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Mini Review

The extostosin family: Proteins with many functions

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ABSTRACT

Heparan sulfates are complex sulfated molecules found in abundance at cell surfaces and in the extracellular matrix. They bind to and influence the activity of a variety of molecules like growth factors, proteases and morphogens and are thus involved in various cell–cell and cell–matrix interactions. The mammalian EXT proteins have glycosyltransferase activities relevant for HS chain polymerization, however their exact role in this process is still confusing. In this review, we summarize current knowledge about the biochemical activities and some proposed functions of the members of the EXT protein family and their roles in human disease.

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1. Introduction

Heparan sulfate (HS) proteoglycans (PGs) are ubiquitous components of the extracellular matrix and play important roles in tissue homeostasis (Kim et al., 2011). They are essential for signal transduction of many signaling molecules driving processes such as cell survival, division, migration, differentiation and cancer development (reviewed in (Li, 2008)). HSPGs are synthesized in a multistep process that is initiated by the formation of a tetrasaccharide linkage region attached to a selected serine residue in the core protein (GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser (where GlcA, Gal and Xyl are glucuronic acid, galactose and xylose, respectively). After the addition of an α1,4-linked N-acetylglucosamine (α1,4-GlcNAc) residue, elongation proceeds by the action of glycosyltransferases which add β1,4-linked GlcA and α1,4-linked GlcNAc units in alternating sequence to the nonreducing end of the growing polymer (Fig. 1). 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, GlcA and iduronic acid (IdoA) units, as well as O-sulfate groups in various positions (Lindahl

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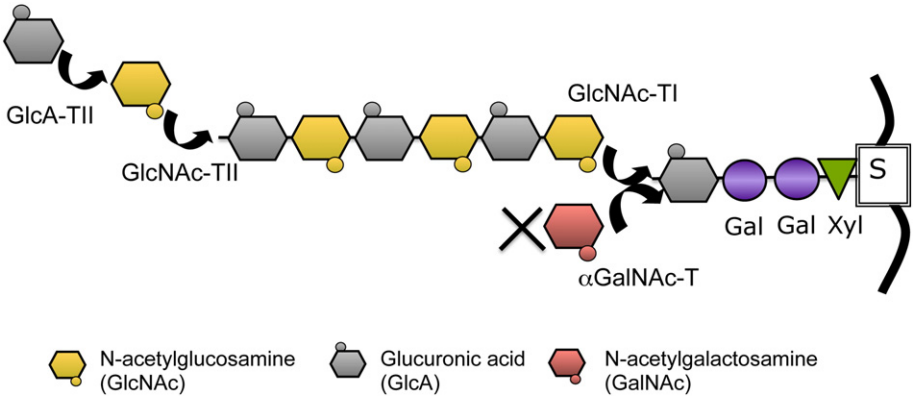


Fig. 1. Schematic illustration of the different glycosyltransferase activities involved in HS chain elongation. HS biosynthesis is initiated by the assembly of a polysaccharide-protein linkage tetrasaccharide (GlcAβ1-3Galβ1-3Galβ1-4Xyl) to a particular serine residue in the core protein. After addition of a single α1,4-linked GlcNAc (GlcNAc-transferase (-T) I activity), the $(GlcA\text{-}GlcNAc)_n$ HS precursor chain is formed by the alternating addition of β1,4-linked GlcA and α1,4-linked GlcNAc residues by glycosyltransferase/s with GlcATII and/or GlcNAcTII activities. If an α1,4-linked GalNAc is added to the tetrasaccharide linkage by αGalNAc-transferase this terminates further chain elongation. It should be noted that αGalNAc-transferase activity has only been detected in *in vitro* studies and that the product has never been identified in native proteoglycans.

et al., 1998; Esko and Selleck, 2002; Bulow and Hobert, 2006; Bishop et al., 2007; Kreuger and Kjellen, 2012). The synthesis of HS backbone is mediated by glycosyltransferases of the exostosin family (EXTs). Five genes encoding EXT proteins have been identified in mammals, *EXT1*, *EXT2*, *EXTL1*, *EXTL2*, and *EXTL3* (Table 1).

EXT1 and *EXT2* were first recognized as two out of three genes that were responsible for the autosomal inherited disorder hereditary multiple osteochondromas (HMO, also called hereditary multiple exostoses, HME), by genetic linkage analysis in 1993–1994 (Cook et al., 1993; Le Merrer et al., 1994; Wu et al., 1994). The three disease genes, located at three distinct chromosomes 8, 11 and 19, were called exostosins and named *EXT1*, *EXT2* and *EXT3*, respectively. The *EXT1* and *EXT2* genes were shortly afterwards cloned and characterized (Ahn et al., 1995; Stickens et al., 1996) whereas *EXT3* has so far not been identified and its linkage to HMO has been questioned. The protein sequence of EXT1 and EXT2 showed structural similarities but their functions remained unknown until two independent studies linked EXT1 and EXT2 to HS synthesis (Lind et al., 1998; McCormick et al., 1998).

The other members of the EXT-family, the EXT-like proteins, EXTL1, EXTL2 and EXTL3, have not been linked to HMO. Instead they were identified in screens for proteins homologous to EXT1 and EXT2 (Wise et al., 1997; Wuyts et al., 1997; Van Hul et al., 1998). EXTL2 and EXTL3 are also referred to as products of EXT-related genes 2 and 1 (*EXTR2* and *EXTR1*), respectively. Since the three EXTL-proteins share amino acid sequence homology with EXT1 and EXT2, it is expected that these proteins also might be involved in HS synthesis.

