# Functional characterization of the novel heparan sulfate 6*O*-endosulfatases Sulf1 and Sulf2

**Zur Erlangung des Doktorgrades der Mathematisch - Naturwissenschaftlichen Fakultäten der Georg-August-Universität zu Göttingen**

**vorgelegt von**

**William Christopher Lamanna**

**aus Chapel Hill, North Carolina, USA**

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# 1 Summary

Sulf1 and Sulf2 (the Sulfs) represent a novel sub-group of sulfatase enzymes which differ significantly from other members of the sulfatase family due to their cell surface localization, unique enzyme domain structure and their endolytic heparan sulfate 6*O*-sulfatase activity. The heparan sulfate (HS) editing activity of these enzymes has been shown to regulate the signaling potential of a number of critical growth factors and morphogens such as FGF, BMP and Wnt. Underlining the importance of these enzymes in modulating cell signaling, Sulf activity has been shown to influence FGF2 induced cellular differentiation and angiogenesis in model systems, while the misregulation of the Sulfs has been associated with a variety of cancer types. To understand the *in vivo* importance of these novel enzymes, Sulf1, Sulf2, and Sulf1/2 knock out mice were generated. Phenotypic analysis revealed mild developmental abnormalities in the single knock out mice as opposed to severe ablation and post-natal fatality in the double deficient mice. Despite the importance of the Sulfs in regulating critical cell signaling events and their involvement in multiple aspects of mammalian development, little is known about the Sulfs endogenous substrate specificity, the molecular impact of Sulf deficiency on HS sulfation or how the Sulfs modulate functionally and topologically distinct heparan sulfate proteoglycan (HSPG) species *in vivo*.

Using human fibrosarcoma cell lines expressing RGS-$His_6$ tagged Sulf1 or Sulf2, the sub-cellular localization of the Sulfs was characterized using immunofluorescence microscopy and differential cellular fractionation under different buffer conditions. This analysis revealed a strong association of the Sulfs with the cell surface and demonstrated an electrostatic association of these enzymes with cellular membranes which was shown to be resistant to detergent extraction. Despite the high affinity membrane association of the Sulfs, low level secretion of both full length and proteolytically processed Sulf was observed which may mediate extracellular endosulfatase activity. By enriching active Sulf from the cellular and secreted fractions of Sulf expressing cell lines, the *in vitro* substrate specificity of Sulf1 and Sulf2 was determined using native HS purified from Sulf1/2 deficient cell lines as a substrate. This analysis revealed a restricted affinity of both Sulfs for the highly sulfated heparan sulfate 6S disaccharides UA(2S)-GlcNS(6S) and, to some extent, UA-GlcNS(6S), within the HS chain, while neither Sulf was able to modify the mono-sulfated UA-GlcNAc(6S) disaccharide.

The *in vivo* effect of Sulf loss was characterized by detailed structural and compositional analysis of HS from primary wild-type, Sulf1 or Sulf2 knock out mouse embryonic fibroblasts. This study revealed dynamic effects of the Sulf knock out on non-substrate *N*-, 2*O*- and 6*O*-sulfate groups in addition to changes in their 6S disaccharide substrates, implicating significant effects of Sulf loss on the HS-

biosynthetic machinery. Further, differences between the Sulf1 and Sulf2 knock out HS sulfation patterns, in conjunction with real-time PCR Sulf expression analysis, revealed a differential ability of Sulf1 and Sulf2 to compensate one-another's loss.

The dynamic effects of the Sulf1 or Sulf2 knock out on HS sulfation, in conjunction with the Sulfs unique cell surface localization and low level secretion, posed important questions regarding the ability of these enzymes to modify different heparan sulfate proteoglycan substrates at the cell surface and within the extracellular matrix (ECM). To address these questions, immortalized cell lines from wild-type, Sulf1, Sulf2 and Sulf1/2 knock out MEFs were generated, allowing the characterization of Sulf deficiency on functionally distinct HSPG fractions. These analyses showed that the dynamic changes in *N*-, 2*O*- and 6*O*-sulfate groups associated with Sulf loss occur across multiple proteoglycan fractions at the cell surface and within the ECM. Additionally, comparison of Sulf single and double knock outs revealed cooperative functions of Sulf1 and Sulf2 in modulating the highly sulfated 6S disaccharide substrate UA(2S)-GlcNS(6S) within the HS chain.

