EXT proteins: Role in heparan sulfate Assembly and in Tumor biology

Lawrence Fred Sembajwe

Thesis for the Degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

The work outlined in this thesis was done under the matrix biology group at the Department of Biomedicine – University of Bergen, under the supervision of Prof. **Marion Kusche-Gullberg**. Most of the experiments were conducted using facilities at the department such as equipment in the general laboratory and the cell-culture room. Flow cytometry experiments were done at the Department of Clinical Laboratory and microarray analyses at the Genomic Core Facility, University of Bergen.

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Finally, I dedicate this dissertation to my Mother – Teopista Nantume for her support throughout my many years of education. It seems her 'Joker' has made yet another surprising achievement! ☺ ☺ ☺

Abbreviations:

APC: Allophycocyanin

C1/C2: Constant or Conserved regions 1 and 2

CS: Chondroitin sulfate

DEAE: Diethyl amino ethyl

DMEM: Dulbecco's modified Eagles medium

ECM: Extracellular matrix

EFYA: Amino acids – Glutamine, Phenylalanine, Tyrosine and Alanine

ER: Estrogen receptor

ERK: Extracellular regulated kinase

EXT: Exostosin

EXTL: Exostosin like

FGF: Fibroblast growth factor

GAGs: Glycosaminoglycans

GlcA: Glucuronic acid

GlcNAc: N-acetylglucosamine

Gt: Gene trap

HA: Hyaluronan

HAS: Hyaluronan Synthase

HER2: Human epidermal growth factor receptor-2

HPRT: Hypoxanthine guanine phosphoribosyl transferase

HS: Heparan sulfate

HSPG: Heparan sulfate proteoglycan

HPLC: High performance liquid chromatography

KO: Knockout

MAPK: Mitogen Activated Protein Kinase

MO: Multiple Osteochondromas

NSCLC: Non-small cell lung cancer

PBS: Phosphate buffered saline

PDZ: Postsynaptic Density (PSD-9) protein/, Drosophila melanogaster-Disc-Large/, Zona Occludens

PG: Proteoglycan

POLR2F: RNA polymerase II subunit F

PR: Progesterone receptor

RPMI: Roswell Park Memorial Institute

RTK: Receptor Tyrosine Kinase

RTqPCR: Reverse transcriptase, quantitative real time polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

VEGFA: Vascular Endothelial Growth Factor-A

VEGFR: Vascular Endothelial Growth Factor Receptor

Wnt: Wint

Wt: Wild type

Abstract

The exostosin family of proteins is necessary for heparan sulfate biosynthesis. Heparan sulfate polysaccharide biosynthesis is one of several different forms of protein-glycosylation processes that take place in the Golgi apparatus. Heparan sulfate modification of a few selected proteins results into the formation of heparan sulfate proteoglycans that are found on cell surfaces, inside the cell and in extracellular matrices. The heparan sulfate chains of the proteoglycans bind a wide range of molecules such as growth factors, serine protease inhibitors and extracellular matrix proteins, thereby influencing a number of cellular processes including cell-signaling and inter-cellular communication. The heparan sulfate chains are important in development, homeostasis and in pathogenesis of various diseases including cancer.

Whereas the activity of the exostosin family of proteins is deemed pivotal for functional heparan sulfate chains, the roles of each of the exostosin members and their mechanisms of action or interaction are not well known. Thus, in this thesis we aimed at generating more knowledge about the role of the exostosin proteins. We used gene expression profiling techniques to study the expression of exostosin genes in both normal and cancer cell-lines. We analyzed the effect of exostosin-1 deficiency in stromal fibroblasts on gene expression in co-cultured A549-carcinoma cells. Our data suggests a new potential role of the exostosin-1 protein in influencing tumor behavior through Tgf-β1. We also investigated the link between the expression levels of the exostosin family of genes and HS-structure in non-malignant and breast cancer epithelial cell-lines. The results of this investigation showed no direct correlation between gene expression of the exostosin family members and heparan sulfate-structure. In addition, we studied the effect of pH on the *in vitro* glycosyltransferase activity of Exostosin-1 and Exostosin-2 proteins. We observed decreased enzyme activities with reduced pH values.

List of publications

Paper I

Kirankumar Katta*, **Lawrence F. Sembajwe***, Marion Kusche-Gullberg

Potential role for Ext1-dependent heparan sulfate in regulating P311 gene expression in A549 carcinoma cells. BBA - General Subjects 1862 (2018) 1472–1481

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Paper II

Lawrence F. Sembajwe, Kirankumar Katta, Mona Gronning and Marion Kusche-Gullberg

The exostosin family of glycosyltransferases: mRNA expression profiles and heparan sulfate structure in human breast carcinoma cell-lines – *Manuscript submitted*

Paper III

Lawrence F. Sembajwe, Mona Grønning and Marion Kusche-Gullberg

Effect of pH on glycosyltransferase activity of heparan sulfate elongating-EXT1 and EXT2 proteins. *Manuscript*

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1. Introduction

1.1 General background

Heparan sulfate (HS), a polysaccharide closely related to heparin, has been implied in a large number of functions in normal physiology and in pathology (Bishop, Schuksz, & Esko, 2007; Gallagher, 2015; J. P. Li & Kusche-Gullberg, 2016). HS polysaccharide chains are covalently attached to different proteins in a proteoglycan (PG) molecule (Couchman, 2010). HSPGs are present throughout evolution and are synthesized by every mammalian cell. HSPGs are very important for normal development and hemostasis through their roles in mediating protein-protein interactions between for example signaling factors and their cognate receptors (Bishop et al., 2007). Heparin is a highly sulfated variant of HS synthesized by a subset of mast cells (Casu, Naggi, & Torri, 2015; Kolset & Pejler, 2011). Heparin has since 1930s been used clinically as an anticoagulant (Casu et al., 2015). The physiological role of heparin in the mammalian body is not known and its function is probably not related to coagulation.

HSPGs are via their HS chains involved in a diverse number of biological events related to intracellular signaling, cell-cell interactions and tissue morphogenesis (Bishop et al., 2007; Blanchette, Thackeray, Perrat, Hekimi, & Benard, 2017; Gallagher, 2015; J. P. Li & Kusche-Gullberg, 2016). In this way, the HS chains influence not only normal cell homeostasis but also the behavior of cancer cells (Lim, Multhaupt, & Couchman, 2015). The diverse functions of HS are dependent on the fine structure of HS chains that are formed during its biosynthesis. HS biosynthesis involves a number of different enzymes with cell and tissue specific expression that is believed to determine the HS structure (J. P. Li & Kusche-Gullberg, 2016).

The HSPGs constitute an important part of the extracellular matrix (ECM), either in the pericellular environment of cells or as solubilized and freely secreted molecules. HSPGs such as agrin, collagen XVIII and perlecan are generally found in the basement membrane (a specialized ECM) but may also be secreted into the interstitial fluid (McCarthy, 2015). The shedding of cell surface HSPGs by proteolytic enzymes generates soluble HSPGs that can inhibit protein interactions at the cell surface or execute their function somewhere else (Manon-Jensen, Itoh, & Couchman, 2010).

The studies presented in this thesis focused on the HS chain elongating enzymes belonging to the so-called exostosin family of proteins and how they influence HS structure and function

in tumor biology. In order to dissect the role of HS chains in pathological conditions, their mode of function in normal physiology need to be well understood. Therefore, we also studied the glycosyltransferase activities of EXT proteins under varying pH conditions.

1.2 Proteoglycans

Proteoglycans (PGs) consist of one or more unbranched sulfated polysaccharide chains called glycosaminoglycans (GAGs) covalently attached to a core protein (J. P. Li & Kusche-Gullberg, 2016).

1.2.1 Glycosaminoglycans [GAGs]

Glycosaminoglycans (GAGs) are long and unbranched polysaccharides consisting of repeating disaccharides, composed of alternating hexuronic acid: glucuronic acid (GlcA) or iduronic acid (IdoA) and hexosamine: glucosamine (GlcNAc) or galactosamine (GalNAc) (Prydz & Dalen, 2000). Depending on the nature of the repeating disaccharides, GAGs are divided into four main groups: heparan sulfate (HS), chondroitin/dermatan sulfate (CS/DS), hyaluronan/ hyaluronic acid (HA) and keratan sulfate (KS) (**Figure 1**). HS chains consist of an alternating sequence of GlcNAc and uronic acid (GlcA or IdoA) (Prydz, 2015). The repeating disaccharide in CS is made of GalNAc and GlcA. A CS chain containing GlcA residues that have been epimerized to IdoA is called DS (Prydz & Dalen, 2000). HA is composed of repeating GlcA and GlcNAc units. Keratan sulfate lacks the hexuronic acid moiety and is composed of alternating galactose and GlcNAc units (Prydz, 2015). With the exception of HA, all the other GAGs are sulfated and found covalently attached to a core-protein forming a PG structure. Hybrid PGs are formed when different types of GAG-chains are attached to the same core protein (Couchman, 2010).

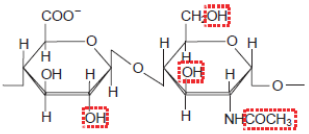
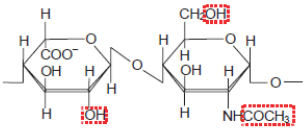
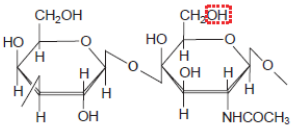
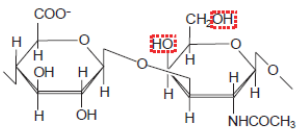
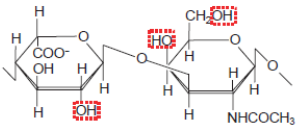
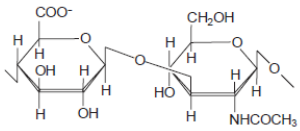
GAG	Hexuronic or Iduronic acid	Galactose	Hexosamine	Disaccharide composition
Heparan sulphate/ Heparin	D-glucuronic acid **(GlcA)** L-iduronic acid **(IdoA)**	-	D-glucosamine **(GlcNAc)**	 **GlcA β(1→4) GlcNAc α((1→4)**  **IdoA α(1→4) GlcNAc α(1→4)**
Keratan sulphate	-	Galactose **(Gal)**	D-glucosamine **(GlcNAc)**	 **Gal β(1→4) GlcNAc β(1→3)**
Chondroitin sulphate	D-glucuronic acid **(GlcA)**	-	D-galactosamine **(GalNAc)**	 **GlcA β(1→3) GalNAc β(1→4)**
Dermatan sulphate	D-glucuronic acid **(GlcA)** L-iduronic acid **(IdoA)**	-	D-galactosamine **(GalNAc)**	 **IdoA β(1→3) GalNAc β(1→4)**
Hyaluronic acid	D-glucuronic acid **(GlcA)**	-	D-glucosamine **(GlcNAc)**	 **GlcA β(1→3) GlcNAc β(1→4)**

Figure 1. Structure of the different glycosaminoglycan chains. The structure of the repeating disaccharides in the different types of glycosaminoglycan chains is drawn without sulphation. The different sulphation positions in each GAG are marked by encircling with a dashed red line. Adopted from *(Prydz & Dalen, 2000)* with permission.

1.2.2 Heparan sulfate proteoglycans (HSPGs)

Heparan sulfate proteoglycans are found distributed on the cell surface with members such as syndecans and glypicans (Multhaupt, Leitinger, Gullberg, & Couchman, 2016), in the ECM (e.g. perlecan and collagen XVIII) (Iozzo, Zoeller, & Nystrom, 2009) and intracellular (serglycin) (Kolset, Prydz, & Pejler, 2004). Collagen XVIII and the cell surface associated PG CD44 are referred to as 'part-time proteoglycans' because they exist with or without covalently added HS chains (Fjeldstad & Kolset, 2005; Ponta, Sherman, & Herrlich, 2003).

The major cell surface proteoglycans - syndecans and glypicans differ in the nature of their association with the cell membrane (**Fig. 2**): the syndecans are transmembrane PGs, (Couchman, 2010) whereas the glypicans are anchored onto the cell membrane by a short glycosylphosphatidylinositol (GPI) anchor (Filmus, Capurro, & Rast, 2008). Perlecan is primarily found in the basement membrane but may also exist outside the basement membrane in the interstitial connective tissue. Perlecan has a complex core protein with HS chains covalently attached toward the N-terminal end, while the C-terminal end contains a short regulatory protein-fragment called endorepellin (Iozzo & Sanderson, 2011). In smooth muscle cells, the HS chains of perlecan are substituted with CS chains (Lord et al., 2014). Examples of PGs that may occur as hybrid PGs are syndecan-1 and syndecan-3 that can carry both HS and CS-chains (Couchman, 2010) and aggrecan that carries both CS and KS chains (Aspberg, 2012).

Serglycin is a unique intracellular PG carrying heparin chains and/or chondroitin sulfate chains. Serglycin is found in secretory granules of hematopoietic cells (Schick et al. 2001).

1.2.3 Glypicans

There are six members of the human glypican family: glypican 1-6 (Filmus et al., 2008). Glypicans are further subdivided into two subfamilies consisting of: (a) glypican-1, -2, -4, -6 and (b) glypican-3 and -5, with about 25% amino-acid identity between the two groups (Filmus et al., 2008). They carry at least one or two HS chains covalently attached to the core protein close to the cell membrane where the carboxyl terminal of the core protein is linked to the cell surface by a GPI anchor (Filmus et al., 2008). Just like other membrane associated HSPGs, glypicans influence growth factor signaling via the HS chains by formation of ternary

complexes with growth factors and corresponding growth factor receptors required for Wnt (Gao et al., 2014), Hedgehog (Capurro et al., 2008) and FGF (Jen, Musacchio, & Lander, 2009) signaling.

1.2.4 Syndecans

There are four members in the human syndecan family, syndecans-1 to -4 (**Fig. 2**) which are characterized by HS or CS chains covalently attached to the ectodomain of the core protein (Mitsou, Multhaupt, & Couchman, 2017). Syndecan-1 and syndecan-3 may carry both HS and CS chains, whereas syndecan-2 and syndecan-4 exclusively have HS chains (Afratis et al., 2017).

Syndecan core proteins consist of a short intracellular domain, a small transmembrane domain and variable length extracellular domains (ectodomains) (Couchman, 2010). The cytoplasmic domain is subdivided into three parts: two conserved regions - C1 and C2, which are similar in all syndecans and are separated by one variable region (V) that is specific for each syndecan isoform (Couchman, 2010). The conserved region C1 is involved in endocytosis and exosome formation whereas C2 has a terminal amino acid sequence - EFYA region (glutamine, phenylalanine, tyrosine and alanine), capable of binding PDZ-domain carrying proteins (Cheng, Montmasson, Terradot, & Rousselle, 2016). There is formation of signaling complexes around the EFYA region including adaptor proteins and kinases such as Src, Rac and Rho. The activation of such proteins triggers important down-stream signaling pathways that may result into cytoskeletal reorganization, cell migration, cell survival and proliferation (Cheng et al., 2016).

The tissue distribution of mammalian syndecans is developmental-stage, cell-type, and tissue-specific (Cheng et al., 2016). Syndecan-1 and -4 have the widest distribution in the body, whereby syndecan-1 is expressed in epithelial cells and syndecan-4 in almost all cells. Syndecan-2 is expressed in endothelial, fibroblasts, liver and neuronal cells (Horiguchi et al., 2013). Syndecan-3 has a limited distribution and is predominantly expressed by nerve and skeletal muscle cells (Cheng et al., 2016; Cornelison, Filla, Stanley, Rapraeger, & Olwin, 2001).

Syndecans may be modified through protease/heparanase activity or internalized into the cytosol where they are degraded in lysosomal vesicles. In addition, translocation of syndecans into the nucleus has been reported (Stewart, Ramani, & Sanderson, 2015). Proteases including members of the matrix metalloproteinase family cleave syndecan core proteins resulting into release (shedding) of the syndecan ectodomain (Manon-Jensen et al., 2010; Manon-Jensen, Multhaupt, & Couchman, 2013). The heparanase enzyme cleaves HS chains rendering them solubilized in the ECM (Stewart et al., 2015; Vlodavsky, Ilan, Naggi, & Casu, 2007; Vlodavsky et al., 2016). Generally, syndecans have been reported to be involved in mediating cell-cell, cell-ECM interactions and cell-pathogen interactions where they facilitate viral attachment to a cell before eventually penetrating the cell membrane (Cheng et al., 2016).

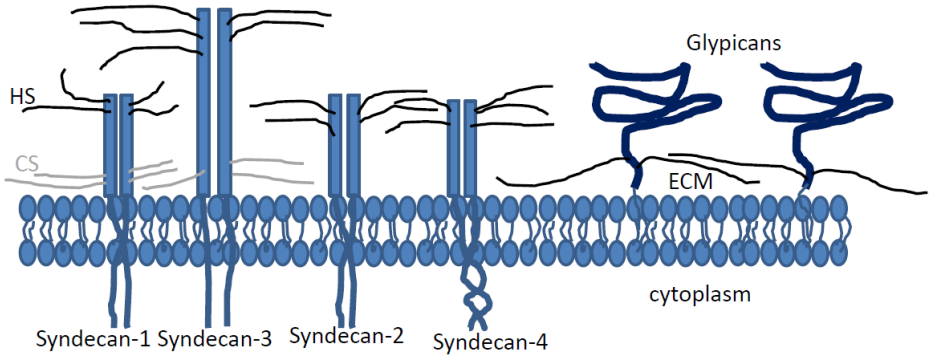


Figure 2. A schematic illustration of cell surface HSPGs: syndecans and glypicans (Modified with permission from a review by *(Couchman, 2010).*

1.2.5 Perlecan

Perlecan is a large basement membrane-associated HSPG composed of five different domains or subunits (Cohen, Grassel, Murdoch, & Iozzo, 1993). Perlecan is the main basement membrane proteoglycan but can also be found in the interstitial space. It is the largest of all proteoglycans, with a core protein of about 500 kDa that can be modified by attachment of up to three HS chains estimated to be 65 kDa each (Kallunki & Tryggvason, 1992; Noonan et al., 1991). Its five domains have various functions and are numbered I-V (**Fig. 3**) starting from

the N-terminal end to the C-terminal end (Kallunki & Tryggvason, 1992; Murdoch, Dodge, Cohen, Tuan, & Iozzo, 1992).

The first domain (I) is the N-terminal end that is commonly modified by HS chains and more rarely with CS chains (Lord et al., 2014). Domain (I) is involved in growth factor sequestration, co-receptor functions and angiogenesis. The second domain (II) is involved in lipid retention. The third domain (III) is involved in attachment to cell surface and secretion into the ECM. The fourth domain (IV) is involved with scaffold formation and maintenance of ECM integrity. The fifth and last domain (V), also known as endorepellin, is involved in cell surface binding, angiogenesis inhibition (Iozzo & Sanderson, 2011), cytoskeleton disassembly and induction of autophagy (Goyal, Gubbiotti, Chery, Han, & Iozzo, 2016; Goyal et al., 2012; Gubbiotti, Neill, & Iozzo, 2017; Poluzzi et al., 2014).

Perlecan is expressed in a wide range of tissues and involved in several physiological processes including cartilage morphogenesis, osteogenesis (Jochmann, Bachvarova, & Vortkamp, 2014), lymph node formation, lipid metabolism and cardiovascular-tissue development (Gubbiotti et al., 2017). Perlecan has been suggested to play a role in embryo implantation and placentation albeit with a significant level of functional redundancy due to presence of other HSPGs in the uterus (Farach-Carson & Carson, 2007). Clinically, perlecan is also involved in several pathological conditions such as inflammation, wound-healing and cancer angiogenesis that potentially define its significance in medicine as a possible drug target (Gubbiotti et al., 2017).

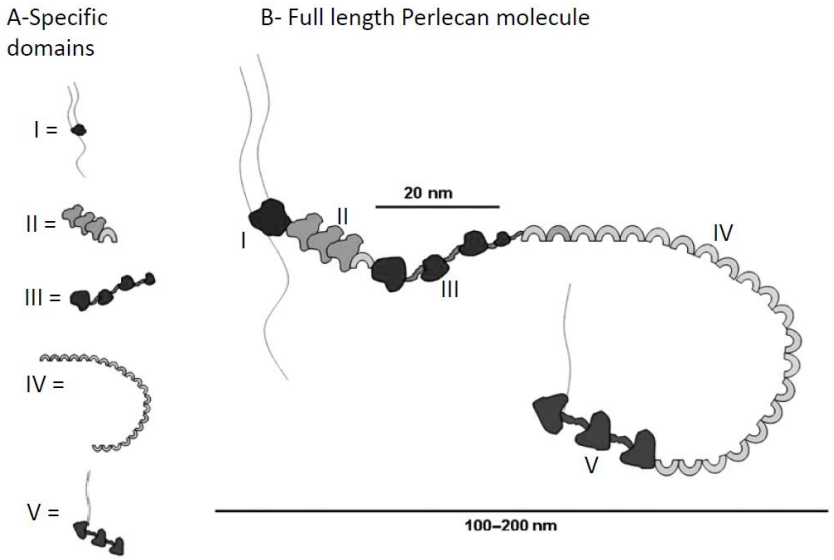


Figure 3. Perlecan structure. Composite scale model for intact human perlecan domains (A) based on available images obtained using rotary shadowing of individually expressed domains and atomic force microscopy. Unlike linear models, the molecule appears as a modular structure with both globular and extended regions (B) that support its function as an extracellular scaffold protein. Specific domains (A) and full-length perlecan molecule (B). (Adapted with permission from *(Farach-Carson & Carson, 2007)*.

1.2.6 Serglycin

Serglycin is an intracellular PG found in secretory vesicles of various cells including mast cells, hematopoietic cells, inflammatory cells, as well as some tumor cells (Korpetinou et al., 2014). Serglycin may also be secreted and become a constituent member of the ECM. The GAG chains that are attached to the serglycin core protein in connective tissue mast cells are heparin, but in other cell types such as mucosal mast cells and active macrophages heparin is substituted with CS (Kolset & Tveit, 2008).

There is continuous synthesis of serglycin in inflammatory and stromal cells in a regulated way. Most of the serglycin synthesized by non-stimulated macrophages is degraded while that synthesized by the activated macrophages is secreted (Scully, Chua, Harve, Bay, & Yip, 2012; Uhlin-Hansen et al., 1993). Serglycin synthesis may be upregulated by factors such as tissue necrosis factor, lipopolysaccharides and interleukin-1 (Imoto-Tsubakimoto et al., 2013; Lemire et al., 2007; Schick, Gradowski, & San Antonio, 2001; Zernichow et al., 2006).

1.2.7 CD44

CD44 is also known as a 'lymphocyte homing receptor' that enables circulating lymphocytes to adhere to specialized endothelial cells. It is a part time proteoglycan because CD44 can exist with or without covalently attached HS or CS chains (Goodison, Urquidi, & Tarin, 1999). It is expressed by various cells including epithelial cells, monocytes and melanocytes (Stamenkovic, Aruffo, Amiot, & Seed, 1991). CD44 can be found in healthy human tissues and in some tumors (Fox et al., 1994). CD44 occurs in several isoforms due to alternative splicing of exons within its ectodomain (Jackson et al., 1995). CD44 is the main hyaluronan receptor (Aruffo, Stamenkovic, Melnick, Underhill, & Seed, 1990).

1.3 Heparan sulfate biosynthesis

The biosynthesis of HS polysaccharide chains takes place in the Golgi apparatus starting with the assembly of a tetrasaccharide GAG-protein linker region composed of one xylose, two galactose units and one GlcA (GlcA-Gal-Gal-Xyl-O) (**Fig. 4**) attached to a selected serine residue in the core protein (Busse-Wicher, Wicher, & Kusche-Gullberg, 2014; Carlsson, Presto, Spillmann, Lindahl, & Kjellen, 2008). The entire assembly process is catalyzed by specific enzymes known as glycosyltransferases which are usually named according to the sugar residue that is added to the growing polysaccharide chain (Sugahara & Kitagawa, 2000). The attachment of xylose onto the serine residue is catalyzed by a xylosyltransferase enzyme; addition of the first galactose by galactosyltransferase I; the second galactose by galactosyltransferase II, and addition of the GlcA is by the glucuronyltransferase I (Sugahara & Kitagawa, 2000).

The addition of a GlcNAc residue to the tetrasaccharide linker region initiates HS chain elongation and is catalyzed by EXTL3 (having GlcNAc-transferase-I activity) (Busse-Wicher et al., 2014). The subsequent elongation of the chain by addition of alternating GlcA and GlcNAc residues is catalyzed by a complex of the exostosin proteins - EXT1 and EXT2, harboring both GlcNAc and GlcA-transferase-II activities (Busse et al., 2007) (**Fig. 4**). The HS chain polymerization is accompanied by a concomitant process of chain modification

reactions that start with the dual enzyme activity of the N-deacetylase/N-sulfotransferase (NDST). This enzyme removes N-acetyl groups from some GlcNAc residues and replaces them with N-sulfate groups (Cheung, Eriksson, Kusche-Gullberg, Lindhal, & Kjellen, 1996). This dual enzyme activity is responsible for creating intervals of N-acetylated (NA), N-sulfated (NS) and mixture of N-acetylated and N-sulfated (NA/NS) regions throughout the entire length of the HS chain (Cheung et al., 1996; Pikas, Eriksson, & Kjellen, 2000).