The EXT proteins are well conserved, especially in their C-terminal parts (Fig. 2). Several conserved cysteine residues are found in all EXTs, suggesting that they share a common structural fold. Except for EXTL1 they all contain one or more conserved aspartic acid-any amino acid-aspartic acid (DXD) motifs (Zak et al., 2002) typical for glycosyltransferases utilizing nucleotide-activated sugars as donor substrates (Breton et al., 1998). EXTL2 is the shortest member of the EXT family consisting of ~330 amino acids whereas EXTL3 is significantly larger than the other members of the EXT family (~900 aa, Table 1) (Van Hul et al., 1998) and contains an N-terminal fragment with no homology with the other family members.

Except for EXTL1, the EXT family members are ubiquitously expressed in mammalian tissues. *EXTL1* mRNA expression in adult human and mouse seems to be limited to a few tissues. Northern blot analyses show high expression of human *EXTL1* in skeletal muscles and brain and lower transcript levels in heart (Wise et al., 1997). Gene profiling shows, that in addition to being highly expressed in skeletal muscle and brain, the human *EXTL1* is expressed in skin (TiGER database: http://bioinfo.wilmer.jhu.edu/tiger/). In the mouse embryo the highest expression is found in the skeleton, limb and nose (Eurexpress: http://www.eurexpress.org/ee/intro.html). Northern blot analyses of the adult mouse *EXTL1* mRNA transcript showed, with one exception, transcript patterns similar to those of the human *EXTL1* mRNA with expression in heart, skeletal muscle and the brain (Stickens et al., 2000). In contrast to analyses of the human Northern blot, *EXTL1* is highly expressed in the adult mouse liver tissues (Stickens et al., 2000), whereas the TiGER database shows low levels of the human *EXTL1* in liver. Future studies will determine if the different expression levels have functional importance.

Little is known about the frequency of structural variants of the EXTs. According to e!Ensemble (http://www.ensembl.org/index.html), all human *EXT*-family members, except for *EXTL1*, can express several putative splice forms resulting in protein products of different sizes. Corresponding data for mouse list protein coding splice variants for *Ext1*, *Ext2* and *Extl2*. However, it is important to note that for the majority of the listed splice variants no experimental confirmation is available. Therefore, their tissue distributions and functional impacts are not known.

2. Proposed functions of the mammalian EXT family of proteins

2.1. EXT1 and EXT2

It has for many years been suggested that the GlcA- and GlcNAc transferase activities required for HS chain elongation are located in a

Table 1
Human and mouse exostosin genes.

Gene	Human			Mouse		
	Chromosome number	mRNA accession	Amino acid	Chromosome number	mRNA accession	Amino acid
EXT1	8	NM_000127	746	15	NM_010162	746
EXT2	11	NM_000401	718	2	NM_010163	718
EXTL1	1	NM_004455	676	4	NM_019578	669
EXTL2	1	NM_001033025	330	3	NM_001163514	330
EXTL3	8	NM_001440	919	14	NM_018788	919

