3.5 Functions Independent of Heparanase Enzymatic Activity

3.5.1 Cell Adhesion Molecule

The first evidence that heparanase performs functions independent of its enzymatic activity was reported in 1995, prior to the cloning and characterization of heparanase [130]. In this study, it was shown that at neutral pH, which is suboptimal for the enzymatic activity of heparanase, the enzyme acted as a cell adhesion molecule for $CD4^+$ T cells. In contrast, at acidic pH that is optimal for the glycolytic activity of the enzyme, heparanase aided $CD4^+$ T cell invasion of the ECM. In a related investigation in 2003, expression of heparanase in non-adherent lymphoma cells resulted in the cells becoming adherent and migratory regardless of whether the cells were transfected with either active or point mutated inactive enzyme, i.e., active site residues Glu^{225} and Glu^{343} were mutated [131]. This observation immediately implied that heparanase is able to act as a cell adhesion molecule, independent of its capacity to be an endoglycosidase, with other analyses correlating enhanced adhesion and migration of heparanase transfected cells with β1-integrin and Rac activation [131–133].

3.5.2 Promoter of Signal Transduction

At the same time that the cell adhesion results were being obtained using heparanase transfected cell lines, it was noted that exogenous addition of heparanase to endothelial cells resulted in enhanced protein kinase B (Akt) phosphorylation that was independent of cell surface HS and heparanase enzymatic activity [134]. Subsequent investigations (reviewed in [135]) found that enzymatically inactive heparanase was able to aid proliferation and survival of cancer cells by not only activating the signaling molecule Akt, but also other molecules such as signal transducer and activator of transcription (STAT), steroid receptor co-activator (Src) and extracellular signal-regulated kinase (Erk), as well as hepatocyte growth factor, insulin-like growth factor and epidermal growth factor receptor (EGFR) [50, 135]. Furthermore, this signaling function is mediated by the C-terminal domain of heparanase, which is totally devoid of endoglycosidase activity, overexpressing this domain in cancer cells augmenting signaling pathways and tumor growth [136–138]. A recent study has also shown that targeting either heparanase or the C-terminal domain of heparanase to mammary epithelium increases both mammary gland

development and tumor growth and metastasis [139]. Another recent report has revealed that heparanase is required for the activation and function of macrophages [140]. Collectively, these studies indicate that heparanase is a remarkably versatile molecule and a major facilitator of many aspects of inflammation and tumor progression, not just endoglycosidase-mediated leukocyte migration, tumor metastasis and angiogenesis.

3.5.3 Transcription Factor

A number of studies have detected heparanase in the nucleus of cells [141–144]. In fact, in patients with lung, neck and gastric cancers, the presence of nuclear heparanase is associated with a favorable prognosis, whereas patients with cytoplasmic heparanase have a poor survival [145]. There is also some evidence that nuclear heparanase expression is linked to cell differentiation [142–144, 146, 147]. Based on these observations we examined whether nuclear heparanase can regulate transcription in resting and activated T lymphocytes, using the human Jurkat T cell line as a well-researched model of T cell activation [148]. Initially, we noted that heparanase associated with transcriptionally active euchromatin, with T cell activation resulting in increased localization of heparanase to the nucleus, and heparanase being recruited to both the promoter and transcribed regions of a unique subset of transcriptionally active genes. Knockdown and overexpression studies showed that heparanase is required for the transcription of a number of immune response genes by associating with the lysine-specific histone demethylase 1 (LSD1), preventing recruitment of the mixed lineage leukemia (MLL) methyltransferase and, consequently, modifying the methylation pattern of histone H3, allowing recruitment of RNA polymerase II and transcription of the genes [148]. A schematic model of this process is depicted in Fig. 3.4. Based on these data it is clear that heparanase can enter the nucleus of cells, associate with both the promoter and transcribed region of a number of transcriptionally active genes, and enhance transcription via changing the methylation state of histone H3.

3.6 Future Perspectives

3.6.1 How Does Heparanase Initiate Signalling Pathways?

Although several studies have shown that the addition of exogenous heparanase to cells results in the induction of signaling pathways the actual cell surface receptor(s) that bind heparanase and initiate this response have not been identified. A number of receptors, however, have been defined that mediate the endocytosis and targeting of exogenous heparanase to lysosomes, namely the HS side chains of syndecan-1 [52], low-density lipoprotein receptor-related protein [149] and the cation-independent

mannose-6-phosphate receptor [150]. The rate of uptake of heparanase via these lysosome-targeting receptors is very high, which raises the possibility that leakage of a small percentage of endocytosed heparanase into the cytoplasm of cells could be sufficient to activate signal transduction pathways. If this hypothesis is correct it implies that heparanase 'hitches a ride' into the cytoplasm of cells and then interacts directly with signaling pathway molecules, rather than binding to and activating a specific cell surface receptor on plasma membranes.

3.6.2 Do Nuclear Heparanase and HS Interact?

It is well established that both heparanase and HS/HSPG can enter the nucleus of cells and, in both cases, influence transcription (see Sect. 3.5.3 and review [44]). There is, however, no information about whether or not these two molecules interact with each other in the nuclei of cells. We have already shown that heparanase is recruited to both the promoter and transcribed regions of a unique subset of genes that are transcriptionally active. Whether HS interacts with a similar subset of genes and either enhances or suppresses transcription would be of particular interest, with changes in the methylation status of histone H3 being the likely outcome of such an interaction. There are also studies showing that the structure of nuclear HS changes during the cell cycle [44]. How such changes influence the effects of HS on transcription would be a research area worth pursuing. It also appears that nuclear heparanase can be enzymatically active [44] and, therefore, would be able to liberate HS fragments from HSPG molecules that have entered the nucleus. It would be interesting to see whether these HS fragments are more able to interact with transcription factor complexes than HS chains linked to HSPGs.