Due to the substrate specificity of the other HS modifying enzymes, all subsequent modifications occur within or in the vicinity of the N-sulfated regions (Kjellen, 2003). The modification by NDST is followed by activity of the C5-epimerase that switches the stereo-chemistry of GlcA at the C-5 position into IdoA (Hagner-Mcwhirter, Lindahl, & Li, 2000). Subsequently, different O-sulfotransferases transfer sulfate groups to the following positions: the sixth position of GlcN-residues, the C-2 position of IdoA and rarely the C-2 position of GlcA and the C-3 position of GlcN residues (Carlsson et al., 2008; El Masri, Seffouh, Lortat-Jacob, & Vives, 2016; Kusche-Gullberg & Kjellen, 2003). The enzymes appear to be expressed in a cell and tissue specific manner, with their activities presumably resulting in distinct HS structures characteristic for each cell and tissue type (Ledin et al., 2004). Because only a fraction of the potential substrates are modified at each modification step, the HS chains are composed of: unmodified NA-domains of -GlcA-GlcNAc-repeats; NS-domains rich in O-sulfate groups; and NA/NS domains with mixed N-acetylated and N-sulfated GlcN residues (Maccarana, Sakura, Tawada, Yoshida, & Lindahl, 1996; Pikas et al., 2000).

Further modifications of the HS chain can occur post biosynthetically by the action of extracellular sulfatases (Sulf1 and Sulf2) removing sulfate groups from 6-O-postions (Morimoto-Tomita, Uchimura, Werb, Hemmerich, & Rosen, 2002) and the heparanase enzymes cleaving the HS chains into smaller oligosaccharides (Hammond, Khurana, Shridhar, & Dredge, 2014; Vives, Seffouh, & Lortat-Jacob, 2014).

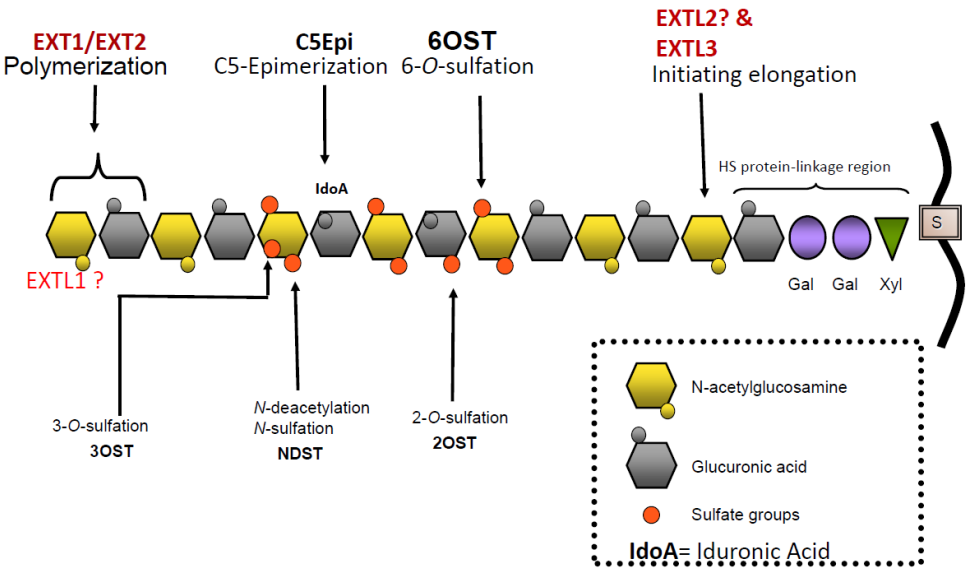


Figure 4. A schematic illustration of HS biosynthesis. The chain elongation from the tetrasaccharide linkage region is initiated by EXTL3 (and possibly EXTL2), and the polymerization is continued by the action of an EXT1/EXT2 complex (possibly also involving EXTL1). There is subsequent chain modification by NDST, C5Epi and O-sulfotransferases (OST) enzymes. This report focused on the chain elongating enzymes indicated in red. *(Figure courtesy of Marion Kusche-Gullberg).*

1.4 The EXT-family

The EXT acronym comes from the word exostosin which is the name used to describe an inherited bone disorder called hereditary multiple exostoses or osteochondromas (HME/MO) (Wuyts & Van Hul, 2000; L. Xu et al., 1999). This bone disorder is associated with autosomal dominant mutations in the gene of either EXT1 or EXT2 (Seki et al., 2001; Wuyts & Van Hul, 2000). The two proteins are part of a family of five members that in addition to EXT1 and EXT2 include the exostosin-like proteins - EXTL1, EXTL2 and EXTL3. The EXTL proteins belong to the EXT-family based on amino acid sequence homology with EXT1 and EXT2, but are not associated with MO (Busse-Wicher et al., 2014).

The genes encoding the EXT-family of proteins are found on the following chromosomal loci: 8q24.1 (*EXT1*) (Cook et al., 1993), 11p11-12 (*EXT2*) (Wuyts et al., 1995) and 19p (*EXTL1*), 1p11-p12 (*EXTL2*) (Wuyts et al., 1997) and 8p12-21 (*EXTL3*) (Van Hul et al., 1998). The expression of the *EXT* genes varies with the developmental stage, cell type and tissue type. The most widely expressed genes are *EXT1*, *EXT2*, *EXTL2* and *EXTL3* that are found in most mammalian tissues, whereas *EXTL1* has a more limited expression profile (Busse-Wicher et al., 2014). *EXTL1* is expressed in brain, heart and skeletal muscles (Wise, Clines, Massa, Trask, & Lovett, 1997).

The EXT-family members are type II trans-membrane proteins with a short N-terminal cytoplasmic region, a trans-membrane domain, a stem region, and a large C-terminal catalytic domain facing the Golgi lumen (**Fig. 5**) (Kellokumpu, Hassinen, & Glumoff, 2016). The physical characteristics of EXTL proteins have been demonstrated based on the crystallographic structures of EXTL2 (Pedersen et al., 2003) and EXTL3 (Awad, Kjellstrom, Svensson Birkedal, Mani, & Logan, 2018) that exhibit similarly conserved cysteine amino acid residues in their catalytic domains (Zak, Crawford, & Esko, 2002). EXTL2 is the smallest member with 330 amino acid (a.a) residues followed by EXTL1 (676 a.a.), EXT2 (718 a.a.), EXT1 (746 a.a.) and EXTL3 is the largest with about 919 a.a. residues (Busse-Wicher et al., 2014). The physiologically active HS chain elongation unit is believed to be a hetero-complex of EXT1/EXT2 that adds alternating GlcNAc and GlcA to the non-reducing end of the growing HS chain (Busse et al., 2007). Co-expressed EXT1 and EXT2 show increased *in vitro* transferase activities compared to the activities observed for EXT1 or EXT2 alone (McCormick, Duncan, Goutsos, & Tufaro, 2000; Senay et al., 2000), indicating the formation of a Golgi-localized hetero-complex. The EXTL proteins have also been suggested to take part in HS biosynthesis (Busse et al., 2007; Zak et al., 2002). EXTL3 initiates HS chain elongation by adding the first GlcNAc residue to the GAG-protein linker region (**Fig. 4**) (Busse et al., 2007). In addition, EXTL3 has been described to transfer a single GlcNAc unit to the non-reducing end of the HS chain, as part of chain elongation or alternatively, the added GlcNAc may serve as a stop signal for further chain elongation (Kim et al., 2001). The roles of EXTL1 and EXTL2 are not clearly defined, with some reports showing that EXTL2 may be involved in both HS chain initiation and chain termination (Moses, Oldberg, & Fransson, 1999; Nadanaka & Kitagawa, 2014; Nadanaka, Zhou, et al., 2013). EXTL2 has been proposed to terminate HS elongation by adding a GlcNAc residue to a transiently phosphorylated xylose on the tetrasaccharide linker region (Nadanaka, Zhou, et al., 2013). EXTL1 has been reported

to have some GlcNAc transferase activity linking it to HS-chain elongation (Kim et al., 2001). In addition to its direct role in HS elongation, EXTL3 has also been suggested to inhibit N-sulfation by forming a complex with NDST1, resulting in the formation of HS chains with N-unsubstituted glucosamine units (Nadanaka, Purunomo, Takeda, Tamura, & Kitagawa, 2014).

Despite the large number of research reports about HS biosynthesis, there are still some unanswered questions regarding (a) the specific role of each of the EXT proteins; (b) the relationship between the EXT1/EXT2 hetero-complex with the other HS-biosynthetic enzymes; and (c) what determines the actual length of an HS chain. Although the EXT1/EXT2 hetero-complex has been postulated as the main co-polymerase responsible for HS chain elongation (Busse et al., 2007), EXT2 has also been reported to interact with the HS modification enzymes NDST1 and NDST2 (Deligny et al., 2016; Presto et al., 2008). The role of EXT2 in HS chain elongation is unclear as its *in vitro* glycosyltransferase activities are weak. Due to its low transferase activities, it was speculated that EXT2 is not involved in the actual elongation of the HS backbone but serves as a chaperon and delivers EXT1 to the Golgi apparatus (Busse et al., 2007). The role of EXT2 in HS biosynthesis is further complicated by results indicating that EXT2 also binds to the modifying enzyme NDST1 (Presto et al., 2008). This has led to the suggestion that NDST1 and EXT1 compete for binding to EXT2 that acts as a transport protein for both enzymes from the ER to the Golgi (Presto et al., 2008). The HS chain elongation seems to be regulated in part by relatively undefined effects/actions of the HS modification enzymes. These include the increased HS-chain length after overexpression of NDST2 (Deligny et al., 2016; Pikas et al., 2000) and binding of EXT2 to both EXT1 and NDST1 (Presto et al., 2008). In addition, overexpression of C-5 epimerase in HEK293 cells increased HS-chain length, but co-expression of the C-5 epimerase with the 2-O-sulfotransferase reversed this effect (Fang, Song, Lindahl, & Li, 2016). It is not known whether other O-sulfotransferases also influence HS-chain length.

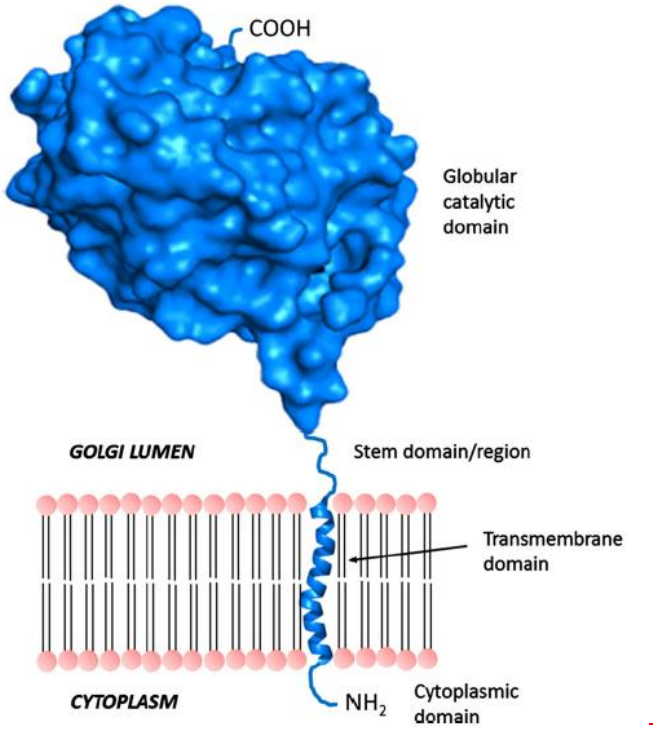


Figure 5. Golgi localized Type II membrane protein illustration. Most of the Golgi localized glycosyltransferases are type II membrane proteins with a short N-terminal cytoplasmic domain, an approx. 20 amino acid α-helical TM-domain, a stem domain and a C-terminal globular catalytic domain facing the lumen of the Golgi (*adapted with permission from (Kellokumpu et al., 2016)*.

1.5 Mutations in *EXT/Ls* using animal models

In order to establish the functions of the various HS biosynthetic enzymes, mutational studies using different model organisms have been performed. Studies using both invertebrate and vertebrate organisms have clearly demonstrated the important role of EXT family of proteins in development. The loss of function of EXT family genes in the various experimental organisms has been reported to cause a number of abnormalities (Busse-Wicher et al., 2014), some of which are described here. Homozygous disruption of the *Ext1* gene in mice results in early mortality. The homozygous mice die at gastrulation before embryonic day 9 (E9), whereas heterozygous mice are phenotypically normal, but with somewhat reduced bone

length (Lin et al., 2000). Embryonic stem (ES) cells from *Ext1*-deficient-mouse embryos are unable to produce HS (Kraushaar, Yamaguchi, & Wang, 2010), and fail to differentiate into β3-tubulin-positive neuronal cells (Johnson et al., 2007) or hematopoietic cell-lineages (Holley et al., 2011). Mouse embryos with a gene trap mutation in *Ext1* (hypomorphic mutation) express approximately 2% of wild type *Ext1* mRNA and live to around embryonic day 14.5 (Koziel, Kunath, Kelly, & Vortkamp, 2004). Embryonic fibroblasts derived from these mouse embryos produce shorter HS-chain than corresponding fibroblasts from wild-type mice (Yamada et al., 2004). Another mouse model with a chondrocyte specific *Ext1* mutation demonstrated that loss of *Ext1*-heterozygosity is contributing to characteristics typical of the MO phenotype (Matsumoto, Irie, Mackem, & Yamaguchi, 2010). Similarly, *Ext2* deficiency is embryonically lethal whereas mice with a heterozygous mutation survive to maturity with development of multiple exostoses (Stickens, Zak, Rougier, Esko, & Werb, 2005).

There is no known *EXTL1* knockout mouse model that possibly could shed light on the function of EXTL1. *EXTL2* knockout (KO) mice develop normally, remain healthy and fertile but show reduced liver regeneration ability following carbon tetrachloride (CCl_4-) induced liver injury due to reduced hepatocyte growth factor (HGF) signaling response (Nadanaka, Kagiyama, & Kitagawa, 2013). Complete deletion of *EXTL3* is incompatible with life and *EXTL3* KO embryos die at about embryonic day 9 (Takahashi et al., 2009). Conditional knockout-mouse models have been generated to study *EXTL3*/HS in selected organs. In a podocyte-specific *EXTL3* KO mouse model, there is reduced HS in the glomerular basement membrane but without significantly increased urinary albumin excretion (Aoki et al., 2018). Targeted deletion of *EXTL3* in pancreatic-β cells leads to abnormal cell morphology, reduced cell proliferation and glucose intolerance due to defective secretion of insulin (Takahashi et al., 2009).

Mutational studies in *Drosophila melanogaster* have also demonstrated the importance of the EXTs. Drosophila *EXT/L* orthologues have been shown to be important: *tout velu* (*ttv, EXT1 orthologue*) in Hedgehog signaling (Bellaiche, The, & Perrimon, 1998); *sister of ttv (sotv*, *EXT2*) and *brother of ttv* (*botv, EXTL3)* in wing development (Takei, Ozawa, Sato, Watanabe, & Tabata, 2004). Other model organisms including *Caenorhabditis elegans (C. elegans)* and zebrafish have also been used to study the impact of mutations in EXTs on morphogenesis and nerve tissue development (Blanchette et al., 2017). Mutations in zebrafish *dackel* (*ext2*) and *boxer* (*extl3*) were shown to be associated with defective axon sorting in the optic nerve tract

(Lee et al., 2004). There is reduced Fgf and Wint signaling in zebrafish carrying an *ext2* mutation that causes decreased HS biosynthesis (Fischer, Filipek-Gorniok, & Ledin, 2011).

1.6 HS-protein interactions

The HS-chains are highly negatively charged molecules that electrostatically attract positively charged amino acid residues (lysine and arginine) in the protein ligands (D. Xu & Esko, 2014). The sulfated NS-domains that are scattered along the HS chains provide the negatively charged binding sites for the interacting proteins and together with the carboxylic groups, contribute to the overall anionic nature of the HS chain (Ori, Wilkinson, & Fernig, 2011). Thus, the NS-domains are important in preserving the functional integrity of the HS chain as they define the binding site for protein ligands (Esko & Selleck, 2002). The HS chains bind to a wide variety of so-called HS-binding proteins including: chemokines, cytokines, morphogens, growth factors, blood coagulation factors, proteins of the complement pathway, ECM structural proteins, single-pass membrane proteins and adhesion molecules (Ori et al., 2011). The ability of HS chains to bind soluble signaling molecules defines their co-receptor role. The HS chains enable bound ligands to attach to their high affinity cognate receptors and thereby influence cell signaling (Gallagher, 2015). The ternary structure formed between the protein ligand, its receptor and the HS chains stabilizes the growth factor-receptor interaction (Gallagher, 2015; Uniewicz et al., 2010). The best known example of this interaction is illustrated by the formation of the FGF2-HS-growth factor receptor ternary complex, which is involved in both physiological (Lin, Buff, Perrimon, & Michelson, 1999) and pathological processes (Lindahl & Kjellen, 2013). Interestingly, HS chains have been demonstrated to mediate the binding of a drug agent – trastuzumab (an antibody that binds to HER2) to its therapeutic target on the surface of breast cancer cells (Suarez et al., 2013).

Factors that affect the HS-chain length and sulfation status also affect the co-receptor function of the HSPG molecule. This has been demonstrated through studies involving overexpression of the heparanase enzyme that cleaves the HS chains (Batool et al., 2017), as well as the effect of sulfatases that selectively remove the 6-O-sulfate groups from the HS molecule (Seffouh et al., 2013). Reduction in fibroblast-*Ext1* expression and hence HS chain length have been shown to diminish FGF2-signaling, as well as reducing the ability of the cell to interact with the ECM (Osterholm et al., 2009). Studies have also demonstrated a reduction in the signaling process, when growth factors are competitively inhibited with antibodies from binding to their

cell surface receptors via the supporting effect of HS chains (Gao et al., 2014; Gao, Kim, & Ho, 2015).

1.7 Human genetic disorders due to mutations in the HS biosynthetic enzymes

Several disorders have been associated with specific mutations in the HS biosynthetic enzymes including both those involved in assembly and modification of the HS chain. Mutations in the gene encoding the GAG initiation enzyme, xylosyl transferase-1 (XYLT1), cause an autosomal recessive short stature syndrome due to reduced enzyme activity (Schreml et al., 2014). Cardiac and joint defects have been reported in patients with a mutation in *β3GALT* (galactosyltransferase I) due to reduced synthesis of HS and CS/DS GAGs (Baasanjav et al., 2011). *GlcATI* mutations resulting in reduced expression of the bilirubin UDP-glucuronosyltransferase enzyme are associated with a chronic non-hemorrhagic liver disorder called Gilbert's syndrome that manifests with jaundice (Bosma et al., 1995; Debinski et al., 1996; Koiwai et al., 1995). Autosomal dominant heterozygous mutations in *EXT1* and *EXT2* cause hereditary multiple osteochondromas (MO) (Pacifici, 2017). MO is characterized by benign cartilaginous tumors at the end of long bones including the femur, humerus, tibia, fibular, as well as ribs and hand bones (Zak et al., 2002). Mutations in *EXT2* have also been implicated in causing another developmental disorder called Scoliosis-macrocephaly-syndrome that is characterized by intellectual disability, hypotonia and scoliosis but without exostoses (Farhan et al., 2015). Mutations in *EXTL3* have been reported to cause a neuro-immuno-skeletal dysplasia syndrome characterized by developmental delay, neuronal, immunological and skeletal abnormalities (Guo et al., 2017; Oud et al., 2017; Volpi et al., 2017). Missense mutations in *NDST1* cause intellectual disability and other disorders such as postnatal growth deficiency (Reuter et al., 2014). NDST1 deficiency has also been associated with developmental delay, ataxia, cranial nerve palsies and respiratory problems in infancy (Armstrong et al., 2017). Mutations in the HS 6-O-sulfotransferase-1 (*HS6ST1*) have been associated with idiopathic hypogonadotropic hypogonadism which is characterized by infertility due to defective gonadotropin-releasing hormone, also as a result of impaired neuronal development and function (Tornberg et al., 2011).

1.8 HS biosynthesis enzymes in cancer.

The involvement of the HS biosynthetic enzymes in cancer was first attributed to EXT1 and EXT2, both of which have been referred to as "tumor suppressor proteins" by several authors (Lind, Tufaro, McCormick, Lindahl, & Lidholt, 1998; McCormick et al., 2000; Ropero et al., 2004; Senay et al., 2000). MO patients are heterozygous for a mutation in either *EXT1* or *EXT2*. Although contradictory reports are present on the cellular origin of osteochondroma, mouse genetic models have shown that MO probably result from a somatic mutation in the remaining wild-type copy of the gene in selected chondrocytes (Jones et al., 2010; Matsumoto et al., 2010). Osteochondromas are benign tumors with a subsequent risk of transformation into malignant osteosarcoma or chondrosarcoma, which occurs in 2-5% of the MO patients. Thus *EXT1* and *EXT2* fit with the classical "two-hit" model characterizing tumor suppressor genes (Knudson, 1971). A recent study however, showed that chondrosarcoma arises from cells only harboring the one-allelic germ-line mutation and not the double hit ones, indicating that osteochondromas and the related chondrosarcomas have different initiating cells (Musso et al., 2015).

Other HS biosynthetic enzymes have also been described to be differently expressed in cancer including NDST-4, C-5 epimerase, sulfotransferases (HS6ST-2, HS3ST and HS2ST-1), heparanase and sulfatases (SULF-1, SULF-2). *NDST-4* has been reported to have a potential tumor suppressor function as loss of *NDST-4* in colorectal cancer was shown to be associated with poor survival (Tzeng et al., 2013). The C-5 epimerase has been suggested to be a potential tumor suppressor in breast and lung cancer cells (Grigorieva et al., 2011; Prudnikova et al., 2010), but a tumor promoter or a prognostic indicator of poor survival in prostate cancer (Rosenberg, Prudnikova, Zabarovsky, Kashuba, & Grigorieva, 2014). Expression of *HS6ST-2* is increased in colorectal cancer tissues as compared to normal tissues with a correlation to poor survival (Hatabe et al., 2013), which suggests that it might be a promoter of tumor growth. Aberrant methylation of *HS3ST* increases the invasive behavior of chondrosarcoma cells indicating a tumor suppressor role for this enzyme (Bui et al., 2010). The extracellular-acting SULF-1 and SULF-2 that post-synthetically modify the HS chains by removing 6-O-sulfate groups from selected GlcN residues have been implicated in tumor progression and metastasis (Hammond et al., 2014; Roy et al., 2017; Vives et al., 2014). An Affymetrix-microarray analysis showed that *EXT2*, *HS2ST-1*, *HPSE* and *SULF-2* are differently expressed in malignant plasma cells (Bret et al., 2009). Upregulation of *HS2ST-1* is associated with better

survival but that of *EXT1* indicates a bad prognosis in patients with multiple myeloma (Bret et al., 2009). The reduced expression of *EXT1* and *EXT2* is presumed to impair proper HS biosynthesis in prostate cancer and glioma (Suhovskih et al., 2014; Ushakov et al., 2017).

1.9 Role of HSPGs in cancer

HSPGs on the cell surface, in the pericellular space and in the ECM of tumors are crucial for tumor cell proliferation and metastasis through their effect on the cells as well as on the tumor microenvironment (Iozzo & Sanderson, 2011). HSPGs have diverse effects in different types of tumors, acting as either promotors or repressors of tumor growth (Iozzo & Sanderson, 2011).

The ability of HSPGs such as glypicans, syndecans and perlecan to bind a multitude of growth factors in the ECM via the HS chains, makes the proteoglycans a major reservoir of growth factors needed to promote tumor growth and metastasis (Gao et al., 2015; Roskams, De Vos, David, Van Damme, & Desmet, 1998). A more direct role of cell surface HSPGs in tumorigenesis is exemplified by syndecans that can influence cell adhesion and migration through physical contacts with focal adhesion proteins and cytoskeleton (Couchman, 2010). Syndecan-4 has been found to be necessary in the formation of stable focal adhesions (Elfenbein & Simons, 2013; Morgan, Humphries, & Bass, 2007) and is considered a good prognostic marker for cancer patients with less migratory estrogen receptor/progesterone receptor (ER- or PR)-positive tumor cells (Lendorf, Manon-Jensen, Kronqvist, Multhaupt, & Couchman, 2011). Syndecan-2 has been reported to promote migratory behavior of the triple negative MDA-MB-231 cells by suppressing the function of syndecan-4 (Lim & Couchman, 2014; Lim et al., 2015). Syndecan-1 is increasingly expressed and shed by myeloma cells, rendering this HSPG to be an indicator of a progressive tumor disease (Khotskaya et al., 2009). Additionally, increased expression of syndecan-1, especially by stromal cells has been reported to be a poor prognostic marker in breast cancer (Barbareschi et al., 2003; Nguyen et al., 2013).