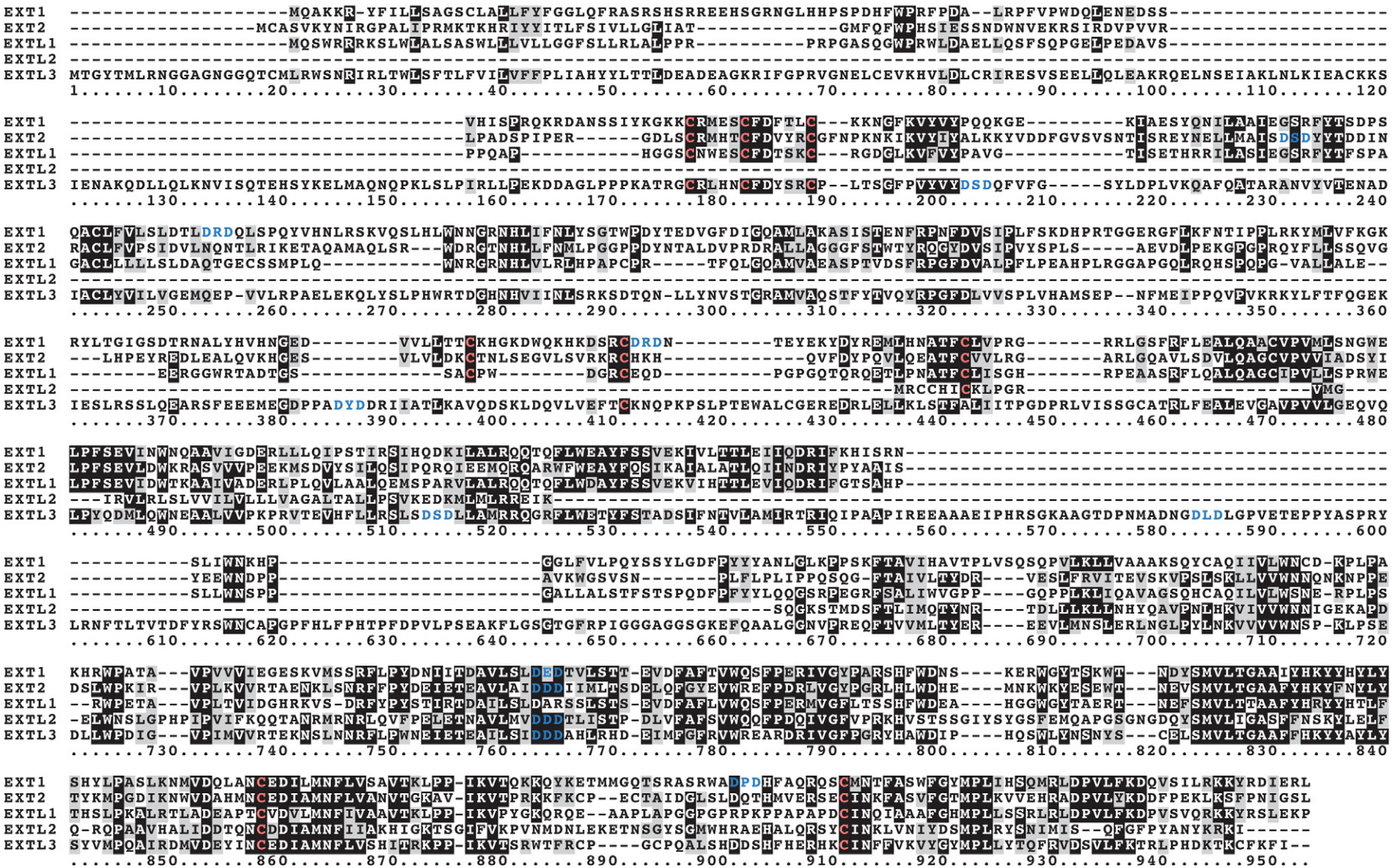


Fig. 2. Amino acid sequence comparison of human EXT/EXTL proteins. Conserved and similar residues are highlighted with black and gray boxes, respectively. Conserved cysteins are labeled in red and DXD motifs in blue. The sequences were aligned using the Clustal W software and the alignments were manually corrected.

single protein (Lidholt et al., 1992). In 1993 a protein that catalyzed the *in vitro* transfer of both GlcA and GlcNAc to the appropriate substrates was purified from bovine serum (Lind et al., 1993). Cloning of the corresponding cDNA identified the putative GlcA/GlcNAc transferase as EXT2 (Lind et al., 1998). At the same time an independent study demonstrated EXT1 to restore HS biosynthesis in an HS-deficient cell line (McCormick et al., 1998).

Early studies that looked at the glycosyltransferase activities of EXT1 and EXT2 tentatively implied that both EXT1 and EXT2 are bifunctional enzymes that harbor GlcA- as well as GlcNAc-transferase (GlcA-TII and GlcA-TII) activities (Fig. 1) involved in HS elongation (Lind et al., 1998; McCormick et al., 2000; Senay et al., 2000). Analyses of mutant Chinese hamster ovary (CHO) cells deficient in HS, mapped the GlcA-transferase domain to the N-terminal half of the EXT1 protein (Wei et al., 2000). So far, the location of the GlcNAc-transferase domain remains unidentified. Even though EXT1 alone is able to polymerize the HS backbone structure *in vitro* (Busse and Kusche-Gullberg, 2003; Kim et al., 2003) it has been clearly recognized that both EXT1 and EXT2 are needed *in vivo* for HS chain elongation. Lack of either EXT1 or EXT2 disrupts HS synthesis indicating that both proteins are required for proper polymerization (McCormick et al., 1998; Lin et al., 2000; Stickens et al., 2005). Furthermore, neither of the two EXT proteins can substitute for the other one. Thus, transfection of EXT2 into cells deficient in EXT1 does not restore HS synthesis (McCormick et al., 2000; Wei et al., 2000). Since co-expression experiments showed that EXT1 and EXT2 form hetero-oligomers and that the EXT1/EXT2 complex possessed substantially higher glycosyltransferase and polymerizing activities than either EXT1 or EXT2 alone it was further suggested that the biologically functional unit in HS polymerization is a co-polymerase in which both EXT1 and EXT2 are essential for activity (McCormick et al., 2000; Senay et al., 2000; Busse and Kusche-Gullberg, 2003; Busse et al., 2007). Interestingly, the interaction and catalytic activities observed after co-expression of EXT1 and EXT2 were not observed by mixing cellular lysates of separately EXT1- and EXT2-expressing cells or separately expressed recombinant EXT1 and EXT2 indicating that EXT1 and EXT2 proteins need to be simultaneously synthesized in the cell in order to attain full catalytic efficacy (Senay et al., 2000).

The EXT1 protein has readily detectable GlcA- and GlcNAc-transferase activities whereas it has been difficult to pinpoint the catalytic activities of EXT2. No increase in glycosyltransferase activities could thus be detected after overexpression of EXT2 in mammalian cells. The problem is further complicated by the tendency of EXT1 and EXT2 to associate with each other and to remain associated also after purification of recombinant proteins (Kobayashi et al., 2000; McCormick et al., 2000; Senay et al., 2000). Therefore, the early studies of the glycosyltransferase activities of EXT2 have been questioned. Instead, the function of EXT2 has been suggested to be assisting in the transport (and maybe also the folding) of EXT1 and the first HS modifying enzyme, a combined N-deacetylase/N-sulfotransferase, from the ER to the Golgi complex (Busse et al., 2007; Presto et al., 2008). Moreover, *in vitro*, a complex of recombinant EXT1 and EXT2 has been shown to polymerize HS directly onto a protein-linkage region lacking the first GlcNAc, implicating additional GlcNAc-TI activity for the EXT1/2 complex (Kim et al., 2003). At present, no *in vivo* data are yet available to confirm the lack of the first GlcNAc residue in the protein-linkage region of *Ext1* or *Ext2* deficient cells. Also, CHO cells deficient in *Ext1* produce a recombinant proteoglycan with a tetrasaccharide linkage region containing one additional GlcNAc residue (Zhang and Esko, 1995), suggesting that also other proteins can add this residue to the linkage region. In fact, another member of the EXT-family, EXTL3, has been shown to perform this activity *in vitro* and mice deficient in *Extl3* are not able to produce HS (Takahashi et al., 2009), implying a more complex regulation of HS biosynthetic machinery *in vivo*. In addition to their involvement in HS synthesis, EXT1 and EXT2 were found to bind to the heat shock protein tumor necrosis factor type I associated protein (TRAP-1) and EXT2 was further shown to strongly interact with polypeptide GalNAc transferase T5 (Simmons et al., 1999). These studies raise the possibility that the function of EXT2 is not only to act as partner in HS biosynthesis but it may also have more complex functions such as being an interacting and/or cellular transporting partner for different molecules.