The biological impact of the dynamic changes in HS sulfation patterning resulting from Sulf deficiency was analyzed by assaying the sensitivity of primary wild-type and Sulf knock out MEFs to the HS dependent growth factors HGF and FGF2. These studies revealed significantly increased mitogenesis in primary Sulf1 knock out MEFs in response to FGF2 while wild-type and Sulf2 knock out response remained moderate. None of the MEF cell lines responded mitogenically to HGF. In addition to mitogenic response assays, FGF2 activation of the down-stream kinase ERK1/2 was analyzed using immortalized wild-type, Sulf1, Sulf2 and Sulf1/2 knock out MEFs. This quantitative signaling analysis revealed a ~4 fold increase in FGF2 response in the Sulf1 and Sulf2 knock outs and an extensive ~11 fold increased response in the Sulf1/2 double knock out. Importantly, these signaling studies demonstrated that the explicit effects of Sulf loss on FGF signaling correlate significantly with the observed changes in 6*O*-sulfation.

The effect of Sulf deficiency on different cell signaling pathways implicates a dynamic influence of these enzymes on a wide range of cellular processes. To obtain a non-biased overview of the cellular impact of Sulf loss, genome-wide microarray analysis of primary wild-type, Sulf1, Sulf2 and Sulf1/2 knock out MEFs was carried out. Examination of statistically relevant data sets revealed a small group of common differentially expressed genes which may represent yet unexplored targets influenced by Sulf activity. These findings are the basis for future analysis of Sulf function and may provide helpful clues in understanding the role of these enzymes in regulating mammalian development.

# 2 Introduction

## 2.1 The sulfatase family

The sulfatases are a large class of enzymes found in both pro- and eukaryotic organisms which catalyze the hydrolysis of sulfate esters (Hanson *et al*., 2004). Currently 17 sulfatase enzymes have been characterized in humans, ten of which have been described to execute distinct catabolic or regulatory functions in the cell (Table 2.1) (Diez-Roux and Ballabio, 2005). This enzyme class can be divided into two categories based on sub-cellular localization and pH optimum (Diez-Roux and Ballabio, 2005). The acidic pH sulfatases represent the majority of the human sulfatases and are classified by their strict localization to the lysosome where they catabolically degrade sulfated glycolipids and glycosaminoglycans. In contrast, the neutral pH sulfatases represent a small sub-group of sulfatases with alternative localizations in the ER, Golgi or at the cell surface where they are involved in regulating the activity of sulfated substrates such as steroid-sulfates and glycosaminogylcans.

*Table 2.1* **The human sulfatases, their sub-cellular localization, substrate specificity and associated genetic disorders**

| Sulfatase | Physiological substrate | Localization | Associated genetic disorder |
|---|---|---|---|
| *arylsulfatase A* | *cerebroside-3-sulfate* | *lysosomal* | *Metachromatic leukodystrophy* |
| *arylsulfatase B* | *CS, DS* | *lysosomal* | *Maroteaux-Lamy syndrome (MPS VI)* |
| **arylsulfatase C** | **3β-hydroxysteriod-sulfate** | **ER** | **X-linked ichthyosis** |
| **arylsulfatase D** | **unknown** | **ER** | |
| **arylsulfatase E** | **unknown** | **Golgi** | **Chondrodysplasia punctata** |
| arylsulfatase F | unknown | ER | |
| *arylsulfatase G* | *unknown* | *lysosomal* | *unknown* |
| arylsulfatase H | unknown | unknown | |
| arylsulfatase I | unknown | unknown | |
| arylsulfatase J | unknown | unknown | |
| arylsulfatase K | unknown | unknown | |
| *galactosamine-6-sulfatase* | *CS, KS* | *lysosomal* | *Morquio A syndrome (MPS IVA)* |
| *glucosamine-6-sulfatase* | *HS, KS* | *lysosomal* | *Sanfilippo D (MPS IIID)* |
| *sulfamidase* | *HS* | *lysosomal* | *Sanfilippo A (MPS IIIA)* |
| *iduronate-2-sulfatase* | *HS, DS* | *lysosomal* | *Hunter's disease (MPS II)* |
| **sulfatase 1 (Sulf1)** | **HS** | **cell surface** | **unknown** |
| **sulfatase 2 (Sulf2)** | **HS** | **cell surface** | **unknown** |

acidic pH sulfatases (cursive); neutral pH sulfatases (bold); HS: heparan sulfate; DS: dermatan sulfate; CS: chondroitin sulfate; KS: keratan sulfate. (Table adapted from Diez-Roux and Ballabio, 2005)

The biological importance of the sulfatases is underlined by the presence of at least eight monogenic diseases associated with specific sulfatase deficiencies (Hanson *et al*., 2004). Indeed, due to the catabolic function of many of these enzymes, sulfatase deficiencies often result in lysosomal storage disorders, a disease class characterized by the lysosomal accumulation of undegraded metabolites resulting in severe pathological symptoms and often death (Futerman and van Meer, 2004). As seen in table 2.1, many sulfatases still remain to be characterized or associated with human disease, indicating that much remains to be learned about the functionality of this enzyme class and the involvement of sulfation in mammalian development and disease.