Glypicans also play important roles in cancer progression, in particular glypican-3 that promotes hepatocellular carcinoma growth through increased Wnt signaling (Gao et al., 2014). There is also increased expression of glypican-1 in certain tumors such as esophageal

squamous cell carcinoma, pancreatic cancers and glioblastomas with a poor prognosis (Hara et al., 2016; Lu et al., 2017; Saito et al., 2017). In contrast, glypican-5 has been suggested to act as a 'tumor suppressor' as it prevented tumor growth in a xenograft model and inhibited phosphorylation of oncogenic cell surface receptor tyrosine kinase (RTK) receptors in non-small cell lung cancer (NSCLC) cells (Guo, Wang, Zhang, & Yang, 2016). Glypican-5 is also described as an 'epigenetically repressed tumor suppressor' due to hypermethylation of its promotor. Glypican-5 blocks tumor growth or epithelial to mesenchymal transition (EMT) by inhibiting Wnt-signaling in lung adenocarcinoma cells (Wang et al., 2016). Glypican-6 mediates the pro-invasive and migratory effects of nuclear factor of activated T-cells (NFAT) in breast cancer cells by activating the JNK and p38-MAPK pathways (Yiu, Kaunisto, Chin, & Toker, 2011).

The basement membrane HSPG, perlecan may promote tumor angiogenesis through its ability to act as a co-receptor for the pro-angiogenic growth factors FGFs and VEGF, via its HS-chains (Gubbiotti et al., 2017; Iozzo & Sanderson, 2011; Iozzo et al., 2009; Lord et al., 2014). Cleavage of the C-terminal component of perlecan by L-cathepsin yields a smaller molecule called endorepellin. Endorepellin may exhibit anti-angiogenic or anti-proliferative effects by binding the integrin α2β1 resulting into dephosphorylation and deactivation of the neighboring receptor tyrosine kinase receptors and autophagy (Goyal et al., 2016; Gubbiotti et al., 2017; Poluzzi et al., 2014).

The effect of cell surface HSPGs can be modified by enzymes secreted in the ECM that target either the HS chains such as heparanase (Batool et al., 2017; Vlodavsky et al., 2007; Vlodavsky et al., 2016) and SULFs (Frese, Milz, Dick, Lamanna, & Dierks, 2009) or core proteins (sheddases) (Grindel et al., 2014). The sheddases together with heparanase tend to potentiate the role of HSPGs in growth factor signaling, through cleavage of either the core protein (Ding, Lopez-Burks, Sanchez-Duran, Korc, & Lander, 2005) or the HS chains that become solubilized and thus release the attached growth factors (Iozzo et al., 2009). This also promotes tumor angiogenesis if the growth factors released are pro-angiogenic like FGF2 and VEGFA (Iozzo & Sanderson, 2011).

2. Rationale of the study

EXT proteins are glycosyltransferases necessary for the polymerization of HS chains. In this study, HS biosynthesis was studied with specific focus on EXT1 and EXT2. Gene deletion studies in mice have shown that EXT1, EXT2 and EXTL3 are necessary for HS chain elongation. *In vitro* studies have ascribed EXTL3 and EXTL2 as initiators and/or terminators of HS chain polymerization. The HS chains assembled by the EXT proteins are important in development, homeostasis and in disease situations. Several questions remain unanswered about how the EXT proteins interact amongst themselves and with other HS biosynthetic proteins. The knowledge obtained in this study will hopefully improve our understanding of HS-biosynthesis and clarify on how the HS structure might change in pathological situations such as cancer.

General objective:

To obtain more knowledge about the molecular mechanism of action of the exostosin (EXT) protein family in normal physiology and in cancer biology

Specific objectives:

A**:** To analyze how stromal fibroblast *Ext1*-expression levels affect gene expression in tumor cells.

B: To determine the gene expression pattern of the EXT family of proteins in breast cancer cell lines and to study if or how their mRNA levels influence HS structure.

C: To determine effect of pH on the glycosyltransferase activity of EXT1 and EXT2.

3. Summary of results

Paper I

Potential role for fibroblast *Ext1*-dependent heparan sulfate in regulating *P311* gene expression in A549 carcinoma cells

In this study, we investigated how the *Ext1* expression levels in stromal fibroblasts affect gene expression in neighboring tumor cells using 2-dimensional co-culture and 3-dimensional hetero-spheroid cell culture models. In these models, human A549 non-small-cell lung cancer cells were co-cultured with either wild type (wt) or *Ext1* mutant ($Ext1^{Gt/Gt}$) mouse embryonic fibroblasts. The $Ext1^{Gt/Gt}$ fibroblasts have very short HS chains and are therefore an excellent model for studying the influence of HS chains on cellular activities. Differential gene expression experiments showed that *P311* expression in the tumor cells was significantly reduced when co-cultured with the mutant fibroblasts. Compared to the wt fibroblasts, the mutant fibroblasts had reduced *tgf-β1* expression and TGF-β activity. Re-introduction of *Ext1* into the *Ext1* deficient fibroblasts rescued *tgf-β1* expression and TGF-β-activity in the fibroblasts and increased *P311* expression in the co-cultured A549 tumor cells. Direct stimulation of the tumor cells with recombinant TGF-β1 increased expression of *P311*, indicating that P311 is a TGFβ1 regulated protein. To confirm that reduced expression of fibroblast *tgf-β1* influences *P311* levels in co-cultured A549 cells, we down-regulated fibroblast *tgf-β1*-levels in wt fibroblasts using siRNA. Our results show that down-regulation of fibroblast *tgf-β1* resulted in reduced *P311* expression in A549 cells. Furthermore, *P311* expression was not affected when A549 cells were cultured in conditioned media isolated from mutated fibroblasts indicating a requirement for physical interaction between the stromal fibroblasts and the tumor cells. Together, these results indicate a previously unknown potential regulatory function of fibroblast-*Ext1* on *tgf-β1* expression in the same cells as well as gene expression of *P311* in adjacent tumor cells.

Paper II

The exostosin family of glycosyltransferases: mRNA expression profiles and heparan sulfate structure in human breast carcinoma cell-lines

In this study, we investigated the mRNA expression profiles of the EXT-family members (*EXT/L)* and how their mRNA levels are reflected in HS structure of various breast cancer cells and a non-tumorigenic mammary gland epithelial cell line. The cell lines used included two triple negative breast cancer-cell lines MDA-MB-231 and HCC38. These cells lack estrogen receptors (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2). One ER/PR-positive cell line - MCF7 and one non-tumorigenic mammary glandular epithelial cell line - MCF10A were also studied. There was significant reduction in expression of all the *EXT/L* genes in the less invasive ER/PR-positive cell line (MCF7), whereas the triple negative cell lines MDA-MB-231 and HCC38 had similar *EXT/L* expression profiles, with significantly elevated *EXTL2* expression as compared to all the other cell lines.

Using the HS-specific 10E4 antibody, flow cytometry analyses showed a strong cell surface staining of HS for the non-tumorigenic MCF10-A cells and the triple negative breast cancer-cell lines. In contrast, the ER/PR-positive MCF7 cell line showed weak staining for 10E4. This suggested that MCF7 cells had less HS as compared to the other cell lines studied.

Cells were radiolabeled with ^{35}S-sulfate to determine the length and fine structure of the HS chains. In agreement with the flow cytometry data, MCF7 had the shortest HS chains of the analyzed cell lines. Surprisingly, the two triple negative cell-lines MDA-MB-231 and HCC38 that exhibited similar *EXT/L* expression profiles had very different chain lengths, with the longest chains found on MDA-MB-231 cells.

HS disaccharide-compositional analyses showed that the major difference in the relative proportions of sulfated disaccharides was that MCF7 cells had a lower proportion of 2-O sulfated disaccharides and the highest proportion of the 6-O sulfated disaccharides as compared to all the other cell lines.

The glycosyltransferase enzyme activity of the EXT proteins varied between the cell-lines with HCC38 cells showing a significantly higher enzyme activity compared to the rest. A major limitation to this study is that we were not able to quantify the amount (as μg protein) of the EXT proteins in the crude cell lysates and thus could not assess the specific transferase

activities in the different cell lines. This is because most of the currently available techniques for protein quantification such as targeted proteomics would not preserve the EXT enzymatic activity during the sample-preparation procedures. Therefore, enzyme activity reported in **paper II** was estimated based on the detected glycosyltransferase activity in the total crude protein sample. Thus, we lack accurate information in order to get a proper correlation between EXT-enzyme activity and HS-chain length among the different cell lines.

Paper III

Effect of pH on the glycosyltransferase activity of EXT1 and EXT2 proteins

In this study, we investigated the effect of pH changes on the glycosyltransferase activity of recombinant full-length EXT1 and EXT2. Using serum as a positive control, we performed glycosyltransferase assays at pH 7.4, 6.5 and 5.0 representing pH approximately similar to that of the endoplasmic reticulum, Golgi complex and lysosome, respectively. Our results indicated that there is a reduction in glycosyltransferase activity at lower pH values. There was an intermediate reduction in enzyme activity for the affinity-purified proteins at a Golgi pH of 6.5, which is a surprising result since this is considered as the optimum pH for glycosyltransferase activity *in vivo*. We also tested a non-radioactive assay-kit that quantifies the glycosyltransferase activity based on the amount of released inorganic phosphate from the leaving UDP-nucleoside of the transferase reaction. The results from using this kit indicated unspecific transfer of the UDP-sugars to the oligosaccharide acceptor and we thus considered it unsuitable for detecting the glycosyltransferase activity of EXT-proteins.

In brief, these results show that a reduction in pH leads to a decline in glycosyltransferase activity of the EXT proteins and the non-radioactive assay is not suitable for determining their enzyme activity.

4. Discussion

4.1 Methodological considerations

The details of the methods are mentioned in the individual manuscripts, but some methodological considerations are explained here:

4.1.1 Cell lines

The reason for selecting cell lines: Cell lines are vital in providing mechanistic details of cellular pathways, which cannot easily be obtained from animal models, clinical data or stored human tissue samples.

The *Ext1* and *Ext2* knockout mice are embryonically lethal (Lin et al., 2000) which makes it unfeasible to study mechanistic changes of these genes using *in vivo* KO animal models. The advantage of using cells with short HS chains ($Ext1^{Gt/Gt}$-mutated fibroblasts) is that we were able to study the cellular effects of *Ext1*/HS deficiency without the need to use HS degradation chemicals or enzymes, which would exert more metabolic stress to the cells. In project I, human A549 lung carcinoma cells were co-cultured with either wild type or $Ext1^{Gt/Gt}$-fibroblasts. Using cells from different species gave us the opportunity to study the cross talk between these two major and mutually dependent components of the tumor microenvironment. In project II, two triple negative breast cancer cell-lines - MDA-MB-231 and HCC38 (negative for ER, PR and HER2 receptors), one ER/PR-positive cell line - MCF7 and one non-tumorigenic human mammary glandular epithelial cell line - MCF10-A were studied. The tumor cell lines were selected based on their different carcinogenic properties.

4.1.2 Gene expression:

RTqPCR and microarray: To study differentially expressed genes in the hetero-spheroids, we used microarray technology in order to get an overview of the most likely affected genes in project I. Then we confirmed those genes that are differentially expressed in the A549 tumor cells using a Real time qPCR technique. Results of gene expression obtained from the RTqPCR may be affected by immortalization of the cells and therefore, we crosschecked our results against primary cells in project I.

4.1.3 Western blots:

Western blotting gives a relative measure of the presence or absence of a protein of interest in a sample, which helps to confirm whether a particular gene has been translated. This requires taking into consideration the specificity of the antibodies used in immunoblotting.

4.1.4 The Glycosyltransferase Assay

The glycosyltransferase assay quantifies the transfer of single sugars to acceptor molecules. We used this method to determine the GlcNAc transferase activity in cells studied in projects II and III (**Fig. 6**) This assay measures EXT1 and to some degree also EXT2 enzymatic activities of cells. GlcA transferase activity is measured using radiolabeled UDP-GlcA and acceptor substrates with a non-reducing end GlcNAc residue.

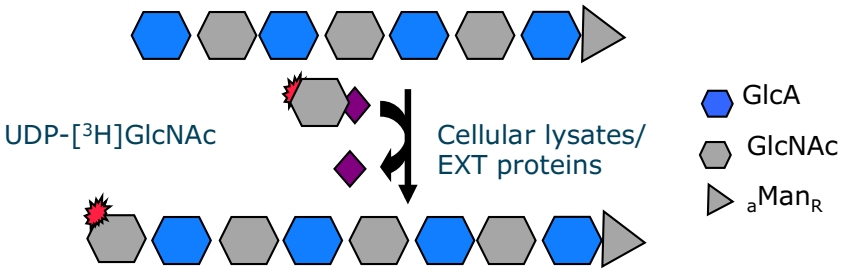


Figure 6. GlcNAc-TII reaction. Cell lysates or affinity captured EXT proteins were incubated with radiolabeled UDP-GlcNAc and $[GlcA\text{-}GlcNAc]_n$ oligosaccharide acceptors. Next, radiolabeled oligosaccharides were separated from unincorporated UDP-sugars by gel chromatography and quantified by scintillation counting.

4.1.5 Metabolic labelling and Analysis of the HS- structure

In order to study the HS-structure, we metabolically labeled cells with ^{35}S-sulfate. The radioactivity is incorporated in the various N- and O-sulfate positions along the entire HS

chain. This makes it possible to estimate the HS size and the composition of the disaccharide units within the NS domains. The procedure summarized in **Figure 7.**

Summary of HS Structural Analysis Procedures

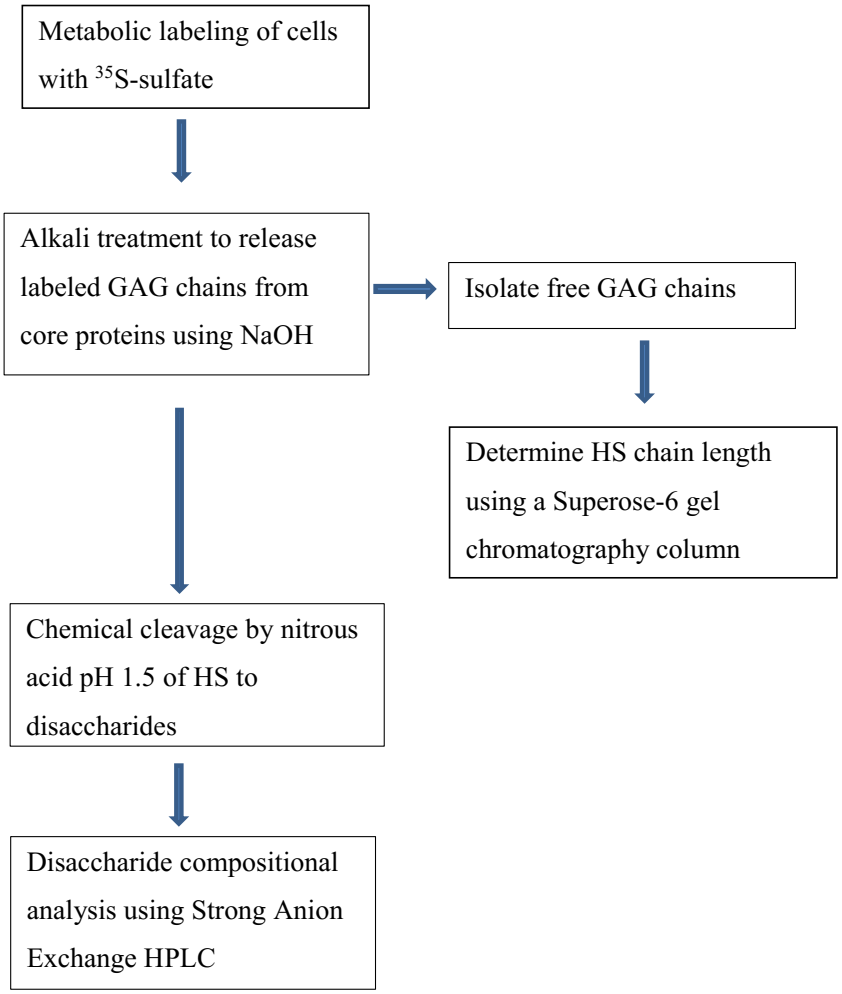


Figure 7. Flow chart summarizing steps taken from ^{35}S labeling of cells to HS-chain length determination and disaccharide analysis.

4.1.6 Fibroblasts *TGF-β1*-siRNA knockdown

In order to confirm the paracrine effect of fibroblast-tgf-β1 on the expression of *P311* in adjacent A549 tumor cells, we first down regulated the expression of *tgf-β1* in wild type fibroblasts. We chose to use siRNA technique-instead of CRISP-CAS9, because we aimed to reduce the levels of *tgf-β1* gene expression comparable to the reduction observed in the mutated fibroblast. Due to the risk of off-target effects, we used two different siRNA primer pairs targeting *tgf-β1* and a non-targeting control siRNA.

4.1.7 Flow cytometry:

This technique can be used to separate or sort out cells of unique characteristic features or surface markers for example. In the first two projects, this technique was used to identify cells stained with an antibody that recognizes cell surface HS-chains or cells that were over expressing EGFP-tagged EXT proteins following transfection.

4.1.8 TGF-β-Activity Assay

This Luciferase based assay is used to quantify the relative amount of secreted active TGF-β in conditioned medium extracted from fibroblasts. The assay relies on the use of transformed Mink lung epithelial cell line (TMLC) which is overexpressing a luciferase-fused plasminogen activator inhibitor-1 (PAI-1) protein. The fact that TGF-β induces the expression PAI-1, we added conditioned media recovered from wt or $Ext1^{Gt/Gt}$-mutated fibroblasts to the TMLC cells in order to quantify the amount of active TGF-β-secreted by the fibroblasts.

4.1.9 3D- Hetero-spheroid cell culture model

The 3D-spheroid models (or composite spheroids) used in this work were made as described before (Osterholm et al., 2012). The 3D-spheroid model mimics better the 3-dimensional microenvironment around the tumor cells, which includes the stromal fibroblasts. This model has been used in our lab to demonstrate how the stromal fibroblast-*Ext1* levels and HS status affect tumor cell migration when co-cultured. The 2D co-culture model is easier to manipulate and was used to study in more details how the fibroblast tgf-β1-paracrine activity was affecting *p311* expression in the A549 tumor cells.

4.1.10 Immunoprecipitation (IP)

We used this technic in Paper 3 to purify transiently overexpressed epitope-tagged EXT1 and EXT2 from the respective crude cell lysates. We were able to confirm successful immunoprecipitation using SDS-PAGE followed by western blotting using antibodies to the protein tags. IP can be done using resin, agarose beads or magnetic beads.

4.2 General discussion

The regulation of HS biosynthesis involves a combination of enzymatic activities during the assembly of a tetrasaccharide-linker region, chain initiation/elongation and modification. There are around 25 different enzymes involved in HS biosynthesis operating in the lumen of the Golgi complex (Prydz, 2015). The overall HS-chain structure is dependent on the expression of the biosynthetic enzymes, the availability of substrates and specificity of the enzymes in recognizing the substrates (J. P. Li & Kusche-Gullberg, 2016). HS-chain initiation/elongation is very important in laying the HS foundation or backbone, which determines the final chain structure in terms of length and distribution of various domains (Busse & Kusche-Gullberg, 2003; Senay et al., 2000). The HS-chain initiation/elongation process is catalyzed by the EXT-proteins that are encoded by the EXT-family of genes (*EXT1, EXT2, EXTL1, EXTL2* and *EXTL3*) (Busse-Wicher et al., 2014). The EXT proteins form hetero-oligomeric complexes, which are also capable of combining with other proteins such as NDSTs in a larger 'GAGosome' complex that regulates the overall HS-biosynthesis (Deligny et al., 2016; McCormick et al., 2000; Presto et al., 2008). Although previous studies have tried to demonstrate the functions of both cloned and endogenous enzymes, some important questions remain unanswered. These are questions regarding the specific roles of individual proteins; how they interact with each other and with other biosynthetic proteins; and how their expression changes in pathological conditions like cancer or if their expression is indicative of the HS structure. The HS-chain elongation involves the transfer of a single nucleotide sugar (e.g. UDP-sugars like UDP-GlcA or UDP-GlcNAc) by the glycosyltransferase enzymes to the non-reducing end of the growing chain (Busse & Kusche-Gullberg, 2003). Thus, there is a need to transport the UDP sugars and other important substrates such as PAPS, across the Golgi membrane from the cytoplasm into the lumen where glycosylation and sulfation of PGs (and other proteins) occur (Prydz, 2015). An UDP-sugar transporter such as HFRC1 that is capable of transporting both UDP-GlcNAc and UDP-GlcA, but not UDP-mannose, influences the HS biosynthesis in this manner (Suda et al., 2004). The activity of the UDP-sugar transporters is dependent on the relative concentration of required UDP sugars in the cytosol and Golgi lumen that is adequate to drive the glycosyltransferase activity forward (Prydz, 2015; Verbert, Cacan, & Cecchelli, 1987).

The overall aim of this study was to investigate the mechanism controlling HS chain elongation and structure in physiological and pathological situations. We used a combination

of biochemical, molecular and cell culture techniques involving both normal and tumorigenic cell-lines. The HSPGs influence interactions between cancer cells and the stromal microenvironment via HS-chains on the cell surface. The *Ext1* levels and HS-chain length of stromal fibroblasts have been demonstrated to affect not only growth factor signaling and fibroblasts' ability to interact with the ECM (Osterholm et al., 2009), but also adjacent-tumor cell migration and proliferation (Osterholm et al., 2012). In paper I, we discovered another potential role of fibroblast *Ext1*-dependent HS in influencing *P311* gene expression in adjacent tumor cells by regulating tgf-β1 levels. Ext1 is essential for HS elongation (Busse et al., 2007) thus; its deficiency results in shorter HS chains (Osterholm et al., 2009). However, *Ext1* also appears to regulate *tgf-β1* expression and secretion in an autocrine manner. In addition, our results as well as previous studies seem to suggest the existence of a positive feedback-regulatory mechanism between TGF-β1 and P311 that influences cell differentiation, proliferation and migration (H. Li et al., 2016; Yue et al., 2014; Zhang et al., 2016). The effect of fibroblast-*Ext1* on tgf-β1 levels and subsequently on *P311* expression in adjacent tumor cells might provide an explanation for the anti-proliferation and anti-migration effects observed previously in *Ext1*-mutated fibroblast/A549 hetero-spheroids (Osterholm et al., 2012). As we also observed reduced Smad3 and Erk1/2 phosphorylation in the *Ext1*-mutated fibroblasts, another explanation for the anti-migratory effect observed previously by (Osterholm et al., 2012), could be that there is no enhancement or synergistic action of FGF2 on the TGF-β-mediated cell migration (Saitoh, 2015). In addition, there is also lack of FGFR-isoform switching from FGFR2/3 to FGFR1 (high-affinity receptor for FGF2) as suggested by (Shirakihara et al., 2011). Due to lack of sufficient HS-chains on the cell surface of stromal fibroblasts in contact with the tumor cells, the subsequent decrease in induction of both FGF2 and TGFβ signaling result in an overall change in cell behavior.

As a conclusion to this part, we can deduce that *Ext1*-dependent fibroblast-HS chains influence *P311* expression in adjacent A549-tumor cells through paracrine TGFβ1 activity.

In the second part (**paper II**), we set out to determine if the *EXT/L* expression profiles can be reflected by the HS structure in breast cancer cell lines. One reason for this investigation was the existence of several reports suggesting specific changes in HS structure based on the dysregulated mRNA levels of HS-biosynthetic enzymes in various cancers (Fernandez-Vega et al., 2015; Okolicsanyi et al., 2014; Suhovskih et al., 2015; Suhovskih et al., 2014; Ushakov et al., 2017).

In our study, the *EXT/L* expression pattern seems to reflect the differences in the tumorigenic characteristics of the breast cancer-cell lines (Neve et al., 2006), whereby MCF7 cells have a gene expression profile that is different from that of the triple negative cell lines. The significantly increased expression of *EXTL2* in the triple negative cell-lines further highlights the enigma surrounding the exact role of this protein in HS chain assembly. Bioinformatics data from www.proteinatlas.org shows that *EXTL2* RNA is expressed in almost all types tumors, with the highest expression in Gliomas (8-fold compared to 3-fold in breast cancer). EXTL2 protein expression is also reported in almost all types of tumors, with the highest expression reported (approx. 100%) in endometrial, breast, ovarian, colorectal, head and neck, liver, stomach and prostate cancers; and the lowest expression (approx. 20%) in skin cancer (www.proteinatlas.org). To our knowledge there are no published papers confirming EXTL2 expression in various cancers. Some *in vitro* studies have indicated that EXTL2 can act either as an initiator or as a terminator of HS-chain elongation (Kitagawa, Shimakawa, & Sugahara, 1999; Nadanaka, Zhou, et al., 2013; Okada, Nadanaka, Shoji, Tamura, & Kitagawa, 2010). In contrast, another study showed that overexpression of *EXTL2* in HEK293 cells did not cause a significant change in the HS-chain length whereas down-regulation of the *EXTL2* resulted in longer HS chains (Katta et al., 2015). The high expression of *EXTL2* in the triple negative cells is difficult to evaluate, as the precise role of EXTL2 in HS synthesis is unclear. It is neither possible to infer on if the observed high *EXTL2* expression levels are reflected by translated protein levels nor its effect on HS biosynthesis.