2.2. EXTL1

EXTL1 has been identified as a GlcNAc transferase catalyzing the addition of a GlcNAc to substrates mimicking the non-reducing end of growing HS chain (GlcNAc-TI activity, Fig. 1) (Kim et al., 2001). There are very few reports regarding the biochemistry of EXTL1, most probably due to its limited expression level. Yet, there are several reports on changes in *EXTL1* mRNA expression levels at different stages of cellular development and in tumors (Liu et al., 2006; Gu et al., 2008). For example, *EXTL1* expression is down-regulated in chorionic villi samples of 10–11 old euploid fetuses with increased nuchal translucency (fluid collection behind the neck of the fetus) (Farina et al., 2006). *Extl1* mRNA levels, but not those of the other *Exts*, are up-regulated during differentiation of C2C12 myoblast into myotubes, indicating that *Extl1* may be involved in myogenic differentiation (Janot et al., 2009). Transgenic mice overexpressing *Extl1* in a B-cell specific manner show a blockage of early B-cell maturation (Duchez et al., 2011). The effect could be attributed to the overexpression of *Extl1* in the B-cell linage, since heparin addition to cells *in vitro* had no effect. Notably, in the above mentioned studies, no attempts were made to analyze HS structure so the link of *Extl1* expression levels to HS synthesis remains to be determined. Furthermore, since expression of HS synthesizing enzymes may be translationally controlled (Grobe and Esko, 2002), information about protein instead of transcript levels will be important. So far, no mutants of EXTL1 have been described so the precise role of EXTL1 *in vivo* remains elusive. Its restricted expression suggests a more specialized function than expected of a glycosyltransferase involved in HS synthesis.

2.3. EXTL2

EXTL2 has been demonstrated to have two *in vitro* glycosyltransferase activities, transfer of either α-linked GlcNAc or α-linked N-acetylgalactosamine (GalNAc) to the tetrasaccharide linkage region (Kitagawa et al., 1999; Okada et al., 2010). The functional significance of the α-linked GalNAc transfer is difficult to understand since the product is not an acceptor for glycosyltransferases involved in glycosaminoglycan synthesis (Fig. 1) and has never been identified in native proteoglycans. Both HS and chondroitin sulfate (CS) polymerization are initiated on the tetrasaccharide linkage region. The addition of the first GlcNAc residue to the linkage tetrasaccharide region is the key step to the initiation of HS polymerization and is carried out by a unique glycosyltransferse, GlcNAc-TI, which differs from the corresponding enzyme/s involved in chain elongation. Although EXTL2 exhibits *in vitro* transferase activity that could correspond to an initiator of HS synthesis (GlcNAc-TI activity, Fig. 1) this activity is weak and has been detected only with a synthetic linkage-region analog as substrate but not with the authentic linkage tetrasacccharide substrate (Kitagawa et al., 1999). In fact, recombinant EXTL2 was found to transfer α-linked GalNAc much more efficiently than α-linked GlcNAc to the synthetic linkage-region analog (Kitagawa et al., 1999; Pedersen et al., 2003) that may suggest a function for this enzyme other than as an initiator of HS biosynthesis. The enzyme could possibly catalyze the formation of a novel glycan, perhaps unrelated to proteoglycan assembly. However, in an *Ext1* deficient mouse fibroblast cell line, gro2C (McCormick et al., 1998), EXTL2 has been proposed to initiate HS synthesis by transferring GlcNAc to the linkage region followed by EXT2-mediated elongation of HS chains (Okada et al., 2010). The EXT2 polymerized chains are considerably shorter than HS chains from wild-type fibroblast cells. Intriguingly, a similar shortening of HS chains is observed in embryonic

fibroblasts carrying a hypomorphic mutation in *Ext1*, transcribing only about 3% of wild type *Ext1* mRNA (Yamada et al., 2004).

In a recent article another function for EXTL2 as a suppressor of glycosaminoglycan (HS and CS) synthesis in the mouse liver was proposed (Nadanaka et al., 2013b). The authors show that EXTL2-mediated transfer of GlcNAc to a linkage region containing a phosphorylated Xyl residue prevents further chain elongation (Nadanaka et al., 2013b). Additionally, mouse liver cells lacking EXTL2 produce significantly more HS and CS during liver repair (Nadanaka et al., 2013a). The increase in sulfated glycosaminoglycan synthesis had no detectable impact on normal animal physiology but impaired the regeneration process of the liver (Nadanaka et al., 2013a). The findings regarding the role of EXTL2 as a terminator of HS chain elongation (when the linkage region contains a phosphorylated xylose) and the results suggesting that, in the absence of EXT1, EXTL2 initiates HS synthesis are puzzling and may indicate a cell specific regulation of HS synthesis. Clearly, additional more refined experiments will be needed to resolve the function of EXTL2 and its role in glycosaminoglycan synthesis.

2.4. EXTL3

EXTL3 has been ascribed two glycosyltransferase activities, GlcNAc-TI and GlcNAc-TII (Fig. 1), and thus may be involved in the initiation and elongation of HS chains (Kim et al., 2001). siRNA mediated gene silencing experiments in mammalian cells demonstrated that reduced EXTL3 levels results in synthesis of longer HS chains whereas overexpression of EXTL3 had no obvious effect on HS chain length (Busse et al., 2007). These results indicated that lowering the amount of EXTL3 results in fewer linkage regions containing the GlcNAc necessary for the start of HS elongation. With less acceptor substrates available, more extensive polymerization of the chains that are being synthesized can occur. Furthermore, no HS was detected in 9-day old mouse embryos lacking EXTL3 (Takahashi et al., 2009) and mutations in corresponding enzymes in *Drosophila melanogaster* (Bornemann et al., 2004; Takei et al., 2004), *Caenorhabditis elegans* (Morio et al., 2003) and *Danio rerio* (Lee et al., 2004) led to reduced HS synthesis. Taken together, these data suggest that EXTL3 is an initiator of HS chains.