3.6.3 Relationship Between Heparanase-1 and Heparanase-2

One of the most exciting recent development in the heparanase field is the realization that heparanase-2 can interact with heparanase-1, both directly and indirectly, and counteract many of the biological effects of heparanase-1 (reviewed in [37]). When heparanase-2 was first cloned in 2000 it was regarded as a distant relative of heparanase-1 and also, based on mRNA expression, appeared to have a different cellular distribution pattern to heparanase-1 [36]. Subsequent studies, however, have revealed that although heparanase-2 has no glycosidase activity it inhibits the enzymatic activity of heparanase-1, suppresses tumor growth and angiogenesis, and maintains cells in a differentiated state. Thus, heparanase-2 appears to counteract the pro-tumorigenic properties of heparanase-1 and behaves like a tumor suppressor. There is much to be done to understand the molecular basis of this intriguing interaction and eventually harness it for the development of new therapies. For further information on Hpa 2, see Chapters by E. Mckenzie and by Roberts and Woolf, Chaps. 34 and 35 in this volume.

3.6.4 Drug Development: Where to Next?

The discovery that there is only one heparanase gene in the mammalian genome and, thus, only one endoglycosidase that can degrade ECM HS, made heparanase a very attractive target for drug development. The subsequent finding, however, that heparanase deficient mice are essentially normal was a surprise, particularly as heparanase has been identified as a major contributor to many biological processes, not just tumor metastasis and angiogenesis. Certainly, these developments have made highly specific inhibitors of heparanase enzymatic activity much less attractive as cancer therapeutics. In fact, in heparanase knockout mice, upregulation of certain matrix metalloproteinases (MMP) occurs which probably aid migration of cells through basement membrane barriers and compensate for heparanase loss [55, 151]. Based on this conclusion, it would be expected that heparanase inhibitors would rapidly select for tumors that exploit this evasion strategy. There are also recent studies that indicate enzymatic degradation of basement membranes is not the only way cells can navigate their way through basement membrane barriers, cells passing through preformed entry and exit sites in basement membranes and/or providing mechanical forces that trigger basement membrane breaches (reviewed in [152]). Nevertheless, there may be specific situations where inhibitors of the enzymatic activity of heparanase are effective therapeutics, the most likely being localized inflammatory responses. Also, it appears that the microenvironment of tumors is a rich source of heparanase so targeting heparanase inhibitors to these sites, which are non-cancerous and, consequently, less prone to developing treatment resistance, is worthy of investigation.

On the other hand, the encouraging clinical trial data obtained with the HS mimetic PI-88 demonstrates that this class of drug has potential as an anti-cancer treatment, a key feature of PI-88 being that multiple HS-dependent processes were simultaneously targeted, not just inhibition of the enzymatic activity of heparanase. Similar considerations hold for PG545 (= Pixatimod) and SST0001 (= Roneparstat). Combining HS-mimetics with other, complementary therapies, such as checkpoint-inhibitors and conventional cytotoxic drugs also deserves attention. The discovery that heparanase can act as a promoter of signaling pathways and as a transcription factor obviously opens up the possibility of completely different heparanase-based therapeutics, although how these will be identified will be a major challenge. Finally, the demonstration that heparanase-2 counteracts the multifaceted pro-tumorigenic properties of heparanase-1 raises the possibility of therapeutics that enhance the tumor suppressive activities of heparanase-2. Inhibition of the pro-inflammatory features of heparanase, which may or may not have anti-tumor effects, is another factor that needs to be considered when developing heparanase-based therapeutics. Thus, despite heparanase inhibitors being developed for almost 40 years, overall heparanase still represents an attractive target for multiple therapeutic strategies.

3.7 Concluding Remarks

There is now overwhelming evidence that heparanase belongs to that rapidly expanding class of proteins that perform multiple tasks and are termed 'multifunctional proteins' [153]. In the case of heparanase, multifunctionality is evident from the ability of the molecule to simultaneously act as an endoglycosidase, a signaling molecule and a transcription factor (Table 3.1). But since the substrate specificity of heparanase is HS this results in the enzymatic activity of the molecule also having multiple functional effects. As discussed earlier in Sect. 3.3, over 300 proteins interact with HS and as a result of this interaction often form oligomers in 3-dimensional space that would be disassembled following exposure to heparanase, resulting in a diverse range of functional consequences. Another unique feature of heparanase is that it is present in multiple locations within and outside cells, namely in the ECM, on cell surfaces, in the cytoplasm, within lysosomes, endosomes and exosomes and, finally, within the nuclei of cells. In fact, an analysis of 235 immune-related proteins that could potentially bind HS revealed that heparanase was *the only HS-binding protein* that is found at intracellular, extracellular and plasma membrane locations [154]. Based on these findings it is not surprising that heparanase influences so many biological processes and, for example, is claimed to promote all aspects of tumour development [37]. Such discoveries indicate that heparanase research has a bright future indeed!

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