Although previous work involving silencing of *EXTL3* with siRNA resulted in longer HS chains (Busse et al., 2007), it is not clear if the reduced *EXTL3* expression observed in the triple negative cancer cells has a similar effect since we do not know how the levels compare to the cells used in (Busse et al., 2007). A very interesting result from this work is that although the two triple negative cell lines (MDA-MB-231 and HCC38) had similar *EXT/L* mRNA expression profiles, they exhibited completely different HS-chain length, again pointing to a more complicated regulation rather than simply the gene expression profiles. This might be due to differences in overall regulation HS biosynthesis and post-synthetic modification processes. In comparison however, the shorter HS chains in MCF7 cells are most probably due to the low *EXT1* expression in this cell line, similar to that observed in the $Ext1^{Gt/Gt}$ cells and after siRNA mediated downregulation of *EXT1* (Busse-Wicher et al., 2014; Busse et al., 2007; Osterholm et al., 2009).

Various studies have investigated the expression of HS-biosynthetic enzymes mainly at mRNA levels but have not been able to show how this relates to the overall HS-structure found on the cancer cells. The HS binding-sites for various proteins are defined by the existence of sulfated regions (N- and O-sulfated domains) scattered along the entire length of the HS polysaccharide (J. P. Li & Kusche-Gullberg, 2016). In addition, this HS-structural organization is known to be modified post-synthetically by heparanase and sulfatases (SULFs) (Hammond et al., 2014). Increased heparanase (HPSE) expression and activity have been reported in various cancers (Vlodavsky et al., 2016) and we therefore, investigated if heparanase activity could explain the difference in the HS-chain length among our breast cancer-cell lines. Interestingly, we observed the highest HPSE (50kDa) protein expression in the MDA-MB-231 cells that have the longest HS-chains. This would suggest that differences in HS-chain length among the various cell-lines are probably due to cell-type specific regulation of HS biosynthesis rather than HS degradation by heparanase. We did not investigate *SULF* mRNA levels or activity and thus, we cannot rule out the possibility that increased expression of *SULFs* in the triple negative cells is responsible for observed lower 6-O-sulfation in these cells.

To conclude this part, we observed no direct correlation between *EXT/L* expression and HS-structure among the breast cancer-cell lines studied, except where *EXT1*-levels were extremely low as seen in MCF7 cells.

In the third part (**paper III**), we investigated the effect of different pH conditions on the glycosyltransferase activity of EXT1 and EXT2. We aimed at investigating if there are clear changes in the EXT enzyme activity as a result of the transitioning from the more neutral pH of ER (approx. pH 7.2) to the more acidic pH of the Golgi (approx. pH 6.5) (Rivinoja, Pujol, Hassinen, & Kellokumpu, 2012). We aimed at generating information that would clarify on whether the EXT1/EXT2 hetero-complex is formed in the ER or in the Golgi. Surprisingly, our *in vitro* assay-based results seem to contradict the widely hypothesized *in vivo* glycosyltransferase activity of EXT proteins, with suggested hetero-complex formation and optimum activity in the acidic pH of the Golgi (McCormick et al., 2000). Interestingly, our results reflect a much earlier report by Lidholt and Lindahl, which first demonstrated a similar trend of increasing glycosyltransferase activity with increasing pH in an *in vitro* setting (Lidholt & Lindahl, 1992). Most *in vitro* studies report higher activity of co-expressed EXT1/EXT2 than that of the individual proteins alone with assays done at the nearly neutral

pH of 7.4 (Busse et al., 2007; Busse & Kusche-Gullberg, 2003; Esko & Selleck, 2002; Senay et al., 2000). Indeed, it might be true that the optimal pH 7.4 used for most *in vitro* glycosyltransferase assays does not correspond to that of the Golgi due to constant monitoring of the pH by active ion pumps in the metabolizing organelle of a live cell (Demaurex, Furuya, D'Souza, Bonifacino, & Grinstein, 1998; Rivinoja et al., 2012). Moreover, Anti Hassinen et al, have also demonstrated that formation of enzymatically relevant and more active glycosyltransferase-hetero-complexes is dependent of the acidity of the Golgi (Hassinen et al., 2011).

Our results in this study are very preliminary and more repeated experiments are needed to draw any conclusions even though we showed that there is a decline in the EXT-glycosyltransferase activity after decreasing the pH.

General Conclusion:

We have demonstrated that:

(a) Fibroblast-*Ext1*-dependent HS chains influence *P311* expression in co-cultured A549 lung adenocarcinoma cell-lines, through a combination of physical mechanisms and paracrine TGF-β activity. There is also a potentially new autocrine regulatory role of fibroblast-*Ext1* on *tgf-β1* expression and secretion of active TGF-β.

(b) There is no direct correlation between *EXT/L* expression and HS structure in the breast carcinoma cell-lines and the non-tumorigenic breast epithelial cell-line investigated. The variations in the HS structure seem to reflect the carcinogenic properties of the individual breast cancer cell-lines.

(c) Decrease in pH seems to reduce the glycosyltransferase activity of EXT1 and EXT2 proteins in *in vitro* experiments.

5. Future perspectives

In order to unravel the exact biological significance of the observed changes in *P311* expression in the tumor cells, future studies would focus on the use of live imaging techniques including employing multicolor FRET, to pin down the specific location or destination of the translated protein in the live cell. This would help to provide additional information regarding the potential interactions between P311 and other structural or functional proteins in the cell. To quantify fibroblast-specific TGF-β1 in our hetero-spheroids, may require use of different fibroblasts overexpressing a tagged cytokine that can be exclusively quantified in this type of 3D co-culture model.

In order to further study the correlation between the expression profiles of the *EXT/L* genes and HS structure in breast cancer cells, future studies should include investigation of possible unique epigenetic regulatory mechanisms. Moreover, even if the effect of heparanase on HS structure has already been demonstrated in other studies, it would be interesting to study if there are epigenetic changes that influence heparanase expression in the cancer cell lines used in our study. Perhaps, a modified quantification method using live imaging of both EXT proteins and HS chains would be a better alternative to obtain an accurate correlation between glycosyltransferase activity and HS-chain length, albeit only applicable to EXT-overexpressing cells with protein tags.

Since there is no information regarding the exact binding sites between EXT1 and EXT2, mutational studies involving the different domains of these proteins are needed in order to identify the most relevant parts necessary for their interaction.

6. References

Afratis, N. A., Nikitovic, D., Multhaupt, H. A., Theocharis, A. D., Couchman, J. R., & Karamanos, N. K. (2017). Syndecans - key regulators of cell signaling and biological functions. *FEBS J, 284*(1), 27-41. doi:10.1111/febs.13940

Aoki, S., Saito-Hakoda, A., Yoshikawa, T., Shimizu, K., Kisu, K., Suzuki, S., . . . Sugawara, A. (2018). The reduction of heparan sulphate in the glomerular basement membrane does not augment urinary albumin excretion. *Nephrol Dial Transplant, 33*(1), 26-33. doi:10.1093/ndt/gfx218

Armstrong, L., Tarailo-Graovac, M., Sinclair, G., Seath, K. I., Wasserman, W. W., Ross, C. J., & van Karnebeek, C. D. (2017). A girl with developmental delay, ataxia, cranial nerve palsies, severe respiratory problems in infancy-Expanding NDST1 syndrome. *Am J Med Genet A, 173*(3), 712-715. doi:10.1002/ajmg.a.37621

Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B., & Seed, B. (1990). CD44 is the principal cell surface receptor for hyaluronate. *Cell, 61*(7), 1303-1313.

Aspberg, A. (2012). The different roles of aggrecan interaction domains. *J Histochem Cytochem, 60*(12), 987-996. doi:10.1369/0022155412464376

Awad, W., Kjellstrom, S., Svensson Birkedal, G., Mani, K., & Logan, D. T. (2018). Structural and Biophysical Characterization of Human EXTL3: Domain Organization, Glycosylation, and Solution Structure. *Biochemistry, 57*(7), 1166-1177. doi:10.1021/acs.biochem.7b00557

Baasanjav, S., Al-Gazali, L., Hashiguchi, T., Mizumoto, S., Fischer, B., Horn, D., . . . Hoffmann, K. (2011). Faulty initiation of proteoglycan synthesis causes cardiac and joint defects. *Am J Hum Genet, 89*(1), 15-27. doi:10.1016/j.ajhg.2011.05.021

Barbareschi, M., Maisonneuve, P., Aldovini, D., Cangi, M. G., Pecciarini, L., Angelo Mauri, F., . . . Doglioni, C. (2003). High syndecan-1 expression in breast carcinoma is related to an aggressive phenotype and to poorer prognosis. *Cancer, 98*(3), 474-483. doi:10.1002/cncr.11515

Batool, T., Fang, J., Barash, U., Moustakas, A., Vlodavsky, I., & Li, J. P. (2017). Overexpression of heparanase attenuated TGF-beta-stimulated signaling in tumor cells. *FEBS Open Bio, 7*(3), 405-413. doi:10.1002/2211-5463.12190

Bellaiche, Y., The, I., & Perrimon, N. (1998). Tout-velu is a Drosophila homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. *Nature, 394*(6688), 85-88. doi:10.1038/27932

Bishop, J. R., Schuksz, M., & Esko, J. D. (2007). Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature, 446*(7139), 1030-1037. doi:10.1038/nature05817

Blanchette, C. R., Thackeray, A., Perrat, P. N., Hekimi, S., & Benard, C. Y. (2017). Functional Requirements for Heparan Sulfate Biosynthesis in Morphogenesis and Nervous System Development in C. elegans. *PLoS Genet, 13*(1), e1006525. doi:10.1371/journal.pgen.1006525

Bosma, P. J., Chowdhury, J. R., Bakker, C., Gantla, S., de Boer, A., Oostra, B. A., . . . et al. (1995). The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *N Engl J Med, 333*(18), 1171-1175. doi:10.1056/nejm199511023331802

Bret, C., Hose, D., Reme, T., Sprynski, A. C., Mahtouk, K., Schved, J. F., . . . Klein, B. (2009). Expression of genes encoding for proteins involved in heparan sulphate and chondroitin sulphate chain synthesis and modification in normal and malignant plasma cells. *Br J Haematol, 145*(3), 350-368. doi:10.1111/j.1365-2141.2009.07633.x

Bui, C., Ouzzine, M., Talhaoui, I., Sharp, S., Prydz, K., Coughtrie, M. W., & Fournel-Gigleux, S. (2010). Epigenetics: methylation-associated repression of heparan sulfate 3-O-

sulfotransferase gene expression contributes to the invasive phenotype of H-EMC-SS chondrosarcoma cells. *FASEB J, 24*(2), 436-450. doi:10.1096/fj.09-136291

Busse-Wicher, M., Wicher, K. B., & Kusche-Gullberg, M. (2014). The exostosin family: proteins with many functions. *Matrix Biol, 35*, 25-33. doi:10.1016/j.matbio.2013.10.001

Busse, M., Feta, A., Presto, J., Wilen, M., Gronning, M., Kjellen, L., & Kusche-Gullberg, M. (2007). Contribution of EXT1, EXT2, and EXTL3 to heparan sulfate chain elongation. *J Biol Chem, 282*(45), 32802-32810. doi:10.1074/jbc.M703560200

Busse, M., & Kusche-Gullberg, M. (2003). In vitro polymerization of heparan sulfate backbone by the EXT proteins. *J Biol Chem, 278*(42), 41333-41337. doi:10.1074/jbc.M308314200

Capurro, M. I., Xu, P., Shi, W., Li, F., Jia, A., & Filmus, J. (2008). Glypican-3 inhibits Hedgehog signaling during development by competing with patched for Hedgehog binding. *Dev Cell, 14*(5), 700-711. doi:10.1016/j.devcel.2008.03.006

Carlsson, P., Presto, J., Spillmann, D., Lindahl, U., & Kjellen, L. (2008). Heparin/heparan sulfate biosynthesis: processive formation of N-sulfated domains. *J Biol Chem, 283*(29), 20008-20014. doi:10.1074/jbc.M801652200

Casu, B., Naggi, A., & Torri, G. (2015). Re-visiting the structure of heparin. *Carbohydr Res, 403*, 60-68. doi:10.1016/j.carres.2014.06.023

Cheng, B., Montmasson, M., Terradot, L., & Rousselle, P. (2016). Syndecans as Cell Surface Receptors in Cancer Biology. A Focus on their Interaction with PDZ Domain Proteins. *Front Pharmacol, 7*, 10. doi:10.3389/fphar.2016.00010

Cheung, W. F., Eriksson, I., Kusche-Gullberg, M., Lindhal, U., & Kjellen, L. (1996). Expression of the mouse mastocytoma glucosaminyl N-deacetylase/ N-sulfotransferase in human kidney 293 cells results in increased N-sulfation of heparan sulfate. *Biochemistry, 35*(16), 5250-5256. doi:10.1021/bi952325b

Cohen, I. R., Grassel, S., Murdoch, A. D., & Iozzo, R. V. (1993). Structural characterization of the complete human perlecan gene and its promoter. *Proc Natl Acad Sci U S A, 90*(21), 10404-10408.

Cook, A., Raskind, W., Blanton, S. H., Pauli, R. M., Gregg, R. G., Francomano, C. A., . . . et al. (1993). Genetic heterogeneity in families with hereditary multiple exostoses. *Am J Hum Genet, 53*(1), 71-79.

Cornelison, D. D., Filla, M. S., Stanley, H. M., Rapraeger, A. C., & Olwin, B. B. (2001). Syndecan-3 and syndecan-4 specifically mark skeletal muscle satellite cells and are implicated in satellite cell maintenance and muscle regeneration. *Dev Biol, 239*(1), 79-94. doi:10.1006/dbio.2001.0416

Couchman, J. R. (2010). Transmembrane signaling proteoglycans. *Annu Rev Cell Dev Biol, 26*, 89-114. doi:10.1146/annurev-cellbio-100109-104126

Debinski, H. S., Lee, C. S., Dhillon, A. P., Mackenzie, P., Rhode, J., & Desmond, P. V. (1996). UDP-glucuronosyltransferase in Gilbert's syndrome. *Pathology, 28*(3), 238-241.

Deligny, A., Dierker, T., Dagalv, A., Lundequist, A., Eriksson, I., Nairn, A. V., . . . Kjellen, L. (2016). NDST2 (N-Deacetylase/N-Sulfotransferase-2) Enzyme Regulates Heparan Sulfate Chain Length. *J Biol Chem, 291*(36), 18600-18607. doi:10.1074/jbc.M116.744433

Demaurex, N., Furuya, W., D'Souza, S., Bonifacino, J. S., & Grinstein, S. (1998). Mechanism of acidification of the trans-Golgi network (TGN). In situ measurements of pH using retrieval of TGN38 and furin from the cell surface. *J Biol Chem, 273*(4), 2044-2051.

Ding, K., Lopez-Burks, M., Sanchez-Duran, J. A., Korc, M., & Lander, A. D. (2005). Growth factor-induced shedding of syndecan-1 confers glypican-1 dependence on mitogenic responses of cancer cells. *J Cell Biol, 171*(4), 729-738. doi:10.1083/jcb.200508010

El Masri, R., Seffouh, A., Lortat-Jacob, H., & Vives, R. R. (2016). The "in and out" of glucosamine 6-O-sulfation: the 6th sense of heparan sulfate. *Glycoconj J*. doi:10.1007/s10719-016-9736-5

Elfenbein, A., & Simons, M. (2013). Syndecan-4 signaling at a glance. *J Cell Sci, 126*(Pt 17), 3799-3804. doi:10.1242/jcs.124636

Esko, J. D., & Selleck, S. B. (2002). Order out of chaos: assembly of ligand binding sites in heparan sulfate. *Annu Rev Biochem, 71*, 435-471. doi:10.1146/annurev.biochem.71.110601.135458

Fang, J., Song, T., Lindahl, U., & Li, J. P. (2016). Enzyme overexpression - an exercise toward understanding regulation of heparan sulfate biosynthesis. *Sci Rep, 6*, 31242. doi:10.1038/srep31242

Farach-Carson, M. C., & Carson, D. D. (2007). Perlecan--a multifunctional extracellular proteoglycan scaffold. *Glycobiology, 17*(9), 897-905. doi:10.1093/glycob/cwm043

Farhan, S. M., Wang, J., Robinson, J. F., Prasad, A. N., Rupar, C. A., Siu, V. M., & Hegele, R. A. (2015). Old gene, new phenotype: mutations in heparan sulfate synthesis enzyme, EXT2 leads to seizure and developmental disorder, no exostoses. *J Med Genet, 52*(10), 666-675. doi:10.1136/jmedgenet-2015-103279

Fernandez-Vega, I., Garcia-Suarez, O., Garcia, B., Crespo, A., Astudillo, A., & Quiros, L. M. (2015). Heparan sulfate proteoglycans undergo differential expression alterations in right sided colorectal cancer, depending on their metastatic character. *BMC Cancer, 15*, 742. doi:10.1186/s12885-015-1724-9

Filmus, J., Capurro, M., & Rast, J. (2008). Glypicans. *Genome Biol, 9*(5), 224. doi:10.1186/gb-2008-9-5-224

Fischer, S., Filipek-Gorniok, B., & Ledin, J. (2011). Zebrafish Ext2 is necessary for Fgf and Wnt signaling, but not for Hh signaling. *BMC Dev Biol, 11*, 53. doi:10.1186/1471-213x-11-53

Fjeldstad, K., & Kolset, S. O. (2005). Decreasing the metastatic potential in cancers--targeting the heparan sulfate proteoglycans. *Curr Drug Targets, 6*(6), 665-682.

Fox, S. B., Fawcett, J., Jackson, D. G., Collins, I., Gatter, K. C., Harris, A. L., . . . Simmons, D. L. (1994). Normal human tissues, in addition to some tumors, express multiple different CD44 isoforms. *Cancer Res, 54*(16), 4539-4546.

Frese, M. A., Milz, F., Dick, M., Lamanna, W. C., & Dierks, T. (2009). Characterization of the human sulfatase Sulf1 and its high affinity heparin/heparan sulfate interaction domain. *J Biol Chem, 284*(41), 28033-28044. doi:10.1074/jbc.M109.035808

Gallagher, J. (2015). Fell-Muir Lecture: Heparan sulphate and the art of cell regulation: a polymer chain conducts the protein orchestra. *Int J Exp Pathol, 96*(4), 203-231. doi:10.1111/iep.12135

Gao, W., Kim, H., Feng, M., Phung, Y., Xavier, C. P., Rubin, J. S., & Ho, M. (2014). Inactivation of Wnt signaling by a human antibody that recognizes the heparan sulfate chains of glypican-3 for liver cancer therapy. *Hepatology, 60*(2), 576-587. doi:10.1002/hep.26996

Gao, W., Kim, H., & Ho, M. (2015). Human Monoclonal Antibody Targeting the Heparan Sulfate Chains of Glypican-3 Inhibits HGF-Mediated Migration and Motility of Hepatocellular Carcinoma Cells. *PLoS One, 10*(9), e0137664. doi:10.1371/journal.pone.0137664

Goodison, S., Urquidi, V., & Tarin, D. (1999). CD44 cell adhesion molecules. *Mol Pathol, 52*(4), 189-196.

Goyal, A., Gubbiotti, M. A., Chery, D. R., Han, L., & Iozzo, R. V. (2016). Endorepellin-evoked Autophagy Contributes to Angiostasis. *J Biol Chem, 291*(37), 19245-19256. doi:10.1074/jbc.M116.740266

Goyal, A., Poluzzi, C., Willis, C. D., Smythies, J., Shellard, A., Neill, T., & Iozzo, R. V. (2012). Endorepellin affects angiogenesis by antagonizing diverse vascular endothelial growth factor receptor 2 (VEGFR2)-evoked signaling pathways: transcriptional repression of hypoxia-inducible factor 1alpha and VEGFA and concurrent inhibition of nuclear factor of activated T cell 1 (NFAT1) activation. *J Biol Chem, 287*(52), 43543-43556. doi:10.1074/jbc.M112.401786

Grigorieva, E. V., Prudnikova, T. Y., Domanitskaya, N. V., Mostovich, L. A., Pavlova, T. V., Kashuba, V. I., & Zabarovsky, E. R. (2011). D-glucuronyl C5-epimerase suppresses small-cell lung cancer cell proliferation in vitro and tumour growth in vivo. *Br J Cancer, 105*(1), 74-82. doi:10.1038/bjc.2011.170

Grindel, B. J., Martinez, J. R., Pennington, C. L., Muldoon, M., Stave, J., Chung, L. W., & Farach-Carson, M. C. (2014). Matrilysin/matrix metalloproteinase-7(MMP7) cleavage of perlecan/HSPG2 creates a molecular switch to alter prostate cancer cell behavior. *Matrix Biol, 36*, 64-76. doi:10.1016/j.matbio.2014.04.005

Gubbiotti, M. A., Neill, T., & Iozzo, R. V. (2017). A current view of perlecan in physiology and pathology: A mosaic of functions. *Matrix Biol, 57-58*, 285-298. doi:10.1016/j.matbio.2016.09.003

Guo, L., Elcioglu, N. H., Mizumoto, S., Wang, Z., Noyan, B., Albayrak, H. M., . . . Ikegawa, S. (2017). Identification of biallelic EXTL3 mutations in a novel type of spondylo-epi-metaphyseal dysplasia. *J Hum Genet, 62*(8), 797-801. doi:10.1038/jhg.2017.38

Guo, L., Wang, J., Zhang, T., & Yang, Y. (2016). Glypican-5 is a tumor suppressor in non-small cell lung cancer cells. *Biochem Biophys Rep, 6*, 108-112. doi:10.1016/j.bbrep.2016.03.010

Hagner-Mcwhirter, A., Lindahl, U., & Li, J. (2000). Biosynthesis of heparin/heparan sulphate: mechanism of epimerization of glucuronyl C-5. *Biochem J, 347 Pt 1*, 69-75.

Hammond, E., Khurana, A., Shridhar, V., & Dredge, K. (2014). The Role of Heparanase and Sulfatases in the Modification of Heparan Sulfate Proteoglycans within the Tumor Microenvironment and Opportunities for Novel Cancer Therapeutics. *Front Oncol, 4*, 195. doi:10.3389/fonc.2014.00195

Hara, H., Takahashi, T., Serada, S., Fujimoto, M., Ohkawara, T., Nakatsuka, R., . . . Naka, T. (2016). Overexpression of glypican-1 implicates poor prognosis and their chemoresistance in oesophageal squamous cell carcinoma. *Br J Cancer, 115*(1), 66-75. doi:10.1038/bjc.2016.183

Hassinen, A., Pujol, F. M., Kokkonen, N., Pieters, C., Kihlstrom, M., Korhonen, K., & Kellokumpu, S. (2011). Functional organization of Golgi N- and O-glycosylation pathways involves pH-dependent complex formation that is impaired in cancer cells. *J Biol Chem, 286*(44), 38329-38340. doi:10.1074/jbc.M111.277681

Hatabe, S., Kimura, H., Arao, T., Kato, H., Hayashi, H., Nagai, T., . . . Nishio, K. (2013). Overexpression of heparan sulfate 6-O-sulfotransferase-2 in colorectal cancer. *Mol Clin Oncol, 1*(5), 845-850. doi:10.3892/mco.2013.151

Holley, R. J., Pickford, C. E., Rushton, G., Lacaud, G., Gallagher, J. T., Kouskoff, V., & Merry, C. L. (2011). Influencing hematopoietic differentiation of mouse embryonic stem cells using soluble heparin and heparan sulfate saccharides. *J Biol Chem, 286*(8), 6241-6252. doi:10.1074/jbc.M110.178483

Horiguchi, K., Syaidah, R., Fujiwara, K., Tsukada, T., Ramadhani, D., Jindatip, D., . . . Yashiro, T. (2013). Expression of the cell-surface heparan sulfate proteoglycan syndecan-2 in developing rat anterior pituitary gland. *Cell Tissue Res, 353*(3), 473-481. doi:10.1007/s00441-013-1641-7

Imoto-Tsubakimoto, H., Takahashi, T., Ueyama, T., Ogata, T., Adachi, A., Nakanishi, N., . . . Matsubara, H. (2013). Serglycin is a novel adipocytokine highly expressed in

epicardial adipose tissue. *Biochem Biophys Res Commun, 432*(1), 105-110. doi:10.1016/j.bbrc.2013.01.078

Iozzo, R. V., & Sanderson, R. D. (2011). Proteoglycans in cancer biology, tumour microenvironment and angiogenesis. *J Cell Mol Med, 15*(5), 1013-1031. doi:10.1111/j.1582-4934.2010.01236.x

Iozzo, R. V., Zoeller, J. J., & Nystrom, A. (2009). Basement membrane proteoglycans: modulators Par Excellence of cancer growth and angiogenesis. *Mol Cells, 27*(5), 503-513. doi:10.1007/s10059-009-0069-0

Jackson, D. G., Bell, J. I., Dickinson, R., Timans, J., Shields, J., & Whittle, N. (1995). Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon. *J Cell Biol, 128*(4), 673-685.