In addition to being a glycosyltransferase, the N-terminal part of the EXTL3 protein has been associated with TNF-α-induced nuclear factor-κB (NF-κB) activity (Mizuno et al., 2001). EXTL3 has also been proposed to be a cell surface receptor for regenerating islet-derived proteins (REGs). The REG family is a group of secreted proteins with a C-type lectin-like domain involved in proliferation and differentiation of several different cell types (Parikh et al., 2012). In 2000, EXTL3 was first described as a cell surface receptor for a rat pancreatic REG-protein (Kobayashi et al., 2000). Following this publication several other publications have claimed EXTL3 to be a cell surface receptor for different members of the REG-family (Levetan et al., 2008; Mueller et al., 2008; Acquatella-Tran Van Ba et al., 2012; Lai et al., 2012). However, it is unclear if EXTL3 binds directly to REG proteins or not. Lai et al. (2012) found that binding of recombinant REG3A to keratinocyte EXTL3 promotes wound re-epithelialization through activation of the phosphatidylinositol 3 kinase (PI3K)-AKT signaling pathway (Lai et al., 2012). Binding of REG1 to rat pancreatic-derived cells, supposedly *via* EXTL3, induces growth by activating a MAPK-cyclin D1 pathway (Mueller et al., 2008). Immunoprecipitation studies of REG3A treated keratinocytes showed association of EXTL3 with REG3A (Lai et al., 2012) whereas in a yeast two-hybrid system no physical association of rat Reg-1 protein with EXTL3 was observed (Mueller et al., 2008). Furthermore, EXTL3 silencing in keratinocytes had no effect on the HS-dependent fibroblast growth factor 7- (FGF7) mediated *in vitro* wound induced re-epithelialization but decreased REG3A induced re-epithelialization (Lai et al., 2012). This result, together with the fact that syndecan-1 expression was unchanged after EXTL3 silencing, was interpreted to suggest that the effect of EXTL3 on REG3A mediated keratinocyte proliferation was independent of HS or HS binding growth factors. However, no attempts were made to quantify HS. At this stage, one cannot rule out that EXTL3 is a receptor for REG-proteins, however, how and if this property relates to HS synthesis is not known. Clearly, further studies of EXTL3 is needed to understand the proposed multiple roles of EXTL3.

3. EXT orthologs and animal mutants

Phylogenetic analyses of EXT family based on amino acid sequence alignments show that *EXT1*, *EXT2* and *EXTL3* most likely have already been present in the last common ancestor of all eumetazoans (Feta et al., 2009)(Fig. 3). In contrast, *EXTL1* and *EXTL2* genes are present only in vertebrates, probably appearing with the emergence of fish. *EXTL1* is more closely related to *EXT1* than the other *EXT* family members and the *EXTL1* gene seems to result from a duplication of the whole *EXT1* gene before the appearance of jawless fish (lampreys, hagfish). *EXTL2* is the most divergent and may have arisen *via* partial duplication of the *EXTL3* gene in the ancestors of the jawed fish.

The importance of EXT1, EXT2, and EXTL3 for HS synthesis and HS-dependent signaling has been shown in several animal models. Loss of function of these genes in mouse, *D. rerio*, *D. melanogaster* and *C. elegans* causes developmental abnormalities (Table 2) most probably due to defective morphogen/growth factor signaling. Below is a short presentation of model organisms with loss of functions caused by mutations in the genes encoding *EXT*-family members.

Ext1 and *Ext2*-deficient mouse embryos exhibit the same developmental abnormalities; they lack HS, fail to gastrulate and die around embryonic day 8.5 (Lin et al., 2000; Stickens et al., 2005). In contrast, mice carrying a hypomorphic mutation in *Ext1* generated by gene trapping ($Ext1^{Gt/Gt}$) (Mitchell et al., 2001) survive to embryonic day 14.5 (Koziel et al., 2004). The longer survival time of the $Ext1^{Gt/Gt}$ mice may be explained by the fact that they still produce small amounts of *Ext1*, which results in synthesis of short but apparently normally sulfated HS chains (Yamada et al., 2004). Conditional disruption of *Ext1* in the developing mouse brain results in severe brain defects (Inatani et al., 2003; Yamaguchi et al., 2010). *Extl3* deficient mice die around E9 and cell specific ablation of *Extl3* in mouse pancreatic islets affects the maturation of postnatal islets due to a reduced HS synthesis (Takahashi et al., 2009).

Zebrafish have three *EXT1* genes (*ext1a*, *ext1b* and *ext1c*) with different expression patterns that may suggest functional divergence (Siekmann and Brand, 2005). Mutations in the zebrafish orthologs of *EXT2* (*ext2*/*dackel*) and *EXTL3* (*extl3*/*boxer*) result in missorting of retinal ganglion cells and drastically reduced HS levels (Lee et al., 2004). Interestingly, the reduction of HS synthesis in the *extl3* mutants are accompanied by increased CS synthesis indicating that linkage regions that are not initiated by extl3 and thus do not form HS, instead are used as primers for CS synthesis (Holmborn et al., 2012).