Jen, Y. H., Musacchio, M., & Lander, A. D. (2009). Glypican-1 controls brain size through regulation of fibroblast growth factor signaling in early neurogenesis. *Neural Dev, 4*, 33. doi:10.1186/1749-8104-4-33

Jochmann, K., Bachvarova, V., & Vortkamp, A. (2014). Reprint of: Heparan sulfate as a regulator of endochondral ossification and osteochondroma development. *Matrix Biol, 35*, 239-247. doi:10.1016/j.matbio.2014.04.001

Johnson, C. E., Crawford, B. E., Stavridis, M., Ten Dam, G., Wat, A. L., Rushton, G., . . . Merry, C. L. (2007). Essential alterations of heparan sulfate during the differentiation of embryonic stem cells to Sox1-enhanced green fluorescent protein-expressing neural progenitor cells. *Stem Cells, 25*(8), 1913-1923. doi:10.1634/stemcells.2006-0445

Jones, K. B., Piombo, V., Searby, C., Kurriger, G., Yang, B., Grabellus, F., . . . Sheffield, V. C. (2010). A mouse model of osteochondromagenesis from clonal inactivation of Ext1 in chondrocytes. *Proc Natl Acad Sci U S A, 107*(5), 2054-2059. doi:10.1073/pnas.0910875107

Kallunki, P., & Tryggvason, K. (1992). Human basement membrane heparan sulfate proteoglycan core protein: a 467-kD protein containing multiple domains resembling elements of the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. *J Cell Biol, 116*(2), 559-571.

Katta, K., Imran, T., Busse-Wicher, M., Gronning, M., Czajkowski, S., & Kusche-Gullberg, M. (2015). Reduced Expression of EXTL2, a Member of the Exostosin (EXT) Family of Glycosyltransferases, in Human Embryonic Kidney 293 Cells Results in Longer Heparan Sulfate Chains. *J Biol Chem, 290*(21), 13168-13177. doi:10.1074/jbc.M114.631754

Kellokumpu, S., Hassinen, A., & Glumoff, T. (2016). Glycosyltransferase complexes in eukaryotes: long-known, prevalent but still unrecognized. *Cell Mol Life Sci, 73*(2), 305-325. doi:10.1007/s00018-015-2066-0

Khotskaya, Y. B., Dai, Y., Ritchie, J. P., MacLeod, V., Yang, Y., Zinn, K., & Sanderson, R. D. (2009). Syndecan-1 is required for robust growth, vascularization, and metastasis of myeloma tumors in vivo. *J Biol Chem, 284*(38), 26085-26095. doi:10.1074/jbc.M109.018473

Kim, B. T., Kitagawa, H., Tamura, J., Saito, T., Kusche-Gullberg, M., Lindahl, U., & Sugahara, K. (2001). Human tumor suppressor EXT gene family members EXTL1 and EXTL3 encode alpha 1,4- N-acetylglucosaminyltransferases that likely are involved in heparan sulfate/ heparin biosynthesis. *Proc Natl Acad Sci U S A, 98*(13), 7176-7181. doi:10.1073/pnas.131188498

Kitagawa, H., Shimakawa, H., & Sugahara, K. (1999). The tumor suppressor EXT-like gene EXTL2 encodes an alpha1, 4-N-acetylhexosaminyltransferase that transfers N-acetylgalactosamine and N-acetylglucosamine to the common glycosaminoglycan-

protein linkage region. The key enzyme for the chain initiation of heparan sulfate. *J Biol Chem, 274*(20), 13933-13937.

Kjellen, L. (2003). Glucosaminyl N-deacetylase/N-sulphotransferases in heparan sulphate biosynthesis and biology. *Biochem Soc Trans, 31*(2), 340-342. doi:10.1042/

Knudson, A. G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A, 68*(4), 820-823.

Koiwai, O., Nishizawa, M., Hasada, K., Aono, S., Adachi, Y., Mamiya, N., & Sato, H. (1995). Gilbert's syndrome is caused by a heterozygous missense mutation in the gene for bilirubin UDP-glucuronosyltransferase. *Hum Mol Genet, 4*(7), 1183-1186.

Kolset, S. O., & Pejler, G. (2011). Serglycin: a structural and functional chameleon with wide impact on immune cells. *J Immunol, 187*(10), 4927-4933. doi:10.4049/jimmunol.1100806

Kolset, S. O., Prydz, K., & Pejler, G. (2004). Intracellular proteoglycans. *Biochem J, 379*(Pt 2), 217-227. doi:10.1042/bj20031230

Kolset, S. O., & Tveit, H. (2008). Serglycin--structure and biology. *Cell Mol Life Sci, 65*(7-8), 1073-1085. doi:10.1007/s00018-007-7455-6

Korpetinou, A., Skandalis, S. S., Labropoulou, V. T., Smirlaki, G., Noulas, A., Karamanos, N. K., & Theocharis, A. D. (2014). Serglycin: at the crossroad of inflammation and malignancy. *Front Oncol, 3*, 327. doi:10.3389/fonc.2013.00327

Koziel, L., Kunath, M., Kelly, O. G., & Vortkamp, A. (2004). Ext1-dependent heparan sulfate regulates the range of Ihh signaling during endochondral ossification. *Dev Cell, 6*(6), 801-813. doi:10.1016/j.devcel.2004.05.009

Kraushaar, D. C., Yamaguchi, Y., & Wang, L. (2010). Heparan sulfate is required for embryonic stem cells to exit from self-renewal. *J Biol Chem, 285*(8), 5907-5916. doi:10.1074/jbc.M109.066837

Kusche-Gullberg, M., & Kjellen, L. (2003). Sulfotransferases in glycosaminoglycan biosynthesis. *Curr Opin Struct Biol, 13*(5), 605-611.

Ledin, J., Staatz, W., Li, J. P., Gotte, M., Selleck, S., Kjellen, L., & Spillmann, D. (2004). Heparan sulfate structure in mice with genetically modified heparan sulfate production. *J Biol Chem, 279*(41), 42732-42741. doi:10.1074/jbc.M405382200

Lee, J. S., von der Hardt, S., Rusch, M. A., Stringer, S. E., Stickney, H. L., Talbot, W. S., . . . Roehl, H. (2004). Axon sorting in the optic tract requires HSPG synthesis by ext2 (dackel) and extl3 (boxer). *Neuron, 44*(6), 947-960. doi:10.1016/j.neuron.2004.11.029

Lemire, J. M., Chan, C. K., Bressler, S., Miller, J., LeBaron, R. G., & Wight, T. N. (2007). Interleukin-1beta selectively decreases the synthesis of versican by arterial smooth muscle cells. *J Cell Biochem, 101*(3), 753-766. doi:10.1002/jcb.21235

Lendorf, M. E., Manon-Jensen, T., Kronqvist, P., Multhaupt, H. A., & Couchman, J. R. (2011). Syndecan-1 and syndecan-4 are independent indicators in breast carcinoma. *J Histochem Cytochem, 59*(6), 615-629. doi:10.1369/0022155411405057

Li, H., Yao, Z., He, W., Gao, H., Bai, Y., Yang, S., . . . Luo, G. (2016). P311 induces the transdifferentiation of epidermal stem cells to myofibroblast-like cells by stimulating transforming growth factor beta1 expression. *Stem Cell Res Ther, 7*(1), 175. doi:10.1186/s13287-016-0421-1

Li, J. P., & Kusche-Gullberg, M. (2016). Heparan Sulfate: Biosynthesis, Structure, and Function. *Int Rev Cell Mol Biol, 325*, 215-273. doi:10.1016/bs.ircmb.2016.02.009

Lidholt, K., & Lindahl, U. (1992). Biosynthesis of heparin. The D-glucuronosyl- and N-acetyl-D-glucosaminyltransferase reactions and their relation to polymer modification. *Biochem J, 287 (Pt 1)*, 21-29.

Lim, H. C., & Couchman, J. R. (2014). Syndecan-2 regulation of morphology in breast carcinoma cells is dependent on RhoGTPases. *Biochim Biophys Acta, 1840*(8), 2482-2490. doi:10.1016/j.bbagen.2014.01.018

Lim, H. C., Multhaupt, H. A., & Couchman, J. R. (2015). Cell surface heparan sulfate proteoglycans control adhesion and invasion of breast carcinoma cells. *Mol Cancer, 14*, 15. doi:10.1186/s12943-014-0279-8

Lin, X., Buff, E. M., Perrimon, N., & Michelson, A. M. (1999). Heparan sulfate proteoglycans are essential for FGF receptor signaling during Drosophila embryonic development. *Development, 126*(17), 3715-3723.

Lin, X., Wei, G., Shi, Z., Dryer, L., Esko, J. D., Wells, D. E., & Matzuk, M. M. (2000). Disruption of gastrulation and heparan sulfate biosynthesis in EXT1-deficient mice. *Dev Biol, 224*(2), 299-311. doi:10.1006/dbio.2000.9798

Lind, T., Tufaro, F., McCormick, C., Lindahl, U., & Lidholt, K. (1998). The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. *J Biol Chem, 273*(41), 26265-26268.

Lindahl, U., & Kjellen, L. (2013). Pathophysiology of heparan sulphate: many diseases, few drugs. *J Intern Med, 273*(6), 555-571. doi:10.1111/joim.12061

Lord, M. S., Chuang, C. Y., Melrose, J., Davies, M. J., Iozzo, R. V., & Whitelock, J. M. (2014). The role of vascular-derived perlecan in modulating cell adhesion, proliferation and growth factor signaling. *Matrix Biol, 35*, 112-122. doi:10.1016/j.matbio.2014.01.016

Lu, H., Niu, F., Liu, F., Gao, J., Sun, Y., & Zhao, X. (2017). Elevated glypican-1 expression is associated with an unfavorable prognosis in pancreatic ductal adenocarcinoma. *Cancer Med, 6*(6), 1181-1191. doi:10.1002/cam4.1064

Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., & Lindahl, U. (1996). Domain structure of heparan sulfates from bovine organs. *J Biol Chem, 271*(30), 17804-17810.

Manon-Jensen, T., Itoh, Y., & Couchman, J. R. (2010). Proteoglycans in health and disease: the multiple roles of syndecan shedding. *FEBS J, 277*(19), 3876-3889. doi:10.1111/j.1742-4658.2010.07798.x

Manon-Jensen, T., Multhaupt, H. A., & Couchman, J. R. (2013). Mapping of matrix metalloproteinase cleavage sites on syndecan-1 and syndecan-4 ectodomains. *FEBS J, 280*(10), 2320-2331. doi:10.1111/febs.12174

Matsumoto, K., Irie, F., Mackem, S., & Yamaguchi, Y. (2010). A mouse model of chondrocyte-specific somatic mutation reveals a role for Ext1 loss of heterozygosity in multiple hereditary exostoses. *Proc Natl Acad Sci U S A, 107*(24), 10932-10937. doi:10.1073/pnas.0914642107

McCarthy, K. J. (2015). The Basement Membrane Proteoglycans Perlecan and Agrin: Something Old, Something New. *Curr Top Membr, 76*, 255-303. doi:10.1016/bs.ctm.2015.09.001

McCormick, C., Duncan, G., Goutsos, K. T., & Tufaro, F. (2000). The putative tumor suppressors EXT1 and EXT2 form a stable complex that accumulates in the Golgi apparatus and catalyzes the synthesis of heparan sulfate. *Proc Natl Acad Sci U S A, 97*(2), 668-673.

Mitsou, I., Multhaupt, H. A. B., & Couchman, J. R. (2017). Proteoglycans, ion channels and cell-matrix adhesion. *Biochem J, 474*(12), 1965-1979. doi:10.1042/bcj20160747

Morgan, M. R., Humphries, M. J., & Bass, M. D. (2007). Synergistic control of cell adhesion by integrins and syndecans. *Nat Rev Mol Cell Biol, 8*(12), 957-969. doi:10.1038/nrm2289

Morimoto-Tomita, M., Uchimura, K., Werb, Z., Hemmerich, S., & Rosen, S. D. (2002). Cloning and characterization of two extracellular heparin-degrading endosulfatases in mice and humans. *J Biol Chem, 277*(51), 49175-49185. doi:10.1074/jbc.M205131200

Moses, J., Oldberg, A., & Fransson, L. A. (1999). Initiation of galactosaminoglycan biosynthesis. Separate galactosylation and dephosphorylation pathways for phosphoxylosylated decorin protein and exogenous xyloside. *Eur J Biochem, 260*(3), 879-884.

Multhaupt, H. A., Leitinger, B., Gullberg, D., & Couchman, J. R. (2016). Extracellular matrix component signaling in cancer. *Adv Drug Deliv Rev, 97*, 28-40. doi:10.1016/j.addr.2015.10.013

Murdoch, A. D., Dodge, G. R., Cohen, I., Tuan, R. S., & Iozzo, R. V. (1992). Primary structure of the human heparan sulfate proteoglycan from basement membrane (HSPG2/perlecan). A chimeric molecule with multiple domains homologous to the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. *J Biol Chem, 267*(12), 8544-8557.

Musso, N., Caronia, F. P., Castorina, S., Lo Monte, A. I., Barresi, V., & Condorelli, D. F. (2015). Somatic loss of an EXT2 gene mutation during malignant progression in a patient with hereditary multiple osteochondromas. *Cancer Genet, 208*(3), 62-67. doi:10.1016/j.cancergen.2015.01.002

Nadanaka, S., Kagiyama, S., & Kitagawa, H. (2013). Roles of EXTL2, a member of the EXT family of tumour suppressors, in liver injury and regeneration processes. *Biochem J, 454*(1), 133-145. doi:10.1042/bj20130323

Nadanaka, S., & Kitagawa, H. (2014). EXTL2 controls liver regeneration and aortic calcification through xylose kinase-dependent regulation of glycosaminoglycan biosynthesis. *Matrix Biol, 35*, 18-24. doi:10.1016/j.matbio.2013.10.010

Nadanaka, S., Purunomo, E., Takeda, N., Tamura, J., & Kitagawa, H. (2014). Heparan sulfate containing unsubstituted glucosamine residues: biosynthesis and heparanase-inhibitory activity. *J Biol Chem, 289*(22), 15231-15243. doi:10.1074/jbc.M113.545343

Nadanaka, S., Zhou, S., Kagiyama, S., Shoji, N., Sugahara, K., Sugihara, K., . . . Kitagawa, H. (2013). EXTL2, a member of the EXT family of tumor suppressors, controls glycosaminoglycan biosynthesis in a xylose kinase-dependent manner. *J Biol Chem, 288*(13), 9321-9333. doi:10.1074/jbc.M112.416909

Neve, R. M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F. L., Fevr, T., . . . Gray, J. W. (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell, 10*(6), 515-527. doi:10.1016/j.ccr.2006.10.008

Nguyen, T. L., Grizzle, W. E., Zhang, K., Hameed, O., Siegal, G. P., & Wei, S. (2013). Syndecan-1 overexpression is associated with nonluminal subtypes and poor prognosis in advanced breast cancer. *Am J Clin Pathol, 140*(4), 468-474. doi:10.1309/ajcpz1d8calhdxcj

Noonan, D. M., Fulle, A., Valente, P., Cai, S., Horigan, E., Sasaki, M., . . . Hassell, J. R. (1991). The complete sequence of perlecan, a basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein-receptor, and the neural cell adhesion molecule. *J Biol Chem, 266*(34), 22939-22947.

Okada, M., Nadanaka, S., Shoji, N., Tamura, J., & Kitagawa, H. (2010). Biosynthesis of heparan sulfate in EXT1-deficient cells. *Biochem J, 428*(3), 463-471. doi:10.1042/bj20100101

Okolicsanyi, R. K., van Wijnen, A. J., Cool, S. M., Stein, G. S., Griffiths, L. R., & Haupt, L. M. (2014). Heparan sulfate proteoglycans and human breast cancer epithelial cell tumorigenicity. *J Cell Biochem, 115*(5), 967-976. doi:10.1002/jcb.24746

Ori, A., Wilkinson, M. C., & Fernig, D. G. (2011). A systems biology approach for the investigation of the heparin/heparan sulfate interactome. *J Biol Chem, 286*(22), 19892-19904. doi:10.1074/jbc.M111.228114

Osterholm, C., Barczyk, M. M., Busse, M., Gronning, M., Reed, R. K., & Kusche-Gullberg, M. (2009). Mutation in the heparan sulfate biosynthesis enzyme EXT1 influences growth factor signaling and fibroblast interactions with the extracellular matrix. *J Biol Chem, 284*(50), 34935-34943. doi:10.1074/jbc.M109.005264

Osterholm, C., Lu, N., Liden, A., Karlsen, T. V., Gullberg, D., Reed, R. K., & Kusche-Gullberg, M. (2012). Fibroblast EXT1-levels influence tumor cell proliferation and migration in composite spheroids. *PLoS One, 7*(7), e41334. doi:10.1371/journal.pone.0041334

Oud, M. M., Tuijnenburg, P., Hempel, M., van Vlies, N., Ren, Z., Ferdinandusse, S., . . . Kuijpers, T. W. (2017). Mutations in EXTL3 Cause Neuro-immuno-skeletal Dysplasia Syndrome. *Am J Hum Genet, 100*(2), 281-296. doi:10.1016/j.ajhg.2017.01.013

Pacifici, M. (2017). Hereditary Multiple Exostoses: New Insights into Pathogenesis, Clinical Complications, and Potential Treatments. *Curr Osteoporos Rep, 15*(3), 142-152. doi:10.1007/s11914-017-0355-2

Pedersen, L. C., Dong, J., Taniguchi, F., Kitagawa, H., Krahn, J. M., Pedersen, L. G., . . . Negishi, M. (2003). Crystal structure of an alpha 1,4-N-acetylhexosaminyltransferase (EXTL2), a member of the exostosin gene family involved in heparan sulfate biosynthesis. *J Biol Chem, 278*(16), 14420-14428. doi:10.1074/jbc.M210532200

Pikas, D. S., Eriksson, I., & Kjellen, L. (2000). Overexpression of different isoforms of glucosaminyl N-deacetylase/N-sulfotransferase results in distinct heparan sulfate N-sulfation patterns. *Biochemistry, 39*(15), 4552-4558.

Poluzzi, C., Casulli, J., Goyal, A., Mercer, T. J., Neill, T., & Iozzo, R. V. (2014). Endorepellin evokes autophagy in endothelial cells. *J Biol Chem, 289*(23), 16114-16128. doi:10.1074/jbc.M114.556530

Ponta, H., Sherman, L., & Herrlich, P. A. (2003). CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol, 4*(1), 33-45. doi:10.1038/nrm1004

Presto, J., Thuveson, M., Carlsson, P., Busse, M., Wilen, M., Eriksson, I., . . . Kjellen, L. (2008). Heparan sulfate biosynthesis enzymes EXT1 and EXT2 affect NDST1 expression and heparan sulfate sulfation. *Proc Natl Acad Sci U S A, 105*(12), 4751-4756. doi:10.1073/pnas.0705807105

Prudnikova, T. Y., Mostovich, L. A., Domanitskaya, N. V., Pavlova, T. V., Kashuba, V. I., Zabarovsky, E. R., & Grigorieva, E. V. (2010). Antiproliferative effect of D-glucuronyl C5-epimerase in human breast cancer cells. *Cancer Cell Int, 10*, 27. doi:10.1186/1475-2867-10-27

Prydz, K. (2015). Determinants of Glycosaminoglycan (GAG) Structure. *Biomolecules, 5*(3), 2003-2022. doi:10.3390/biom5032003

Prydz, K., & Dalen, K. T. (2000). Synthesis and sorting of proteoglycans. *J Cell Sci, 113 Pt 2*, 193-205.

Reuter, M. S., Musante, L., Hu, H., Diederich, S., Sticht, H., Ekici, A. B., . . . Kahrizi, K. (2014). NDST1 missense mutations in autosomal recessive intellectual disability. *Am J Med Genet A, 164a*(11), 2753-2763. doi:10.1002/ajmg.a.36723

Rivinoja, A., Pujol, F. M., Hassinen, A., & Kellokumpu, S. (2012). Golgi pH, its regulation and roles in human disease. *Ann Med, 44*(6), 542-554. doi:10.3109/07853890.2011.579150

Ropero, S., Setien, F., Espada, J., Fraga, M. F., Herranz, M., Asp, J., . . . Esteller, M. (2004). Epigenetic loss of the familial tumor-suppressor gene exostosin-1 (EXT1) disrupts

heparan sulfate synthesis in cancer cells. *Hum Mol Genet, 13*(22), 2753-2765. doi:10.1093/hmg/ddh298

Rosenberg, E. E., Prudnikova, T. Y., Zabarovsky, E. R., Kashuba, V. I., & Grigorieva, E. V. (2014). D-glucuronyl C5-epimerase cell type specifically affects angiogenesis pathway in different prostate cancer cells. *Tumour Biol, 35*(4), 3237-3245. doi:10.1007/s13277-013-1423-6

Roskams, T., De Vos, R., David, G., Van Damme, B., & Desmet, V. (1998). Heparan sulphate proteoglycan expression in human primary liver tumours. *J Pathol, 185*(3), 290-297. doi:10.1002/(sici)1096-9896(199807)185:3<290::aid-path91>3.0.co;2-i

Roy, D., Mondal, S., Khurana, A., Jung, D. B., Hoffmann, R., He, X., . . . Shridhar, V. (2017). Loss of HSulf-1: The Missing Link between Autophagy and Lipid Droplets in Ovarian Cancer. *Sci Rep, 7*, 41977. doi:10.1038/srep41977

Saito, T., Sugiyama, K., Hama, S., Yamasaki, F., Takayasu, T., Nosaka, R., . . . Kurisu, K. (2017). High Expression of Glypican-1 Predicts Dissemination and Poor Prognosis in Glioblastomas. *World Neurosurg*. doi:10.1016/j.wneu.2017.05.165

Saitoh, M. (2015). Epithelial-mesenchymal transition is regulated at post-transcriptional levels by transforming growth factor-beta signaling during tumor progression. *Cancer Sci, 106*(5), 481-488. doi:10.1111/cas.12630

Schick, B. P., Gradowski, J. F., & San Antonio, J. D. (2001). Synthesis, secretion, and subcellular localization of serglycin proteoglycan in human endothelial cells. *Blood, 97*(2), 449-458.

Schreml, J., Durmaz, B., Cogulu, O., Keupp, K., Beleggia, F., Pohl, E., . . . Ozkinay, F. (2014). The missing "link": an autosomal recessive short stature syndrome caused by a hypofunctional XYLT1 mutation. *Hum Genet, 133*(1), 29-39. doi:10.1007/s00439-013-1351-y

Scully, O. J., Chua, P. J., Harve, K. S., Bay, B. H., & Yip, G. W. (2012). Serglycin in health and diseases. *Anat Rec (Hoboken), 295*(9), 1415-1420. doi:10.1002/ar.22536

Seffouh, A., Milz, F., Przybylski, C., Laguri, C., Oosterhof, A., Bourcier, S., . . . Vives, R. R. (2013). HSulf sulfatases catalyze processive and oriented 6-O-desulfation of heparan sulfate that differentially regulates fibroblast growth factor activity. *FASEB J, 27*(6), 2431-2439. doi:10.1096/fj.12-226373

Seki, H., Kubota, T., Ikegawa, S., Haga, N., Fujioka, F., Ohzeki, S., . . . Fukushima, Y. (2001). Mutation frequencies of EXT1 and EXT2 in 43 Japanese families with hereditary multiple exostoses. *Am J Med Genet, 99*(1), 59-62.

Senay, C., Lind, T., Muguruma, K., Tone, Y., Kitagawa, H., Sugahara, K., . . . Kusche-Gullberg, M. (2000). The EXT1/EXT2 tumor suppressors: catalytic activities and role in heparan sulfate biosynthesis. *EMBO Rep, 1*(3), 282-286. doi:10.1093/embo-reports/kvd045

Shirakihara, T., Horiguchi, K., Miyazawa, K., Ehata, S., Shibata, T., Morita, I., . . . Saitoh, M. (2011). TGF-beta regulates isoform switching of FGF receptors and epithelial-mesenchymal transition. *Embo j, 30*(4), 783-795. doi:10.1038/emboj.2010.351

Stamenkovic, I., Aruffo, A., Amiot, M., & Seed, B. (1991). The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. *Embo j, 10*(2), 343-348.

Stewart, M. D., Ramani, V. C., & Sanderson, R. D. (2015). Shed syndecan-1 translocates to the nucleus of cells delivering growth factors and inhibiting histone acetylation: a novel mechanism of tumor-host cross-talk. *J Biol Chem, 290*(2), 941-949. doi:10.1074/jbc.M114.608455

Stickens, D., Zak, B. M., Rougier, N., Esko, J. D., & Werb, Z. (2005). Mice deficient in Ext2 lack heparan sulfate and develop exostoses. *Development, 132*(22), 5055-5068. doi:10.1242/dev.02088

Suarez, E. R., Paredes-Gamero, E. J., Del Giglio, A., Tersariol, I. L., Nader, H. B., & Pinhal, M. A. (2013). Heparan sulfate mediates trastuzumab effect in breast cancer cells. *BMC Cancer, 13*, 444. doi:10.1186/1471-2407-13-444

Suda, T., Kamiyama, S., Suzuki, M., Kikuchi, N., Nakayama, K., Narimatsu, H., . . . Nishihara, S. (2004). Molecular cloning and characterization of a human multisubstrate specific nucleotide-sugar transporter homologous to Drosophila fringe connection. *J Biol Chem, 279*(25), 26469-26474. doi:10.1074/jbc.M311353200

Sugahara, K., & Kitagawa, H. (2000). Recent advances in the study of the biosynthesis and functions of sulfated glycosaminoglycans. *Curr Opin Struct Biol, 10*(5), 518-527.

Suhovskih, A. V., Domanitskaya, N. V., Tsidulko, A. Y., Prudnikova, T. Y., Kashuba, V. I., & Grigorieva, E. V. (2015). Tissue-specificity of heparan sulfate biosynthetic machinery in cancer. *Cell Adh Migr, 9*(6), 452-459. doi:10.1080/19336918.2015.1049801

Suhovskih, A. V., Tsidulko, A. Y., Kutsenko, O. S., Kovner, A. V., Aidagulova, S. V., Ernberg, I., & Grigorieva, E. V. (2014). Transcriptional Activity of Heparan Sulfate Biosynthetic Machinery is Specifically Impaired in Benign Prostate Hyperplasia and Prostate Cancer. *Front Oncol, 4*, 79. doi:10.3389/fonc.2014.00079

Takahashi, I., Noguchi, N., Nata, K., Yamada, S., Kaneiwa, T., Mizumoto, S., . . . Sugawara, A. (2009). Important role of heparan sulfate in postnatal islet growth and insulin secretion. *Biochem Biophys Res Commun, 383*(1), 113-118. doi:10.1016/j.bbrc.2009.03.140

Takei, Y., Ozawa, Y., Sato, M., Watanabe, A., & Tabata, T. (2004). Three Drosophila EXT genes shape morphogen gradients through synthesis of heparan sulfate proteoglycans. *Development, 131*(1), 73-82. doi:10.1242/dev.00913

Tornberg, J., Sykiotis, G. P., Keefe, K., Plummer, L., Hoang, X., Hall, J. E., . . . Bulow, H. E. (2011). Heparan sulfate 6-O-sulfotransferase 1, a gene involved in extracellular sugar modifications, is mutated in patients with idiopathic hypogonadotrophic hypogonadism. *Proc Natl Acad Sci U S A, 108*(28), 11524-11529. doi:10.1073/pnas.1102284108

Tzeng, S. T., Tsai, M. H., Chen, C. L., Lee, J. X., Jao, T. M., Yu, S. L., . . . Yang, Y. C. (2013). NDST4 is a novel candidate tumor suppressor gene at chromosome 4q26 and its genetic loss predicts adverse prognosis in colorectal cancer. *PLoS One, 8*(6), e67040. doi:10.1371/journal.pone.0067040

Uhlin-Hansen, L., Wik, T., Kjellen, L., Berg, E., Forsdahl, F., & Kolset, S. O. (1993). Proteoglycan metabolism in normal and inflammatory human macrophages. *Blood, 82*(9), 2880-2889.

Uniewicz, K. A., Ori, A., Xu, R., Ahmed, Y., Wilkinson, M. C., Fernig, D. G., & Yates, E. A. (2010). Differential scanning fluorimetry measurement of protein stability changes upon binding to glycosaminoglycans: a screening test for binding specificity. *Anal Chem, 82*(9), 3796-3802. doi:10.1021/ac100188x

Ushakov, V. S., Tsidulko, A. Y., de La Bourdonnaye, G., Kazanskaya, G. M., Volkov, A. M., Kiselev, R. S., . . . Grigorieva, E. V. (2017). Heparan Sulfate Biosynthetic System Is Inhibited in Human Glioma Due to EXT1/2 and HS6ST1/2 Down-Regulation. *Int J Mol Sci, 18*(11). doi:10.3390/ijms18112301

Van Hul, W., Wuyts, W., Hendrickx, J., Speleman, F., Wauters, J., De Boulle, K., . . . Willems, P. J. (1998). Identification of a third EXT-like gene (EXTL3) belonging to the EXT gene family. *Genomics, 47*(2), 230-237. doi:10.1006/geno.1997.5101

Verbert, A., Cacan, R., & Cecchelli, R. (1987). Membrane transport of sugar donors to the glycosylation sites. *Biochimie, 69*(2), 91-99.

Vives, R. R., Seffouh, A., & Lortat-Jacob, H. (2014). Post-Synthetic Regulation of HS Structure: The Yin and Yang of the Sulfs in Cancer. *Front Oncol, 3*, 331. doi:10.3389/fonc.2013.00331

Vlodavsky, I., Ilan, N., Naggi, A., & Casu, B. (2007). Heparanase: structure, biological functions, and inhibition by heparin-derived mimetics of heparan sulfate. *Curr Pharm Des, 13*(20), 2057-2073.

Vlodavsky, I., Singh, P., Boyango, I., Gutter-Kapon, L., Elkin, M., Sanderson, R. D., & Ilan, N. (2016). Heparanase: From basic research to therapeutic applications in cancer and inflammation. *Drug Resist Updat, 29*, 54-75. doi:10.1016/j.drup.2016.10.001

Volpi, S., Yamazaki, Y., Brauer, P. M., van Rooijen, E., Hayashida, A., Slavotinek, A., . . . Notarangelo, L. D. (2017). EXTL3 mutations cause skeletal dysplasia, immune deficiency, and developmental delay. *J Exp Med, 214*(3), 623-637. doi:10.1084/jem.20161525

Wang, S., Qiu, M., Xia, W., Xu, Y., Mao, Q., Wang, J., . . . Yin, R. (2016). Glypican-5 suppresses Epithelial-Mesenchymal Transition of the lung adenocarcinoma by competitively binding to Wnt3a. *Oncotarget, 7*(48), 79736-79746. doi:10.18632/oncotarget.12945

Wise, C. A., Clines, G. A., Massa, H., Trask, B. J., & Lovett, M. (1997). Identification and localization of the gene for EXTL, a third member of the multiple exostoses gene family. *Genome Res, 7*(1), 10-16.

Wuyts, W., Ramlakhan, S., Van Hul, W., Hecht, J. T., van den Ouweland, A. M., Raskind, W. H., . . . et al. (1995). Refinement of the multiple exostoses locus (EXT2) to a 3-cM interval on chromosome 11. *Am J Hum Genet, 57*(2), 382-387.

Wuyts, W., & Van Hul, W. (2000). Molecular basis of multiple exostoses: mutations in the EXT1 and EXT2 genes. *Hum Mutat, 15*(3), 220-227. doi:10.1002/(sici)1098-1004(200003)15:3<220::aid-humu2>3.0.co;2-k

Wuyts, W., Van Hul, W., Hendrickx, J., Speleman, F., Wauters, J., De Boulle, K., . . . Willems, P. J. (1997). Identification and characterization of a novel member of the EXT gene family, EXTL2. *Eur J Hum Genet, 5*(6), 382-389.

Xu, D., & Esko, J. D. (2014). Demystifying heparan sulfate-protein interactions. *Annu Rev Biochem, 83*, 129-157. doi:10.1146/annurev-biochem-060713-035314

Xu, L., Xia, J., Jiang, H., Zhou, J., Li, H., Wang, D., . . . Deng, H. X. (1999). Mutation analysis of hereditary multiple exostoses in the Chinese. *Hum Genet, 105*(1-2), 45-50.

Yamada, S., Busse, M., Ueno, M., Kelly, O. G., Skarnes, W. C., Sugahara, K., & Kusche-Gullberg, M. (2004). Embryonic fibroblasts with a gene trap mutation in Ext1 produce short heparan sulfate chains. *J Biol Chem, 279*(31), 32134-32141. doi:10.1074/jbc.M312624200

Yiu, G. K., Kaunisto, A., Chin, Y. R., & Toker, A. (2011). NFAT promotes carcinoma invasive migration through glypican-6. *Biochem J, 440*(1), 157-166. doi:10.1042/bj20110530

Yue, M. M., Lv, K., Meredith, S. C., Martindale, J. L., Gorospe, M., & Schuger, L. (2014). Novel RNA-binding protein P311 binds eukaryotic translation initiation factor 3 subunit b (eIF3b) to promote translation of transforming growth factor beta1-3 (TGF-beta1-3). *J Biol Chem, 289*(49), 33971-33983. doi:10.1074/jbc.M114.609495

Zak, B. M., Crawford, B. E., & Esko, J. D. (2002). Hereditary multiple exostoses and heparan sulfate polymerization. *Biochim Biophys Acta, 1573*(3), 346-355.

Zernichow, L., Abrink, M., Hallgren, J., Grujic, M., Pejler, G., & Kolset, S. O. (2006). Serglycin is the major secreted proteoglycan in macrophages and has a role in the regulation of macrophage tumor necrosis factor-alpha secretion in response to

lipopolysaccharide. *J Biol Chem, 281*(37), 26792-26801. doi:10.1074/jbc.M512889200

Zhang, L., Li, H., Yao, Z., Yang, S., He, W., Wu, J., & Luo, G. (2016). [Interaction between P311 and transforming growth factor beta 1 and its effect on the function of murine fibroblasts]. *Zhonghua Shao Shang Za Zhi, 32*(4), 208-215. doi:10.3760/cma.j.issn.1009-2587.2016.04.005

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Potential role for *Ext1*-dependent heparan sulfate in regulating *P311* gene expression in A549 carcinoma cells

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ABSTRACT

Background: Exostosin-1 (EXT1), a member of the EXT protein family, is indispensable for synthesis of heparan sulfate (HS) chains that bind to and modulate the signaling efficiency of numerous growth factor activities. We have previously shown that *Ext1* mutated mouse embryonic fibroblasts produce short sulfated HS chains which dramatically influence tumor cell behavior in a 3-dimensional (3D) heterospheroid system composed of tumor cells and fibroblasts.

Methods: In this study, we have used both 2D co-culture and 3D heterospheroid models, consisting of human A549 carcinoma cells co-cultured with wild-type or *Ext1*-mutated mouse embryonic fibroblasts.

Results and conclusions: Gene expression profiling of differentially expressed genes in fibroblast/A549 heterospheroids identified *P311* as a gene substantially down-regulated in A549 cells co-cultured with *Ext1*-mutated fibroblasts. In addition, we observed that the *Ext1* mutants displayed reduced *Tgf-β1* mRNA levels and lower levels of secreted active TGF-β protein. *Re*-introduction of *Ext1* in the *Ext1* mutant fibroblasts rescued the levels of *Tgf-β1* mRNA, increased the amounts of secreted active TGF-β in these cells, as well as *P311* mRNA levels in adjacent A549 cells. Accordingly, small interfering RNAs (siRNAs) against fibroblast *Tgf-β1* reduced *P311* expression in neighboring A549 tumor cells. Our data raises the possibility that fibroblast *Ext1* levels play a role in *P311* expression in A549/fibroblast co-culture through TGF-β1.

General significance: This study considers a possible novel mechanism of *Ext1*-regulated heparan sulfate structure in modifying tumor-stroma interactions through altering stromal *tgf-β1* expression.

1. Introduction

Exostosin (EXT)-1 and EXT2 are glycosyltransferases involved in heparan sulfate (HS) proteoglycan (PG) biosynthesis [1–3]. HSPGs, composed of one or more HS chains covalently attached to various protein cores, are ubiquitous components of the extracellular matrix (ECM) and play many important roles in tissue homeostasis [4,5]. They are essential for signal transduction of a multitude of signaling molecules thus driving processes such as cell survival, division, migration, differentiation and cancer development [5,6]. HSPGs are synthesized in a multistep process that involves chain elongation by the action of an EXT1/EXT2 *co*-polymerase complex. Concomitant with chain elongation, several modifications occur through an epimerase and various sulfotransferases that generate a complex polysaccharide containing *N*-acetylated and N-sulfated glucosamine residues, glucuronic acid and iduronic acid units, as well as O-sulfate groups in various positions [7]. Five genes encoding EXT proteins have been identified in mammals, *EXT1*, *EXT2*, and the *EXT*-like genes *EXTL1*, *EXTL2*, and *EXTL3*. *EXT1* and *EXT2* were first recognized as genes responsible for the autosomal inherited disorder hereditary multiple osteochondromas (also called hereditary multiple exostoses), by genetic linkage analysis [8–10]. The EXT-like proteins, EXTL1, EXTL2 and EXTL3, have not been linked to hereditary multiple osteochondromas. Instead, they were identified in screens for proteins homologous to EXT1 and EXT2 [11–13].

We have previously shown that *Ext1* mutated mouse embryonic fibroblasts ($Ext1^{Gt/Gt}$) produce short sulfated HS chains with relatively intact sulfation pattern [14]. The average molecular sizes of the HS chains from wild-type ($Ext1^{wt/wt}$) and mutated ($Ext1^{Gt/Gt}$) mouse embryonic fibroblasts were estimated to be 70 and 12.5 kDa, respectively. The mutated fibroblasts show a reduced proliferation rate, a reduced FGF2 signaling response and a reduced ability to adhere to and to remodel collagen gels [15]. We further used a three-dimensional (3D) mini-tumor spheroid model of human tumor cell lines and mouse fibroblasts to study the cross-talk between these two major and mutually

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dependent components by providing a microenvironment more similar to a primary tumor. In mixed multicellular 3D-spheroids, composed of human tumor cells and *Ext1*$^{wt/wt}$ or *Ext1*$^{Gt/Gt}$ fibroblasts, the *Ext1* mutation in stromal fibroblasts strongly influences tumor cell behavior and the interstitial fluid pressure [16]. *Ext1*$^{Gt/Gt}$/A549 lung adenocarcinoma cell heterospheroids have a homogenous low interstitial fluid pressure throughout the spheroid, whereas the corresponding data for *Ext1*$^{wt/wt}$/A549 spheroids vary greatly with the depth of measurements. After 6 days in culture, the wild-type fibroblasts form an inner core and the tumor cells an outer layer of cells. For spheroids containing *Ext1*$^{Gt/Gt}$ fibroblasts, this segregation is less obvious, indicating impaired tumor cell migration that coincides with a lower proliferation rate. The *Ext1*-dependent migration behavior was not restricted to A549 cells, as similar cell behaviors were observed also with H460- and HeLa cell-containing heterospheroids [16].

To further investigate the role of *Ext1* expressed by stromal fibroblasts in modulating the lung adenocarcinoma cells in *Ext1*$^{Gt/Gt}$/tumor heterospheroids we in this study screened for differently expressed human genes in human A549 tumor cells interacting with *Ext1*$^{Gt/Gt}$ fibroblasts. Using microarray analysis we identified the *P311* (*C5ORF13*) gene as downregulated in *Ext1*$^{Gt/Gt}$/A549 heterospheroids. *P311* encodes the P311 protein, also known as NREP (Neuronal Regeneration Related Protein) or PTZ17 (pentylenetetrazol-17) [17]. P311 is a PEST [rich in proline (P), glutamic acid (E), serine (S), and threonine (T)]-domain containing 8-kDa intracellular protein with a short half-life of approximately 5 min [18]. It was originally found in developing neurons and muscles but is expressed ubiquitously in several other tissues as e.g. muscle, lung, regenerating tissues [19] and invasive glioma cells [20]. We further show that the mRNA expression of *P311* in A549 tumor cells is regulated by the cytokine transforming growth factor beta-1 (TGF-β1).

2. Material and methods

2.1. Cell culture

No experiments on live vertebrates were performed; the cells/cell lines described below were either purchased or given to us as a gift and have been used in our lab for > 12 years. Mouse embryonic fibroblasts derived from wild-type and *Ext1* gene trapped *Ext1*$^{Gt/Gt}$ (official designation, *Ext1*$^{Gt(pGT2TMpfs)064Wcs}$, [21]) (kindly given by Dr. Andrea Vortkamp, University of Duisburg-Essen, Essen, Germany) were Sv40-immortalized as described in [14]. A549, a human non-small cell lung adenocarcinoma cell line, was purchased from the American Type Culture Collection (ATCC). Mink lung epithelial cells (TMLC) were kindly provided by Dr. Dean Sheppard (UCSF Medical Center, CA, USA). Monolayer cell cultures and multicellular heterospheroids were cultured in Dulbecco's modified Eagles medium (DMEM) with Glutamax (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 units/ml of penicillin and 0.1 mg/ml of streptomycin (Sigma) and cultured at 37 °C and 5% CO_2.

2.2. Construction of Ext1 expression plasmid and transfection of HEK 293 cells

For reintroduction of *Ext1* into *Ext1*$^{Gt/Gt}$ fibroblasts, a full-length mouse *Ext1* cDNA was excised from pBudCE4.1 vector [22] using *Eco*RI restriction sites and subcloned into the corresponding site of retroviral pBABE plasmid vector (Addgene) carrying the puromycin resistant gene (pBabe puro IRES-EGFP). Enzyme restriction digestions confirmed the orientation of the insert with *Ext1* in frame with the C-terminal EGFP tag. For stable expression, the vector carrying the *Ext1* cDNA or the empty vector (mock) were transfected into retroviral packaging Phoenix cells (HEK293T cells constructs capable of producing gag-pol, and envelope protein for viruses). Subsequently, the culture medium from the transfected Phoenix cells containing the retrovirus particles was collected to infect the *Ext1*$^{Gt/Gt}$ cells. After infection, cells were cultured in the presence of 2 μg/ml of puromycin (Sigma). Three cellular populations (referred to as cl.1, cl.2 and cl.3) stably expressing *Ext1* at relatively low, medium and high levels were isolated by flow cytometry based on the level of EGFP expression.

2.3. Gene silencing with siRNA

Pre-designed siRNAs for mouse TGF-β1 were from Ambion and non-targeting control siRNA was from Dharmacon. Sequences of primers are listed in Supplementary Table S1. Wild-type *Ext1* (*Ext1*$^{wt/wt}$) cells were transfected with the siRNA (50 nM of each) using lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). After 48 h cells were trypsinized and co-cultured with A549 cells in 1:1 ratio for an additional 24 h. mRNA levels were evaluated by real time PCR using human and mouse specific primers as described below.

2.4. 2D co-culture experiments

Ext1$^{wt/wt}$ or *Ext1*$^{Gt/Gt}$ cells and A549 cells were seeded at 1:1 ratios in a 6-well plate (125,000 cells of each cell type per well). After co-culturing for 48 h mRNA levels were evaluated by real time PCR using human and mouse specific primers (listed in Supplementary Table S1) as described below.

2.5. Preparation and culturing of spheroids

Composite spheroids were prepared by co-culturing *Ext1*$^{wt/wt}$ or *Ext1*$^{Gt/Gt}$ mouse embryonic fibroblasts with A549 tumor cells using the hanging drop method [23,24] as described in [16]. Briefly, sub-confluent cells were trypsinized and suspended in culture medium to a concentration of 1×10^6 cells/ml. Single cell suspension of fibroblasts and tumor cells were mixed in a ratio of 9:1 and 25 μl of the composite cell suspension (2.5×10^4 cells) were pipetted onto the lid of a cell culture dish to form one drop. The lid was then inverted and placed over a cell culture dish containing DMEM for humidity and cultured under standard conditions for 6 days.

2.6. Microarray analysis in composite spheroids

Human specific microarray analysis was performed on 6-day old composite spheroids. Five different replicates were made for each spheroid type. For RNA preparation, the spheroids were grinded in RLT buffer using a mixer mill homogenizer (Retsch, Germany). Total RNA was prepared from the homogenized spheroids using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Extracted total RNA was quality tested on Agilent Bioanalyzer 2100 and a human specific microarray was performed at the NMC-UoB Microarray Core Facility using the Illumina Bead Array Technology (HumanHT-12 v4 Expression Bead Chip). The raw microarray data was quality examined in GenomeStudio and SampleProbeProfile-text file was exported from GenomeStudio, during which, control probes were removed. The resulting gene expression table was imported into J-Express 2012 (http://jexpress.bioinfo.no/site/) for further quality control and analysis. Differentially expressed human genes between the two groups of samples (RNAs from *Ext1*$^{wt/wt}$/A549 and *Ext1*$^{Gt/Gt}$/A549) were analyzed using SAM method and only the genes with q-value < 10 were considered to be valid. The datasets generated and analyzed during the current study are available in the ArrayExpress repository, ID: E-MTAB-5874.

2.7. Quantitative real time PCR (RT-PCR)

Total RNA was isolated from cells in monolayer cultures and heterospheroids using RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. Aliquots of 1 μg of total RNA were reverse transcribed

to cDNA using random primers (iScript cDNA synthesis kit, Bio-Rad) according to the manufacturer's instructions. mRNA expression analyses were conducted using iQ SYBR green supermix (Bio-Rad) in a LightCycler 480 (Roche Applied Science). Data for human and mouse genes were normalized to the reference genes, *HPRT* or *POLRF2* and β-*actin*, respectively. Each cDNA sample was run in triplicates and the relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method [25]. The primers used (Supplementary Table S1) were selected using Primer-BLAST NCBI.

2.8. TGF-β bioassay

The amount of active TGF-β secreted by the fibroblasts was measured by addition of conditioned media to mink lung epithelial reporter cells (TMLC cells) expressing a luciferase reporter gene linked to a truncated plasminogen activator inhibitor-1 (PAI-1) promoter [26]. *Ext1*$^{wt/wt}$ and *Ext1*$^{Gt/Gt}$ fibroblasts (6×10^6 cells) were plated in 75 cm^2 culture flasks (Falcon) and incubated at 37 °C in complete DMEM medium. After overnight incubation, the cells were washed three times and thereafter starved in serum-free DMEM. Conditioned media were collected after 48 h, centrifuged and the supernatants used to analyze the secretion of active TGF-β. TMLC cells were plated in a 6-well plate (1×10^6 cells/well) and allowed to attach overnight in complete DMEM. Next day, the cells were washed three times with PBS and incubated with the conditioned media at 37 °C. 5 ng/ml of recombinant human TGF-β1 (Peprotech Inc.) was used as a positive control. After 18 h, cells were washed with PBS and lysed with 100 μl lysis buffer (Tropix® Lysis Solution, from Applied Biosystems). 25 μl-aliquots of each culture supernatants were transferred to microtiter plates to which 100 μl of luciferase substrate (Luciferase Assay System, from Promega) was added. The luciferase activity was measured using a Wallac 1420 VICROR3 Multilabel Counter (PerkinElmer). All samples were measured in triplicate.

2.9. Flow cytometry

For flow cytometry analyses, subconfluent cell cultures were harvested using cell dissociation buffer (Gibco) and resuspended in PBS. To detect cell surface HS, 1×10^6 cells were incubated with mouse anti-HS mAb 10E4 primary antibody (Seikagaku) at 1:50 dilution for 30 min at 4 °C. After three washes with PBS, the cells were stained with the secondary antibody; an allophycocyanin (APC) conjugated goat-anti mouse IgG, 1:75 dilution (Jackson Immuno research Laboratories, Inc.). Cells incubated with secondary antibody only served as negative controls. Fluorescence was measured by flow cytometry using an AccuriC-6 system (Accuri Cytometers Inc.) and data was analyzed using FlowJo software (Tree star, Inc.).

2.10. Immunocytofluorescence staining

For cell staining with the 10E4 antibody, cells were grown on fibronectin coated (2 μg/ml) coverslips, fixed with 4% formaldehyde, washed three times with PBS and then blocked with 10% goat serum diluted in PBS for 1 h at room temperature. After blocking, cells were incubated with 10E4, at 1:50 dilution, for 1 h, washed three times with PBS and then incubated with Goat Anti-mouse Alexa Fluor 594 (Jackson ImmunoResearch), diluted 1:400, for 45 min. The coverslips were washed three times with PBS stained for nuclei with DAPI (Invitrogen) and mounted using Thermo Scientific™ Shandon™ Immu-Mount (Fisher Scientific). Cells stained with secondary antibody only served as negative control. Images were visualized under a Zeiss Axioscope microscope equipped with optics for observing fluorescence, and captured using a digital AxioCam MRm camera.