The *Drosophila* orthologs of the mammalian EXT genes, *tout velu* (*ttv*), *sister of ttv* (*sotv* or *dEXT2*) and *brother of ttv* (*botv* or *dEXTL3*), correspond to mammalian *EXT1*, *EXT2* and *EXTL3*, respectively (Fig. 3). The *Drosophila EXT/L* genes were identified in genetic screens for mutations affecting morphogen signaling and mutations in any of these genes lead to reduction or abolishing of HS synthesis (The et al., 1999; Tsuda et al., 1999; Toyoda et al., 2000; Bornemann et al., 2004; Takei et al., 2004). Interestingly, due to translational control of HS biosynthetic enzymes, including *ttv*, HS synthesis in *Drosophila* is temporally regulated (Bornemann et al., 2008), indicating that translational regulation may represent an important control of HS synthesis.

C. elegans genome has only two *EXT* family members, *rib-1* and *rib-2*. Based on amino acid sequence homology, RIB-1 and RIB-2 appear to correspond to mammalian *EXT1* and *EXTL3*, respectively (Fig. 3B). Complete loss of function mutations of *rib-1* or *rib-2* result in embryonic lethality and reduced HS synthesis (Morio et al., 2003; Kitagawa et al., 2007).

Lowering the amounts of maternally derived *X.EXT1* mRNA in *Xenopus* embryos leads to abnormal dorsal–ventral axis formation (Tao et al., 2005).

3.1. Enzyme activities of non-mammalian EXT-proteins

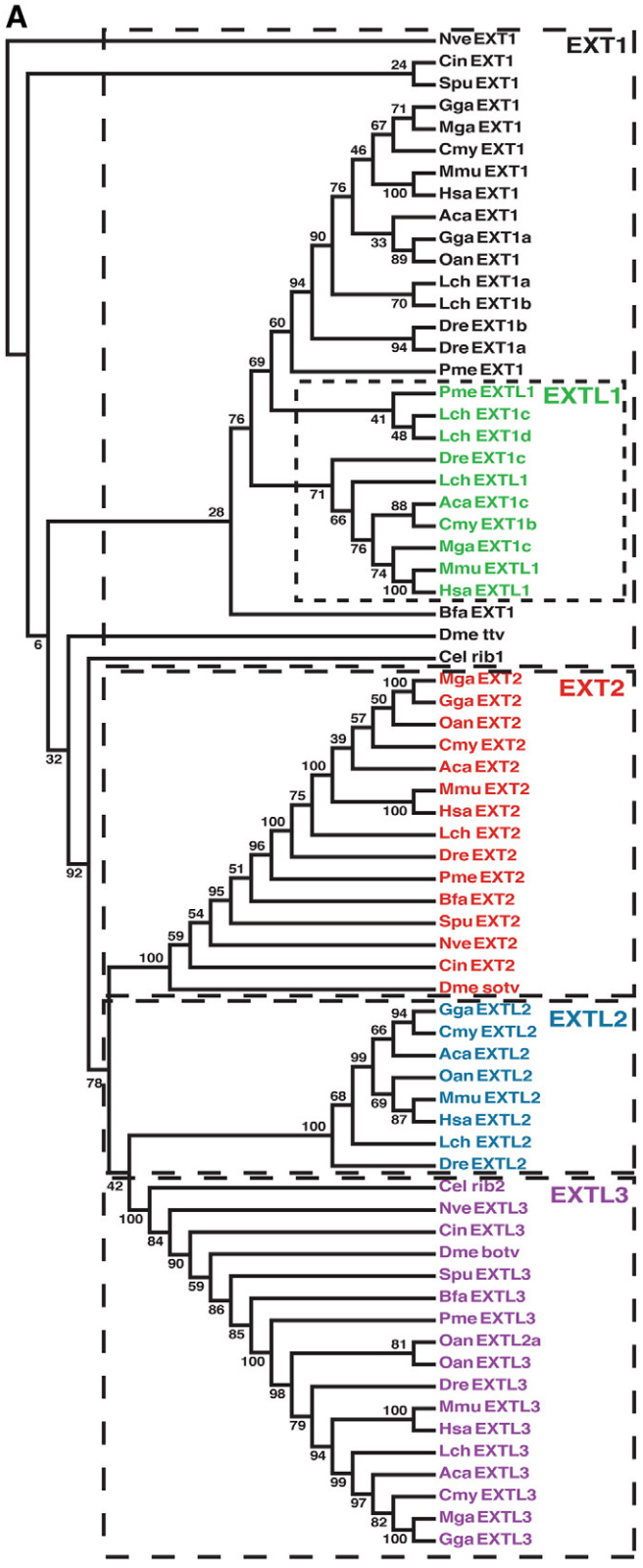
So far biochemical enzyme activity studies of non-mammalian species have been performed on recombinantly expressed *Drosophila* and *C. elegans* EXT-proteins.

In vitro enzyme assays of soluble recombinant forms of the *Drosophila* enzymes expressed in COS-1 cells revealed similar enzyme activities to those of their mammalian counterparts. BOTV has both GlcNAc-TI and -TII activities involved in initiation and polymerization (Kim et al., 2002). TTV and SOTV have GlcA- and GalNAc-TII activities *in vitro* and enhanced activities upon co-expression suggesting that, similar to their mammalian counterparts, both proteins participate in HS polymerization (Izumikawa et al., 2006).