2.11. Analysis of growth factor signaling

Subconfluent cell cultures grown in a 6-well plate were washed with serum deprived DMEM and starved for 18 h in the same medium. After changing to fresh starvation medium, FGF2 (R&D systems) final concentrations 1 ng/ml or 10 ng/ml, or TGF-β1 (Peprotech) final concentration 5 ng/ml were added to the cells for 10 min at 37 °C. Cells incubated with serum-free DMEM alone served as negative controls. Cell extracts were separated on SDS 10% polyacrylamide gels and electrotransferred onto a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 5% BSA in Tris buffered saline, 0.05% Tween20 for 1 h at room temperature. The membrane was incubated with rabbit anti-phospho-SMAD-3 (1:1000, Cell Signaling Technology) recognizing the activated, phosphorylated kinase, and after stripping, the membrane was incubated with rabbit anti-total SMAD-3 (1:1000, Cell Signaling Technology) recognizing both the non-phosphorylated and the phosphorylated kinase and β-actin (1:5000, Santa Cruz Biotechnology). For FGF2 mediated ERK phosphorylation, equal amounts of cell lysates was separated on two separate SDS-10% acrylamide gels. One membrane was incubated with rabbit anti-phospho-ERK (1:1000, Cell Signaling Technology) recognizing the activated, phosphorylated kinase, the other membrane was incubated with rabbit anti-total ERK (1:1000, Cell Signaling Technology) recognizing both the unphosphorylated and the phosphorylated kinase. The bound primary antibodies were detected by incubating with HRP-conjugated goat anti-rabbit (1:5000, Santa Cruz Biotechnology) for 1 h at room temperature. The phospho-ERK membrane was then stripped and also stained with the anti-total ERK antibody. The membranes were developed using ECL reagent (Pierce), and the bands were visualized using ChemiDoc XRS device (Bio-Rad) and quantified by using ImageJ software (National Institutes of Health, Bethesda, MD).

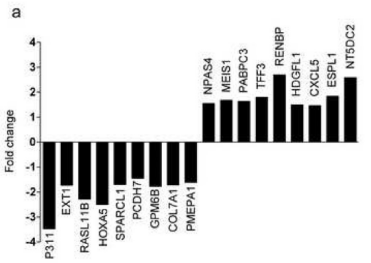
2.12. Statistical analysis

Statistical significance between groups was determined using the two-tailed paired Student *t*-test. $p < 0.05$ was considered statistically significant. Graphs and statistical analysis were done with GraphPad Prism v.6 (Graphpad software, Inc., La Jolla, CA).

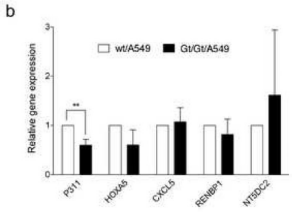
3. Results

3.1. Gene expression profiling of differentially expressed human genes in *Ext1*$^{Gt/Gt}$/A549 heterospheroids

To further investigate the contribution of fibroblast *Ext1* in tumor cell-fibroblast interactions we took advantage of the fact that our mini-tumor heterospheroid models are composed of human tumor cells and mouse embryonic fibroblasts. In the current study, we used this model system to compare the gene expression profiles of human A549 cells in *Ext1*$^{wt/wt}$/A549 and *Ext1*$^{Gt/Gt}$/A549 heterospheroids using a human specific microarray. Microarray analysis revealed differentially expressed human genes in the mouse fibroblast/human (A549) tumor cell spheroids. A total of 57 genes were differentially expressed in *Ext1*$^{Gt/Gt}$/A549 spheroids, in comparison with *Ext1*$^{wt/wt}$/A549 spheroids (24 genes were down-regulated and 33 genes were up-regulated, calculated by SAM method, q-value < 10). A few genes suggested being involved in tumor cell proliferation and invasion, for example, *CXCL5* and *TFF3*, were up-regulated in tumor cells in *Ext1*$^{Gt/Gt}$/A549 heterospheroids. In contrast, several genes encoding for proteins found in the ECM and genes involved in regulation of cell motility were suppressed in the tumor cells. The top most up- or down-regulated genes are shown in Fig. 1a. The expression of five selected down- or up-regulated molecules was analyzed by real-time PCR of fibroblast/A549 spheroids using human specific primers. The specificities of the primers were validated by lack of products when used for real-time PCR of mouse genes. *Ext1*



Symbol	Gene name
P311	Chromosome 5 open reading frame 13
EXT1	Exostosin1
RASL11B	RAS like family 11
HOXA5	Homeobox A5
SPARCL1	SPARC like 1
PCDH7	Procadherin 7
GPM6B	Glycoprotein M6B
COL7A1	Collagen type VII, alpha 1
PMEPA1	Prostrate transmembrane protein
NPAS4	Neuronal PAS domain protein 4
MEIS1	Meis homeobox 1
PABC3	Poly(A) binding protein, cytoplasmic 3
TFF3	Trefoil factor 3
RENBP	Renin binding protein
HDGFL1	Hepatoma derived growth factor like 1
CXCL5	Chemokine CXC motif ligand 1
ESPL1	Extra spindle pole bodies homolog 1
NT5DC2	5'- nucleotidase domain containing 2



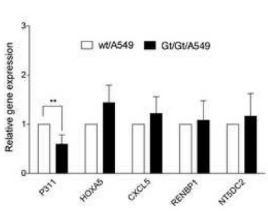


Fig. 1. Differentially expressed human genes in $Ext1^{Gt/Gt}$/A549 heterospheroids. mRNA was isolated from 6-day-old heterospheroids and used for microarray analysis and real time PCR. a The top most up and down regulated genes in A549 cells in $Ext1^{Gt/Gt}$/A549 spheroids compared to $Ext1^{wt/wt}$/A549 spheroids. Fold changes represent the average values from 5 individual samples of each type of spheroid. b, c Validation of selected differentially expressed genes from the microarray were determined by real time PCR and normalized to those of *HPRT*. The mRNA levels are expressed as fold changes relative to $Ext1^{wt/wt}$/A549 spheroid expression that was set to 1. In b the same RNAs that were used for the microarray were used for real time PCR and in c RNA was extracted from newly prepared heterospheroids under identical conditions as those used for microarray (n = 5 for each spheroid type). The error bars represent mean ± SD mRNA levels of values from b four and c five independent experiments performed in triplicate. In b and c the white bars represents $Ext1^{wt/wt}$/A549 heterospheroids and the black bars $Ext1^{Gt/Gt}$/A549 heterospheroids. $^{*}p < 0.05$, $^{**}p < 0.01$.

expression was used as a positive control. Genes for which we could not guarantee human specific primers were excluded from the real-time PCR analyses. Using total RNA from spheroids used for microarray analysis and total RNA from newly made spheroids, that were distinct from the spheroids used for microarray, only *P311* mRNA was verified to be differently expressed in A549 cells in $Ext1^{Gt/Gt}$/A549 spheroids (Fig. 1b,c). Based on our PCR results, we cannot exclude that some genes that are highly conserved between mouse and human were detected by the array, although the analyses were performed using a human specific microarray.

Next, we compared *P311* mRNA levels in fibroblast/A549 tumor cells in 2D-co-cultures and in A549 treated with conditioned media derived from the wild type or mutant fibroblasts. When A549 cells were co-cultured with $Ext1^{Gt/Gt}$ fibroblasts, we again observed significantly decreased A549 - *P311* mRNA levels (Fig. 2a). Interestingly, this change in *P311* mRNA levels was not observed using conditioned medium from $Ext1^{Gt/Gt}$ fibroblasts (Fig. 2b), indicating that cell-cell physical interactions are important in influencing *P311* gene expression in the neighboring tumor cells.

Since a recent study demonstrated that addition of TGF-β1 to fibroblasts significantly influenced *P311* mRNA levels [27], *P311* mRNA levels in A549 cells were assessed after TGF-β1 addition. In agreement with [27], addition of 5 ng/ml TGF-β1 induced *P311* mRNA expression in A549 cells (Fig. 2c), supporting that TGF-β1 may be one factor secreted from fibroblasts that influences tumor cell behavior in our spheroid system. We further used a human specific P311 antibody to stain A549 cells alone or in co-culture with $Ext1^{wt/wt}$ or $Ext1^{Gt/Gt}$ fibroblasts to determine P311 protein levels. However, we were not successful in visualizing the P311 protein after immunostaining of cells. It is possible this was due to low overall concentration of P311 in A549 cells owing to low protein synthesis and/or rapid degradation of the protein by multiple proteolytic pathways, resulting in an extremely short protein half-life [18].

3.2. Decreased *Tgf-β1* mRNA and active TGF-β protein levels in $Ext1^{Gt/Gt}$ fibroblasts

We next investigated whether the mutation in *Ext1* affected *Tgf-β1* expression levels. Real time PCR revealed that the *Tgf-β1* mRNA expression levels were lower in $Ext1^{Gt/Gt}$ fibroblasts as compared to $Ext1^{wt/wt}$ fibroblasts. Similar results were obtained both for primary and SV40 immortalized fibroblasts (Fig. 3a,b). To confirm this in *Ext1*/A549 heterospheroids, we performed real-time PCR using mouse specific *Tgf-β1* primers (with threshold cycle (Ct) values of 40 or no signal using A549 cDNA). Similar to the results using monolayer cultures *Tgf-β1* mRNA levels were reduced in $Ext1^{Gt/Gt}$*/A549* heterospheroids (Fig. 3c). To explore whether the decreased mRNA expression of *Tgf-β1* was directly associated with the amount of secreted active TGF-β1 protein levels, the amounts of active TGF-β1 protein levels were assessed in the conditioned medium from fibroblast cultures. The TGF-β reporter assay showed that $Ext1^{Gt/Gt}$ cells secreted less soluble active TGF-β compared to the $Ext1^{wt/wt}$ fibroblasts (similar results were obtained for primary and SV40 immortalized fibroblasts) (Fig. 3d,e). Thus, our results indicate that *Ext1* levels influenced *Tgf-β1* mRNA levels and the amount of secreted active TGF-β. Since TGF-β1 can induce its own gene expression and this autoinduction involves the canonical TGF-β signaling pathway with Smad3 [28] we determined the level of activated Smad3 in $Ext1^{Gt/Gt}$ and $Ext1^{wt/wt}$ fibroblasts after stimulation with TGF-β1. Smad3 phosphorylation, induced by transient stimulation with TGF-β1 activated both wild-type and $Ext1^{Gt/Gt}$ mutant cells but pSmad3 levels were reduced in $Ext1^{Gt/Gt}$ cells as compared to wild-type cells (Fig. 3f).

3.3. Re-expression of Ext1 in $Ext1^{Gt/Gt}$ fibroblasts rescued HS, restored *Tgf-β1* mRNA and TGF-β protein levels in fibroblasts and *P311* mRNA expression in A549 cells

To corroborate that the changes in TGF-β and *P311* expression were specifically caused by the mutation in the *Ext1* gene, $Ext1^{Gt/Gt}$

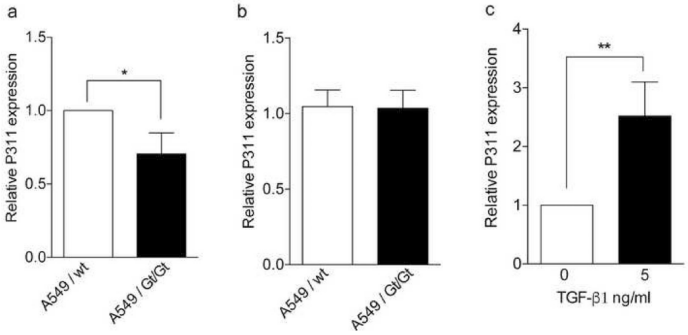


Fig. 2. Fibroblast *Ext1*-levels regulate *P311* gene expression in A549 tumor cells but only when there is physical interaction between the two cell types. A549 cells were: a co-cultured with $Ext1^{wt/wt}$ or $Ext1^{Gt/Gt}$ fibroblasts; b serum starved overnight and then incubated with conditioned media from fibroblasts or c treated with 5 ng/ml TGF-β1. After 48 h treatment human *P311* gene expression levels in A549 cells were determined by real time PCR. The expression levels were normalized to those of *HPRT*. The error bars represent mean ± SD mRNA levels of values from a three and c four independent experiments performed in triplicate. In b the values are given as means ± mean deviation from one experiment performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$.

fibroblasts were stably transfected with a GFP-tagged retroviral vector carrying the full-length mouse *Ext1* cDNA ($Ext1^{Gt/Gt+Ext1}$). The *Ext1* transfected cells were sorted by flow cytometry into low (cl.1), intermediate (cl.2) and high (cl.3) expressing $Ext1^{Gt/Gt+Ext1}$ populations with approximately 4, 7 and 16 fold increased *Ext1* mRNA levels, respectively, in comparison with non-transformed $Ext1^{Gt/Gt}$ fibroblasts (Fig. 4a).

Previously, we have shown that the $Ext1^{Gt/Gt}$ cells have shorter HS side chains and as a consequence display poor binding of the 10E4 antibody. *Re*-introduction of *Ext1* in the *Ext1* mutant fibroblasts rescued both HS chain length and 10E4 binding [15,16]. The relative amount of cell surface HS expressed by $Ext1^{wt/wt}$, $Ext1^{Gt/Gt}$ and $Ext1^{Gt/Gt+Ext1}$ fibroblasts was determined by flow cytometry and immunocytochemistry using the 10E4 antibody recognizing the N-sulfated glucosamine residues [29]. In agreement with our previous results, wild-type fibroblasts stained strongly with 10E4 antibody whereas the $Ext1^{Gt/Gt}$ cells, that have very short HS chains, stained poorly with the antibody [15]. Transfection of *Ext1* into $Ext1^{Gt/Gt}$ fibroblasts resulted in more binding epitopes for the 10E4 antibody (Supplementary Fig. 1a) demonstrating that the $Ext1^{Gt/Gt+Ext1}$ cells had regained longer HS chains that were biologically functional as revealed by an increased FGF2 signaling response (Supplementary Fig. 1b).

Importantly, transfection of *Ext1* into $Ext1^{Gt/Gt}$ fibroblasts increased *Tgf-β1* mRNA levels and, the amounts of active TGF-β (Fig. 5). However, there was no strict correlation between *Tgf-β1* expression levels in $Ext1^{Gt/Gt+Ext1}$ clones and the appreciable increase in *Ext1* mRNA levels, pointing to other factors influencing *Tgf-β1* levels. *Re*-expression of *Ext1* also restored *P311* mRNA levels in A549 cells in $Ext1^{Gt/Gt+Ext1}$/A549 heterospheroids (Fig. 6a) and in 2D-co-cultures with $Ext1^{Gt/Gt+Ext1}$ (Fig. 6b).

3.4. siRNA-mediated silencing of *Tgf-β1* in $Ext1^{wt/wt}$ fibroblasts reduced *P311* expression in adjacent A549 tumor cells

Next, to confirm that down-regulation of fibroblast *Tgf-β1* expression levels indeed influences *P311* levels in adjacent A549 cells, we down-regulated fibroblast *Tgf-β1* using siRNA. We chose siRNA instead of CRISPR-Cas9 as we did not want a complete down-regulation of *Tgf-β1* expression but rather a similar reduction as in the mutant $Ext1^{Gt/Gt}$ fibroblasts. $Ext1^{wt/wt}$ fibroblasts were transiently transfected with two different siRNAs targeting mouse *Tgf-β1* and one non-targeting control siRNA. siRNA- or control treated fibroblasts were co-cultured with A549 cells for 24 h and then analyzed for *Tgf-β1* mRNA expression in fibroblasts and *P311* expression in adjacent A549 cells. Both siRNAs reduced *Tgf-β1*expression level (Fig. 7a,c) and a corresponding reduction in *P311* expression level, further confirming that the mRNA expression of *P311* in A549 tumor cells is regulated by fibroblast TGF-β1 (Fig. 7b,d).

Taken together, we have demonstrated that reduction in *Ext1* in mouse embryonic fibroblasts resulted in decreased *Tgf-β1* mRNA amounts and a reduced ability to produce the active TGF-β protein. Another consequence of the reduced *Ext1* expression was that under co-culture conditions, the *P311* expression in adjacent A549 human tumor cells was affected.

4. Discussion

It has become increasingly recognized that the growth and malignancy of a tumor is influenced by the microenvironment i.e. the tumor stroma [30]. Activated fibroblasts are the predominant cells in the stroma and are responsible for the synthesis, deposition and remodeling of the ECM as well as the production of growth factors, cytokines and ECM-degrading proteases [31]. The cross talk between tumor cells and the surrounding stroma is crucial for the tumor cell migration and metastasis [32]. HSPGs, on stromal cells and on tumor cells, influence tumor cell development [33,34]. We have previously shown that the *Ext1* mutant fibroblasts ($Ext1^{Gt/Gt}$) have short sulfated HS chains [14] and as a consequence they also display decreased MAPK signaling induced by FGF2 and decreased interactions with collagen [15]. Furthermore, using a 3D mini-tumor spheroid model composed of human tumor cell lines and $Ext1^{wt/wt}$ or $Ext1^{Gt/Gt}$ fibroblasts, we have shown that fibroblast *Ext1* levels strongly influence tumor cell behavior, migration and the interstitial fluid pressure [16]. In an attempt to understand the role of fibroblast *Ext1*/HS in regulation of tumor cell behavior, we analyzed the effect of the *Ext1* mutation on the gene expression profile of A549 cells in the 3D mini-tumor spheroid model. In this study, we found a correlation of *Ext1* expression levels in mouse fibroblasts with the levels of *P311* in co-cultured A549 tumor cells. Our results indicated that the regulation of *P311* levels in the tumor cells is controlled by mechanisms that require both cell-cell contacts and paracrine fibroblast-mediated signaling.

P311 has been identified to be differently regulated in a diverse range of cellular settings, regulating cell differentiation, cell migration and tumor cell invasion [20,35–37]. P311 has been ascribed several

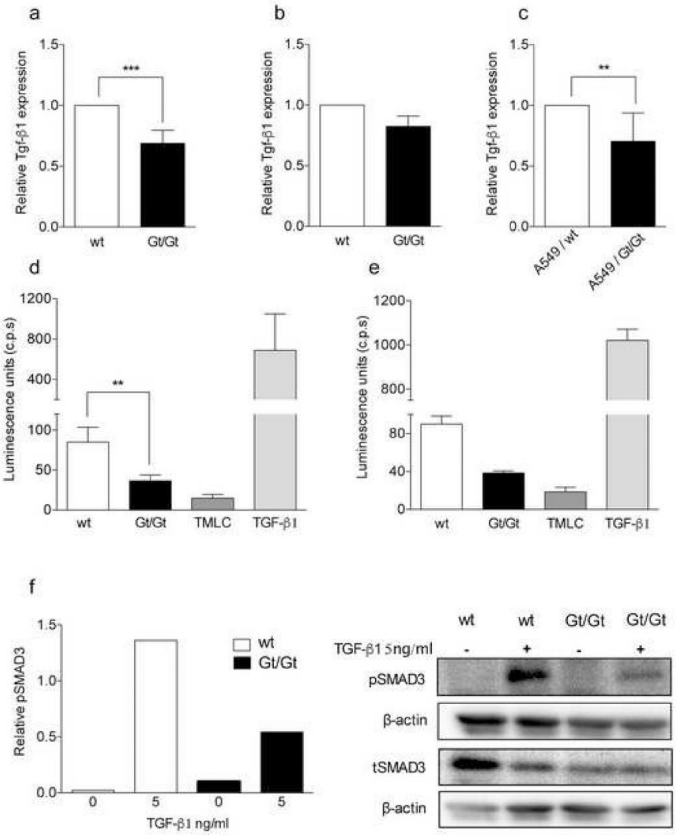


Fig. 3. *Tgf-β1* mRNA expression and active TGF-β levels are reduced in $Ext1^{Gt/Gt}$ cells. In a the bars represent the average *Tgf-β1* mRNA levels for two different sets of separately isolated and immortalized fibroblasts. The change in *Tgf-β1* mRNA levels was confirmed in b primary fibroblasts and c $Ext1^{Gt/Gt}$/A549 spheroids ($n = 3$ of each type of spheroid) normalized to β –actin. In a and c, the error bars represent mean ± S.D. mRNA levels of values from a five and c two independent experiments. In b, the values are given as means ± mean deviation from one experiment. Each measurement was performed in triplicate. d, e Conditioned media isolated from d immortalized and e primary fibroblasts were added to TMLC reporter cells. After 24 h active TGF-β was measured as luminescence. In d the error bars represent mean ± S.D. mRNA levels of values from four independent experiments and in e, the values are given as means ± mean deviation from one representative experiment. Each measurement was performed in duplicate. Addition of 5 ng/ml active TGF-β1 to TMLC reporter cells was used as a positive control. f Serum starved $Ext1^{wt/wt}$ and $Ext1^{Gt/Gt}$ fibroblasts were stimulated with 5 ng/ml of TGF-β1 for 10 min. The bar graph shows the band intensity of phosphorylated versus total SMAD3 normalized to β-actin. The figure shows one representative experiment out of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

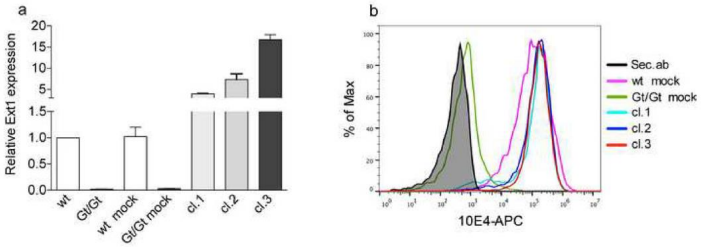


Fig. 4. Re-expression of *Ext1* in $Ext1^{Gt/Gt}$ fibroblasts rescues HS chain length and HS-protein interactions. Stable transfection of $Ext1^{Gt/Gt}$ cells with *Ext1* resulted in: a three cell populations with different degrees of *Ext1* expression levels (cl.1, cl.2 and cl.3). The error bars represent mean ± S.D. mRNA levels of values from two independent experiments performed in triplicate. b The cell surface levels of HS determined by flow cytometry using the 10E4 antibody. The histogram in b shows the intensity of the fluorescence (% of max) and is representative of three independent experiments.

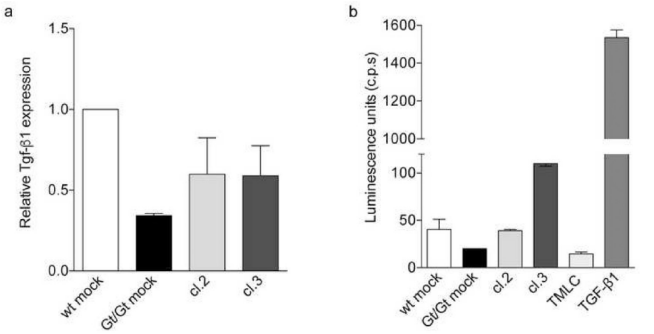


Fig. 5. Re-expression of *Ext1* in $Ext1^{Gt/Gt}$ fibroblasts rescues *Tgf-β1* mRNA levels and the amount of secreted active TGF-β. a mRNA levels for $Ext1^{Gt/Gt+Ext1}$ cl.2 and cl.3 were determined by real time PCR and normalized to those of β-actin. The error bars represent mean ± S.D. of values from two independent experiments done in triplicate. b TGF-β bioassay using TMLC reporter cells was as described in the figure legend to Fig. 3. The graphs show results from one representative experiment out of three independent experiments, each experiment measured in duplicate.

functions such as: influencing the reorganization of the actin cytoskeleton, interacting with Filamin 1 [38] and integrin β4 binding protein (also known as eIF6) [39], regulation of retinoic-acid lipid-droplet biogenesis [40], *TGF-β1*-independent differentiation of fibroblasts into myofibroblasts [41] and to enhance glial cell motility [20,38]. Recently, P311 protein was reported to be an RNA-binding protein that inhibits the transcription of *Tgf-β1* mRNA but stimulates TGF-β1–3 protein translation in human fibroblasts [19]. In another, more recent study a TGF-β1 concentration dependent effect on fibroblast *P311* mRNA levels was observed [27]. We observed strongly reduced *P311* levels in A549 cells when co-cultured with $Ext1^{Gt/Gt}$ cells that are secreting low amounts of TGF-β1. In agreement with this result, *P311* mRNA levels were highly increased after addition of TGF-β1 to A549 cells indicating a specific stimulatory role of TGF-β1 in regulating *P311* mRNA levels in A549 cells (Figs. 2 and 3). The role of TGF-β1 in regulating *P311*mRNA levels in co-cultured A549 cells was further demonstrated after down regulation of fibroblast derived TGF-β1 by siRNA (Fig. 7). Recently it was shown that αvβ6 integrin is essential in activating TGF-β on epithelial cells and that several non-small cell lung cancer (NSCLC) cell lines including A549 cells express low levels of αvβ6 [42]. Thus, the A549 cell line is a good model for studies of tumor-stroma interactions without the involvement of carcinoma-derived active TGF-β. There is a possibility that our results may be specific for A549 cells. However, we have previously shown for composite 3D-heterospheroids, composed of our fibroblast ($Ext1^{wt/wt}$ or $Ext1^{Gt/Gt}$) and three different tumor cell lines (A549, H460 or HeLa cells), that stromal *Ext1*-levels, and thus also HS chain length, modulate tumor-cell migration, tumor cell proliferation and the interstitial fluid pressure in a similar way for the three different tumor cell lines [16]. Although we believe our present results possibly could be applied to other tumor cell lines, future studies are required to clarify this issue.