The lack of an *EXT2* homolog indicates that in *C. elegans* the activities of RIB-1 and RIB-2 are sufficient for the production of HS chains. Soluble RIB-2 has similar *in vitro* glycosyltransferase activities as the other EXTL3 proteins, it catalyzes the transfer of GlcNAc *in vitro* onto acceptors mimicking HS chain initiation and elongation (Kitagawa et al., 2001). RIB-1 exhibits no transferase activities meaning that neither of the worm transferases shows any GlcA-transferase activity as single proteins. Still, when co-expressed, the RIB-1/RIB-2 complex exhibits both GlcA- and GlcNAc-T activities and is able to polymerize HS chains on a linkage region (Kitagawa et al., 2007). RIB-1 lacks a signal sequence, a transmembrane domain and has only one of the conserved DXD-motifs. RIB-1 is about half as long as RIB-2 and the human EXT1, the protein corresponds to the N-terminal part of EXT1, which potentially comprises the GlcA-T domain (Wei et al., 2000; Zak et al., 2002). *rib-1* and *rib-2* mutants both exhibit reduced amounts of HS demonstrating *in vivo* evidence for their requirement for HS synthesis in *C. elegans* (Morio et al., 2003; Kitagawa et al., 2007) (Table 2).

4. Pathologies associated with the human EXT family of proteins

Mutations in either *EXT1* or *EXT2* cause hereditary multiple osteochondromas (HMO), an autosomal dominant disorder characterized by bone deformities and cartilage-capped bony outgrowths, called exostoses or osteochondromas, at the ends of the long bones (reviewed in (Jennes et al., 2009)). HMO is one of the most common inherited skeletal disorders with an estimated incidence of 1–2 per 100 000 live births. The most serious complication is malignant degeneration to chondro- or osteosarcomas, which occurs in 2–5% of the patients. The patients are heterozygous for the mutation and a number of mutations in *EXT1* and *EXT2* have been reported in HMO patients (Jennes et al., 2009). The mutations are randomly distributed over the entire EXT1 gene, whereas EXT2 mutations appear to be concentrated towards the N-terminal part of the protein. Most mutations result in premature termination of translation and loss of function. Less common are missense mutations affecting single amino acids resulting in loss of EXT activity (Jennes et al., 2009). Many theories on the mechanism by which osteochondroma develop have been proposed and contradictory reports are present on the cellular origin of the osteochondroma. Proposed candidates at the growth-plate include chondrocytes, perichondrial cells and cells of the groove of Ranvier. Although tumor suppressor properties were ascribed early to *EXT1* and *EXT2*, it remains unclear if *EXT1* and *EXT2* fit the classic two-hit model for tumor suppressor genes, which implies that osteochondroma results from a somatic mutation in the remaining wild-type copy of the gene (Knudson, 1971). Another possibility is that osteochondromas may arise from haploinsuffiency in the *EXT1* or *EXT2* genes, resulting in reduced HS synthesis. For these aspects of HMO and the description of several animal models that have been developed to understand the pathogenesis and the underlying molecular events of HMO the readers are referred to (Bovee, 2010; Roehl and Pacifici, 2010; Sarrazin et al., 2011; de Andrea and Hogendoorn, 2012; Huegel et al., 2013) and references therein. In addition to skeletal dysfunction, HMO patients complain about other problems, such as childhood autism-like symptoms, digestive problems, muscle weakness, and atherosclerosis (Roehl and Pacifici, 2010) which all may be caused by a reduced HS content. In fact, autism has been suggested to be associated with HMO (Li et al.,



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	Rib1[1]	Rib2	TTV	SOTV	BOTV	zEXT1a	zEXT1b	zEXT1c	Dackel	Boxer	hEXT1	hEXT2	hEXTL1	hEXTL2
Rib2[1]	11													
TTV[2]	45	19												
SOTV[2]	21	21	29											
BOTV[2]	18	34	26	25										
zEXT1a[3]	45	18	54	29	25									
zEXT1b[3]	44	16	52	28	23	86								
zEXT1c[3]	43	21	52	28	27	63	62							
Dackel[3]	26	24	31	45	31	33	32	31						
Boxer[3]	16	33	25	27	48	25	24	25	31					
hEXT1[4]	45	19	52	30	23	84	84	63	33	26				
hEXT2[4]	27	25	32	44	27	33	32	31	84	28	33			
hEXTL1[4]	26	14	39	28	27	46	45	44	28	27	46	27		
hEXTL2[4]	3	25	20	25	27	24	23	22	26	27	24	27	22	
hEXTL3[4]	18	34	25	28	48	25	24	25	31	82	26	29	23	26

Fig. 3. A) Phylogenetic tree of EXT/L orthologs from various metazoan species: The tree was generated using the maximum parsimony algorithm implemented by the Mega 5 software. The turkey (*MgaEXT1c*), lizard (*AcaExt1c*), turtle (*CmyExt1b*) and zebrafish (*DreExt1c*) group with the EXTL1. They may represent *Extl1* genes. However, we can currently not exclude that these genes and the jawless fish latimeria (*LchExt1c/d*) and lamprey (*PmeExtl1*) represent *Ext1* genes. Nve — *Nematostella vectensis* (anemone), Dme — *Drosophila melanogaster* (fly), Cel — *Caenorhabditis elegans* (nematode), Spu — *Strongylocentrotus purpuratus* (urchin), Cin — *Ciona intestinalis* (tunicate), Bfa — *Branchiostoma floridae* (lancelet), Pma — *Petromyzon marinus* (lamprey), Dre — *Danio rerio* (fish), Lch — *Latimeria chalumnae* (coelacanth), Aca — *Anolis carolinensis* (lizard), Gga — *Gallus gallus* (chicken), Mga — *Meleagris gallopavo* (turkey), Oan — *Ornithorhynchus anatinus* (platypus), Mmu — *Mus musculus* (mouse), and Hsa — *Homo sapiens* (human). B) Comparison of amino acid sequence identities between EXT-family members in different species. The numbers show the percent of the identical amino acids in pair-wise aligned sequences using the ClustalW software. Notice the relatively low overall degree of sequence conservation between the human EXT-protein family members.

2002) and mice with a tissue specific deletion of *Ext1* in mature neurons exhibit autism-like behavioral phenotypes (Irie et al., 2012). Multiple osteochondromas are also observed in patients with two contiguous gene syndromes mapped to chromosomes 8 (Langer–Giedion syndrome) (Cook et al., 1993; Ludecke et al., 1995) and 11 (defect 11 syndrome) (Wu et al., 1994; Wuyts et al., 1995) due to deletions or mutations in *EXT1* and *EXT2*, respectively, in addition to loss of neighboring genes.

In addition to a role in HMO, epigenetic inactivation of *EXT1* by promoter hypermethylation resulting in inhibition of HS synthesis is found in leukemia and non-melanoma cancer cells and re-introduction of EXT1 into cancer cells induced a tumor suppressing effect (Ropero et al., 2004). In contrast, high expression of *EXT1* in patients with multiple myeloma was reported to be associated with a poor prognosis (Bret et al., 2009). *EXT2* is mutated in breast carcinoma patients (Yoneda et al., 2012) and both EXT1 and EXT2 have altered N-glycosylation in human aggressive breast cancer cell lines (Drake et al., 2012). However, it is not known how or if these latter modifications affect the activity of the EXT-proteins and thus HS structure. Additionally, in hepatocellular carcinoma, frequent inactivation of one allele (loss of heterozygosity) of *EXT1* has been described (Piao et al., 1997).

The *EXTL*-genes are not linked to HMO and have so far not been associated with any diseases although their chromosomal locations may indicate some involvements in certain types of cancer. *EXTL1* maps to chromosome 1p36, a region that often is deleted in neuroblastoma. Based on its localization, *EXTL1* has been implicated as a candidate involved in the etiology of these tumors. However, detailed molecular analysis of *EXTL1* in neuroblastoma patients and cell lines could not confirm that *EXTL1* is directly involved in the development of neuroblastoma (Mathysen et al., 2004). *EXTL2* has been assigned to a chromosome region (1p11–p12) that is frequently rearranged in sporadic

Table 2
Animal models with loss of function mutations in EXT genes.

Model	Gene	Mutant phenotype	References
Mus musculus (mouse)	*Ext1*$^{-/-}$	Embryonic lethality (~E 7.5). Failure to gastrulate. No HS chains.	Lin et al. (2000)
	Ext1$^{Gt/Gt}$	Embryonic lethality around E 14.5. Short HS chains.	Koziel et al. (2004), Yamada et al. (2004)
	Ext2$^{-/-}$	Embryonic lethality (E 6–7.5). Failure to gastrulate. No HS chains.	Stickens et al. (2005)
	Extl1	No mutants reported	
	Extl2$^{-/-}$	Viable, normal life span, impaired liver regeneration, increased synthesis of GAGs.	Nadanaka et al. (2013a,b)
	Extl3$^{-/-}$	Embryonic lethality (~E 7.5–9). No detectable HS chains.	Takahashi et al. (2009)
	Extl3^{cond}	Conditional mutant — cell specific ablation of *Extl3* in pancreatic islet. Impaired postnatal islet maturation. Reduced HS levels.	Takahashi et al. (2009)
Drosophila melanogaster (fruit fly)	*tout-velu* (*ttv*) (*Ext1*)	Segment polarity phenotype, impaired morphogen distribution. Reduced HS synthesis.	Bellaiche et al. (1998), Bornemann et al. (2004)
	sister of tout-velu (*sotv*) (*Ext2*)	Segment polarity phenotype, impaired morphogen distribution. Reduced HS synthesis.	Bornemann et al. (2004), Han et al. (2004), Takei et al. (2004)
	brother of tout-velu (*botv*) (*Extl3*)	Segment polarity phenotype, impaired morphogen distribution. Reduced HS synthesis.	Han et al. (2004), Takei et al. (2004)
Danio rerio (zebrafish)	*dackel* (*dak*) (*Ext1*)	Partial mutant (due to maternal contribution). Retinal ganglion cell axons missorting in the optic tract, fin defects. Reduced HS levels.	Lee et al. (2004)
	Boxer (*box*) (*Extl3*)	Partial mutant (due to maternal contribution). Retinal ganglion cell axons missorting in the optic tract, fin defects. Reduced HS levels.	Lee et al. (2004)
Caenohabditis elegans (nematode)	*rib-1* (*Ext1*)	Embryonic lethality. Impaired HS synthesis.	Kitagawa et al. (2007)
	rib-2 (*Extl3*)	Embryonic lethality. Impaired HS synthesis.	Morio et al. (2003)

cancer (Wuyts et al., 1997) but so far, there are no reports of *EXTL2* mutations involved in any human disorder. *EXTL3* is located on chromosome 8p21 a frequent target for chromosomal aberrations in breast cancer, is mutated in 25% of early colorectal carcinoma cell lines (Arai et al., 1999) and is epigenetically down-regulated in certain subtypes of colorectal cancers (Karibe et al., 2008), suggesting that *EXTL3* gene alterations may be involved in the pathology of different human carcinomas.

5. Concluding remarks

The requirement of HS for the development of metazoans is undisputable, as seen in *D. melanogaster*, *C. elegans*, *Mus musculus*, *D. rerio* and other model organisms (Bulow and Hobert, 2006). The EXT proteins are conserved between species, and *in vivo* data strongly indicate common roles of EXT orthologs in HS biosynthesis. Yet *in vitro* experiments have produced conflicting data regarding the molecular mechanism of their mode of action. Still little is known about the mechanisms governing HS chain elongation and the interactions of the participating enzymes or to what extent these proteins have other functions. Clearly, more studies are needed, not only to unravel the biology of these molecules but also to understand how mutations in *EXT1* and *EXT2*, but not in the other *EXT* gene-family members, result in hereditary multiple osteochondromas.

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