The diverse functions ascribed to HS include matrix organization and co-receptor functions in various signaling systems [5,43]. TGF-β1 is a heparin/HS-binding growth factor and in cultured cells, HS increases effectiveness of TGF-β1 signaling [44,45]. In mammals, there are three members of the TGF-β family, TGF-β1, TGF-β2 and TGF-β3. The members of the TGF-β family are multifunctional growth factors that regulate a wide range of cellular and physiological processes including cellular differentiation and proliferation and they are critically involved in embryonic development and adult tissue homeostasis [46]. Both *Tgf-β1* mRNA expression level and the amounts of secreted active TGF-β were significantly reduced in the mutant fibroblasts (Fig. 3). If cell surface HS chains on fibroblasts are required for *Tgf-β1* mRNA levels and the amounts of secreted active TGF-β, it is expected that exogenously added heparin would increase *Tgf-β1* mRNA levels in $Ext1^{Gt/Gt}$ fibroblasts. Thus competing with HS on $Ext1^{wt/wt}$ cells and thereby decreasing the *Tgf-β1*

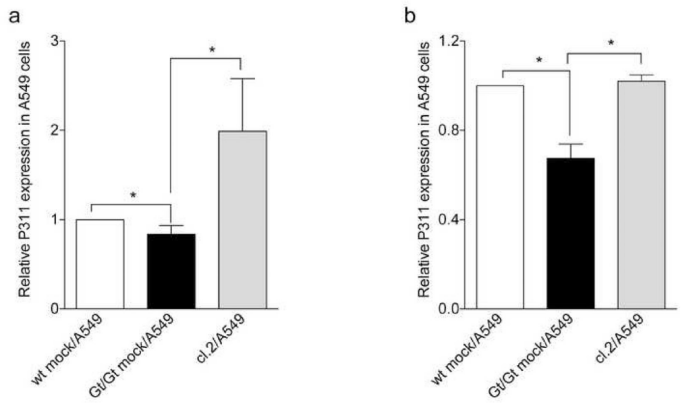


Fig. 6. Re-expression of *Ext1* in $Ext1^{Gt/Gt}$ fibroblasts rescues *P311*mRNA expression in A549 cells. mRNA levels for human *P311* in a $Ext1^{Gt/Gt+Ext1}$/A549 heterospheroids (cl.2) in b co-cultured with $Ext1^{wt/wt/mock}$, $Ext1^{Gt/Gt/mock}$ or $Ext1^{Gt/Gt+Ext1}$ (cl.2) fibroblasts were determined by real time PCR and normalized to those of HPRT. The error bars represent mean ± S.D. mRNA levels of values from three independent experiments measured in triplicates. $^{*}p < 0.05$.

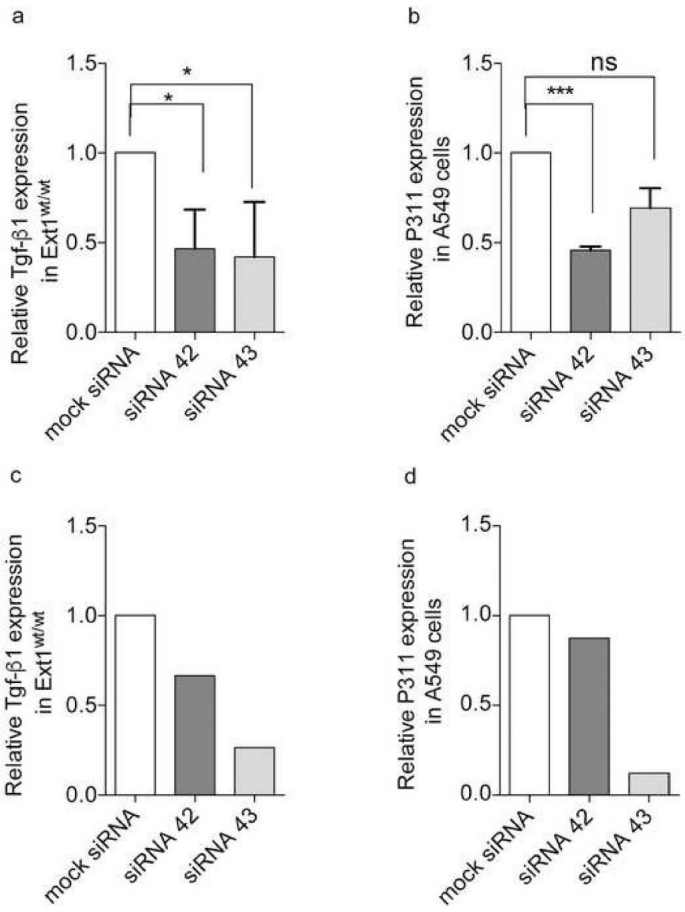


Fig. 7. siRNA down-regulation of fibroblast *Tgf-β1* down-regulates *P311* mRNA levels in adjacent A549 cells. $Ext1^{wt/wt}$ fibroblasts were treated with control siRNA or two different siRNAs (siRNA 42 and siRNA 43) targeting *Tgf-β1*. After 48 h, siRNA treated $Ext1^{wt/wt}$ fibroblasts were trypzinised, washed, counted and added to the A549 cells. After 24 h of co-culturing, total RNA was isolated and subjected to real time PCR using mouse (a, c) and human specific (b, d) primers, respectively. *Tgf-β1* levels were normalized to those of β-*actin* and *P311* normalized to *POLRF2* in b and to *HPRT* in d. In a and b the error bars represent mean ± S.D. mRNA levels of values from three independent experiments measured in triplicates. c and d show average values from one experiment measured in triplicate. ns, non-significant; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

mRNA levels. Addition of heparin to the media decreased the amount of *Tgf-β1* mRNA in wild-type fibroblasts with normal levels of *Ext1* and cell surface HS, but was unable to restore the mRNA levels in the $Ext1^{Gt/Gt}$ cells at the concentrations examined. However, there was a hint that low concentrations of heparin may slightly increase *Tgf-β1* mRNA levels in the $Ext1^{Gt/Gt}$ cells (Supplementary Fig. 2). This suggests that the soluble polysaccharide is not adequate and that *Tgf-β1* expression is dependent on the presence of *Ext1* and/or cell surface associated HS chains.

Ext1 has previously been shown to be necessary for TGF-β2 signaling in neural crest cells [47]. Genetic disruption of *Ext1* and thus lack of HS chains in neural crest cells reduces TGF-β2-induced signaling in a cell autonomous fashion, most probably due to impaired interaction between HS and TGF-β2 [47]. Another HS modifying enzyme, the extracellular HS endo-6-*O*-sulfatase (SULF-1), has also been shown to modify TGF-β levels. A transgenic mouse model overexpressing SULF-1, and thus synthesizing less sulfated HS chains, showed increased TGF-β1 protein levels in liver tissues [48]. Furthermore, cells overexpressing SULF1 showed increased TGF-β1 activity attributed to the fact that de-sulfation of cell surface HS by SULF1 releases TGF-β1 from the HS chains and thereby enhances TGF-β1 binding to its receptor [48]. Our findings have further highlighted the indirect role of EXT proteins in influencing TGF-β activity through their involvement in regulating the synthesis of HS chains. Moreover, deletion of the HS-side chains from the HSPG perlecan has been reported to interfere with TGF-β co-localization in areas of high cellular proliferation such as skin follicular stem cells [49]. Finally, structural changes in HS sulfation pattern have recently been shown to affect TGF-β signaling activity in tumor cells [50].

In the present study, we demonstrate that *Ext1* is a modulator of the expression of *Tgf-β1*, a potent regulator of cellular differentiation. Our results suggest that the regulation of *P311* mRNA in A549 tumor cells is mediated in a cell non-autonomous manner by fibroblast TGF-β1 levels, which in turn is influenced by the amount of fibroblast *Ext1*. *Ext1* most

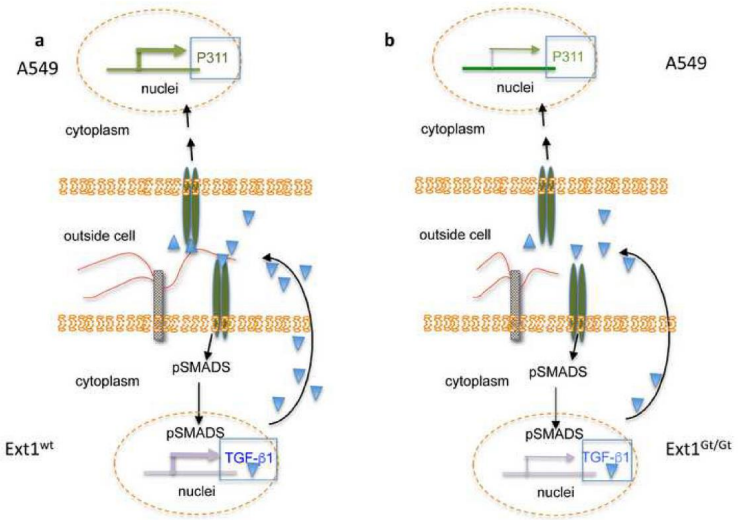


Fig. 8. Hypothetical scheme of the EXT1 and HS–dependent regulation of TGF-β1 that in turn, in a paracrine fashion, regulates the expression levels of tumor cell *P311*. a HS has affinity for TGF-β1, which enhances the ligand presentation to the TGF-β receptors. Subsequently, transduction of the signal to the nucleus via phosphorylation of the Smads induces the expression of *TGF-β1*. b The *Ext1* mutation leads to an HS defect resulting in low efficiency of the interaction between TGF-β1 and the receptors, which results in disturbed Smad phosphorylation and less autoinduction of TGF-β and less *P311* expression. The thickness of the lines and the intensity of the text indicate level of expression.

probably executes its influence via cell surface HS chains (Fig. 8). This paracrine effect of fibroblast produced TGF-β1 on tumor cell *P311* levels has, to our knowledge, not been shown before. The, in comparison, low increase in *Tgf-β1* mRNA levels after re-introduction of *Ext1* into $Ext1^{Gt/Gt}$ cells was surprising. Extracellular matrix HSPGs may bind to and restrict the amount of TGF-β1 available for binding to cell surface associated HSPGs, thus preventing TGF-β1 interaction with its receptor and the autoinduction of TGF-β1. Alternatively, the requirement of TGF-β1 for specific relatively rare HS binding sequences [44,45] could have affected the ability of TGF-β1 to signal and thereby decreasing its autoinduction.

Interestingly, addition of soluble TGF-β1 to A549 cells increased *P311* levels, but our results also showed that the regulation of *P311* levels in the tumor cells is controlled by mechanisms that require both cell-to-cell contact and secreted soluble factors by fibroblasts. This indicates distinct contribution of direct cell surface-dependent interactions and soluble factors to tumor cell gene regulation. The molecular mechanism for the cell-cell-dependent regulation of P311 mRNA levels remains to be elucidated.

Even though we lack corroborating P311 protein data confirming the biological relevance, this study has highlighted one possible mechanism of *Ext1*-regulated HS structure in modifying stroma-tumor interactions via alteration of *Tgf-β1* expression. Given the importance of HS fine structure for cellular signaling the role of HS biosynthesis enzymes in modulating HS structure is increasingly important to understand. Clearly, further studies are needed to establish the link between Ext1, TGF-β1 and the gene expression in neighboring tumor cells.

Abbreviations

A549 cells	adenocarcinomic human alveolar basal epithelial cells
DMEM	Dulbecco's modified Eagle's medium
EXT	Exostosin
$Ext1^{wt/wt}$	EXT1 wild type
$Ext1^{Gt/Gt}$	Ext1 with a gene trap mutation
$Ext1^{Gt/Gt+Ext1}$	$Ext1^{Gt/Gt}$ with reintroduced EXT1
EXTL	Exostosin-like
Erk	Extracellularly regulated kinase
FGF2	Fibroblast growth factor-2
HEK	Human embryonic kidney
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
Tgf-β	Transforming growth factor-beta
TMLC	Mink lung epithelial reporter cells

Transparency document

The Transparency document associated with this article can be found, in online version.

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Author contributions

MKG conceived and designed the experiments; KK, LFS performed the experiments; KK, LFS and MKG analyzed the data; MKG, LFS and KK wrote the paper. MKG and KK prepared the figures. All authors reviewed the results and approved the final version of the manuscript.

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Competing financial interests

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2018.03.024.

References

[1] M. Busse-Wicher, K.B. Wicher, M. Kusche-Gullberg, The exostosin family: proteins with many functions, Matrix Biol. 35 (2014) 25–33.
[2] J.P. Li, M. Kusche-Gullberg, Heparan sulfate: biosynthesis, structure, and function, Int. Rev. Cell Mol. Biol. 325 (2016) 215–273.
[3] B.M. Zak, B.E. Crawford, J.D. Esko, Hereditary multiple exostoses and heparan sulfate polymerization, Biochim. Biophys. Acta 1573 (2002) 346–355.
[4] J.R. Bishop, M. Schuksz, J.D. Esko, Heparan sulphate proteoglycans fine-tune mammalian physiology, Nature 446 (2007) 1030–1037.
[5] J. Gallagher, Fell-Muir lecture: heparan sulphate and the art of cell regulation: a polymer chain conducts the protein orchestra, Int. J. Exp. Pathol. 96 (2015) 203–231.
[6] S.A. Ibrahim, H. Hassan, M. Gotte, MicroRNA regulation of proteoglycan function in cancer, FEBS J. 281 (2014) 5009–5022.
[7] J.D. Esko, S.B. Selleck, Order out of chaos: assembly of ligand binding sites in heparan sulfate, Annu. Rev. Biochem. 71 (2002) 435–471.
[8] A. Cook, W. Raskind, S.H. Blanton, R.M. Pauli, R.G. Gregg, C.A. Francomano, E. Puffenberger, E.U. Conrad, G. Schmale, G. Schellenberg, E. Wijsman, J.T. Hecht, D. Wells, M.J. Wagner, Genetic heterogeneity in families with hereditary multiple exostoses, Am. J. Hum. Genet. 53 (1993) 71–79.
[9] M. Le Merrer, L. Legeai-Mallet, P.M. Jeannin, B. Horsthemke, A. Schinzel, H. Plauchu, A. Toutain, F. Achard, A. Munnich, P. Maroteaux, A gene for hereditary multiple exostoses maps to chromosome 19p, Hum. Mol. Genet. 3 (1994) 717–722.
[10] Y.Q. Wu, P. Heutink, B.B. de Vries, L.A. Sandkuijl, A.M. van den Ouweland, M.F. Niermeijer, H. Galjaard, E. Reyniers, P.J. Willems, D.J. Halley, Assignment of a second locus for multiple exostoses to the pericentromeric region of chromosome 11, Hum. Mol. Genet. 3 (1994) 167–171.
[11] W. Van Hul, W. Wuyts, J. Hendrickx, F. Speleman, J. Wauters, K. De Boulle, N. Van Roy, P. Bossuyt, P.J. Willems, Identification of a third EXT-like gene (EXTL3) belonging to the EXT gene family, Genomics 47 (1998) 230–237.
[12] C.A. Wise, G.A. Clines, H. Massa, B.J. Trask, M. Lovett, Identification and localization of the gene for EXTL, a third member of the multiple exostoses gene family, Genome Res. 7 (1997) 10–16.
[13] W. Wuyts, W. Van Hul, J. Hendrickx, F. Speleman, J. Wauters, K. De Boulle, N. Van Roy, T. Van Agtmael, P. Bossuyt, P.J. Willems, Identification and characterization of a novel member of the EXT gene family, EXTL2, Eur. J. Hum. Genet. 5 (1997) 382–389.
[14] S. Yamada, M. Busse, M. Ueno, O.G. Kelly, W.C. Skarnes, K. Sugahara, M. Kusche-Gullberg, Embryonic fibroblasts with a gene trap mutation in Ext1 produce short heparan sulfate chains, J. Biol. Chem. 279 (2004) 32134–32141.
[15] C. Osterholm, M.M. Barczyk, M. Busse, M. Gronning, R.K. Reed, M. Kusche-Gullberg, Mutation in the heparan sulfate biosynthesis enzyme EXT1 influences growth factor signaling and fibroblast interactions with the extracellular matrix, J. Biol. Chem. 284 (2009) 34935–34943.
[16] C. Osterholm, N. Lu, A. Liden, T.V. Karlsen, D. Gullberg, R.K. Reed, M. Kusche-Gullberg, Fibroblast EXT1-levels influence tumor cell proliferation and migration in composite spheroids, PLoS One 7 (2012) e41334.
[17] N. Miura, A. Naganuma, Metallothionein mediates gene expression of 3.1 mRNA (PTZ17) related to epileptic seizure, FEBS Lett. 479 (2000) 146–148.
[18] G.A. Taylor, E. Hudson, J.H. Resau, G.F. Vande Woude, Regulation of P311 expression by met-hepatocyte growth factor/scatter factor and the ubiquitin/proteasome system, J. Biol. Chem. 275 (2000) 4215–4219.
[19] M.M. Yue, K. Lv, S.C. Meredith, J.L. Martindale, M. Gorospe, L. Schuger, Novel RNA-binding protein P311 binds eukaryotic translation initiation factor 3 subunit b (eIF3b) to promote translation of transforming growth factor beta1-3 (TGF-beta1-3), J. Biol. Chem. 289 (2014) 33971–33983.
[20] L. Mariani, W.S. McDonough, D.B. Hoelzinger, C. Beaudry, E. Kaczmarek, S.W. Coons, A. Giese, M. Moghaddam, R.W. Seiler, M.E. Berens, Identification and validation of P311 as a glioblastoma invasion gene using laser capture microdissection, Cancer Res. 61 (2001) 4190–41906.
[21] K.J. Mitchell, K.I. Pinson, O.G. Kelly, J. Brennan, J. Zupicich, P. Scherz, P.A. Leighton, L.V. Goodrich, X. Lu, B.J. Avery, P. Tate, K. Dill, E. Pangilinan, P. Wakenight, M. Tessier-Lavigne, W.C. Skarnes, Functional analysis of secreted and transmembrane proteins critical to mouse development, Nat. Genet. 28 (2001) 241–249.
[22] M. Busse, A. Feta, J. Presto, M. Wilen, M. Gronning, L. Kjellen, M. Kusche-Gullberg, Contribution of EXT1, EXT2, and EXTL3 to heparan sulfate chain elongation, J. Biol. Chem. 282 (2007) 32802–32810.
[23] J.M. Kelm, N.E. Timmins, C.J. Brown, M. Fussenegger, L.K. Nielsen, Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types, Biotechnol. Bioeng. 83 (2003) 173–180.
[24] N.E. Timmins, L.K. Nielsen, Generation of multicellular tumor spheroids by the hanging-drop method, Methods Mol. Med. 140 (2007) 141–151.
[25] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[26] M. Abe, J.G. Harpel, C.N. Metz, I. Nunes, D.J. Loskutoff, D.B. Rifkin, An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct, Anal. Biochem. 216 (1994) 276–284.
[27] L. Zhang, H.S. Li, Z.H. Yao, S.S. Yang, W.F. He, J. Wu, G.X. Luo, Interaction between P311 and transforming growth factor beta 1 and its effect on the function of murine fibroblasts, Zhonghua Shao Shang Za Zhi 32 (2016) 208–215.
[28] E. Piek, W.J. Ju, J. Heyer, D. Escalante-Alcalde, C.L. Stewart, M. Weinstein, C. Deng, R. Kucherlapati, E.P. Bottinger, A.B. Roberts, Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts, J. Biol. Chem. 276 (2001) 19945–19953.
[29] G. David, X.M. Bai, B. Van der Schueren, J.J. Cassiman, H. Van den Berghe, Developmental changes in heparan sulfate expression: in situ detection with mAbs, J. Cell Biol. 119 (1992) 961–975.
[30] D.F. Quail, J.A. Joyce, Microenvironmental regulation of tumor progression and metastasis, Nat. Med. 19 (2013) 1423–1437.
[31] R. Kalluri, M. Zeisberg, Fibroblasts in cancer, Nat. Rev. Cancer 6 (2006) 392–401.
[32] J. Odenthal, R. Takes, P. Friedl, Plasticity of tumor cell invasion: governance by growth factors and cytokines, Carcinogenesis 37 (2016) 1117–1128.
[33] Z. Piperigkou, B. Mohr, N. Karamanos, M. Gotte, Shed proteoglycans in tumor stroma, Cell Tissue Res. 365 (2016) 643–655.
[34] A.D. Theocharis, S.S. Skandalis, G.N. Tzanakakis, N.K. Karamanos, Proteoglycans in health and disease: novel roles for proteoglycans in malignancy and their pharmacological targeting, FEBS J. 277 (2010) 3904–3923.
[35] J. Shi, K.R. Badri, R. Choudhury, L. Schuger, P311-induced myofibroblasts exhibit ameboid-like migration through RalA activation, Exp. Cell Res. 312 (2006) 3432–3442.
[36] M. Fujitani, S. Yamagishi, Y.H. Che, K. Hata, T. Kubo, H. Ino, M. Tohyama, T. Yamashita, P311 accelerates nerve regeneration of the axotomized facial nerve, J. Neurochem. 91 (2004) 737–744.
[37] J.M. Studler, J. Glowinski, M. Levi-Strauss, An abundant mRNA of the embryonic brain persists at a high level in cerebellum, hippocampus and olfactory bulb during adulthood, Eur. J. Neurosci. 5 (1993) 614–623.
[38] W.S. McDonough, N.L. Tran, M.E. Berens, Regulation of glioma cell migration by serine-phosphorylated P311, Neoplasia 7 (2005) 862–872.
[39] X. Peng, S. Yuan, J. Tan, B. Ma, X. Bian, C. Xu, W. He, H. Cao, Z. Huang, Y. Cui, C. Gan, X. Wang, J. Zhou, J. Hu, S. Yang, G. Luo, J. Wu, Identification of ITGB4BP as a new interaction protein of P311, Life Sci. 90 (2012) 585–590.
[40] J.K. Leung, S. Cases, T.H. Vu, P311 functions in an alternative pathway of lipid accumulation that is induced by retinoic acid, J. Cell Sci. 121 (2008) 2751–2758.
[41] D. Pan, X. Zhe, S. Jakkaraju, G.A. Taylor, L. Schuger, P311 induces a TGF-beta1-independent, nonfibrogenic myofibroblast phenotype, J. Clin. Invest. 110 (2002) 1349–1358.
[42] C. Eberlein, C. Rooney, S.J. Ross, M. Farren, H.M. Weir, S.T. Barry, E-cadherin and EpCAM expression by NSCLC tumour cells associate with normal fibroblast activation through a pathway initiated by integrin alphavbeta6 and maintained through TGFbeta signalling, Oncogene 34 (2015) 704–716.
[43] D. Xu, J.D. Esko, Demystifying heparan sulfate-protein interactions, Annu. Rev. Biochem. 83 (2014) 129–157.
[44] J. Lee, S. Wee, J. Gunaratne, R.J. Chua, R.A. Smith, L. Ling, D.G. Fernig, K. Swaminathan, V. Nurcombe, S.M. Cool, Structural determinants of heparin-transforming growth factor-beta1 interactions and their effects on signaling, Glycobiology 25 (2015) 1491–1504.
[45] M. Lyon, G. Rushton, J.T. Gallagher, The interaction of the transforming growth factor-betas with heparin/heparan sulfate is isoform-specific, J. Biol. Chem. 272 (1997) 18000–18006.
[46] D. Yadin, P. Knaus, T.D. Mueller, Structural insights into BMP receptors: specificity, activation and inhibition, Cytokine Growth Factor Rev. 27 (2016) 13–34.
[47] K. Iwao, M. Inatani, Y. Matsumoto, M. Ogata-Iwao, Y. Takihara, F. Irie, Y. Yamaguchi, S. Okinami, H. Tanihara, Heparan sulfate deficiency leads to Peters anomaly in mice by disturbing neural crest TGF-beta2 signaling, J. Clin. Invest. 119 (2009) 1997–2008.
[48] R. Dhanasekaran, I. Nakamura, C. Hu, G. Chen, A.M. Oseini, E.S. Seven, A.G. Miamen, C.D. Moser, W. Zhou, T.H. van Kuppevelt, J.M. van Deursen, T. Mounajjed, M.E. Fernandez-Zapico, L.R. Roberts, Activation of the transforming growth factor-beta/SMAD transcriptional pathway underlies a novel tumor-promoting role of sulfatase 1 in hepatocellular carcinoma, Hepatology 61 (2015) 1269–1283.
[49] C. Shu, S.M. Smith, J. Melrose, The heparan sulphate deficient Hspg2 exon 3 null mouse displays reduced deposition of TGF-beta1 in skin compared to C57BL/6 wild type mice, J. Mol. Histol. 47 (2016) 365–374.
[50] T. Batool, J. Fang, U. Barash, A. Moustakas, I. Vlodavsky, J.P. Li, Overexpression of heparanase attenuated TGF-beta-stimulated signaling in tumor cells, FEBS Open Bio 7 (2017) 405–413.

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