The sulfatases represent a conserved family of enzymes which, despite sharing a common reaction mechanism, show distinct substrate specificities for a number of different sulfo-substrates including steroid sulfates, sulfolipids and glycosaminoglycans (Diez-Roux and Ballabio, 2005). Crystal structure comparison shows a high degree of structural similarity between the sulfatases, especially within the catalytic domain (Sardiello *et al*., 2005). Importantly, all sulfatases contain a unique post-translationally generated amino acid residue within their catalytic domain, $C_\alpha$-formylglycine (FGly), which is essential for their catalytic activity (Schmidt *et al*., 1995). The importance of FGly for sulfatase activity is evident in the molecular mechanism of sulfate ester hydrolysis (von Bülow *et al*., 2001; Boltes *et al*., 2001). As seen in figure 2.1, the geminal diol of FGly-hydrate mediates a nucleophilic attack on the sulfate ester substrate, followed by a trans-sulfation/elimination reaction to release the covalently bound sulfate group. FGly is regenerated to FGly-hydrate by the addition of water.

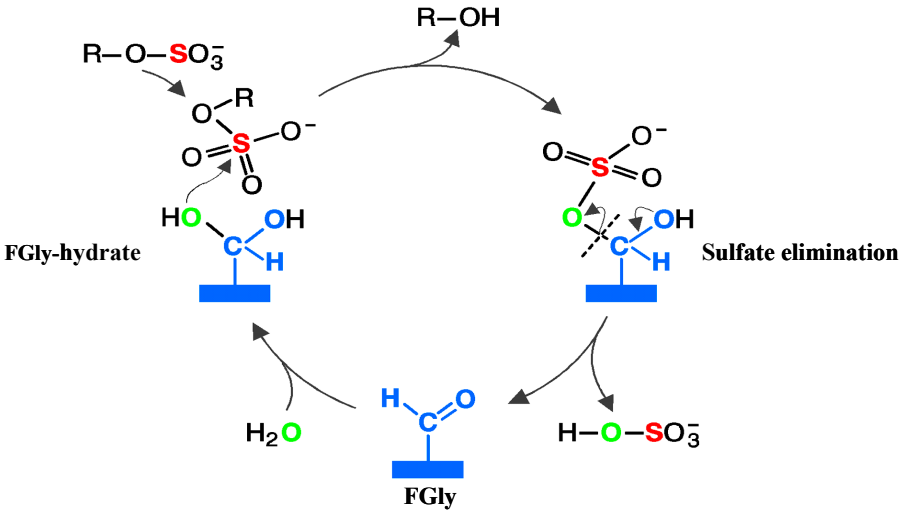


*Figure 2.1* **The Sulfatase trans-sulfation/elimination reaction**

The geminal diol of the sulfatase FGly-hydrate carries out a nucleophilic attack on the sulfate ester resulting in the dissociation of the sulfate from its substrate side group and transient covalent association of the sulfate moiety with the enzyme. The sulfate group is then eliminated by the reconstitution of the FGly carbonyl. Finally, FGly is regenerated to FGly-hydrate by the addition of a water molecule.

In mammals, FGly is generated by the formylglycine generating enzyme (FGE) via post translational oxidation of a critical cysteine residue in the sulfatase active site (von

Figura *et al*., 1998; Dierks *et al*., 2003; Dierks *et al*., 2005). *In vitro* analysis has demonstrated that FGly is generated prior to protein folding in the ER and is dependent on recognition of the minimal sulfatase consensus sequence CXPXR by FGE (Dierks *et al*., 1997; Dierks *et al*., 1999). Deficiency of FGE in humans is the molecular basis of multiple sulfatase deficiency (MSD), a rare but fatal disorder in which all 17 sulfatases are inactive due to a lack of FGly (Dierks *et al*., 2003).

## 2.2 Heparan sulfate proteoglycans

Due to their high sulfate content at multiple sugar positions, glycosaminoglycans (GAGs) compose a major class of substrates for a wide variety of catabolic sulfatases in the lysosome as well as for the regulatory cell surface sulfatases Sulf1 and Sulf2 discussed in this study. GAGs are linear polysaccharides made up of repeating *N*-acetylhexosamine - hexose / hexuronic acid disaccharide units which can be sulfated at different positions and which can extend up to 200 disaccharides in length. Based on differences in sugar composition, GAGs can be classified into four primary families: keratan sulfate (KS), chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS) (Bülow and Hobert, 2006) (Fig. 2.2). At the cell surface and within the extracellular matrix (ECM), these sulfated glycopolymers are found covalently attached to different proteins, forming a functionally diverse family of glycoproteins known as proteoglycans (PGs).

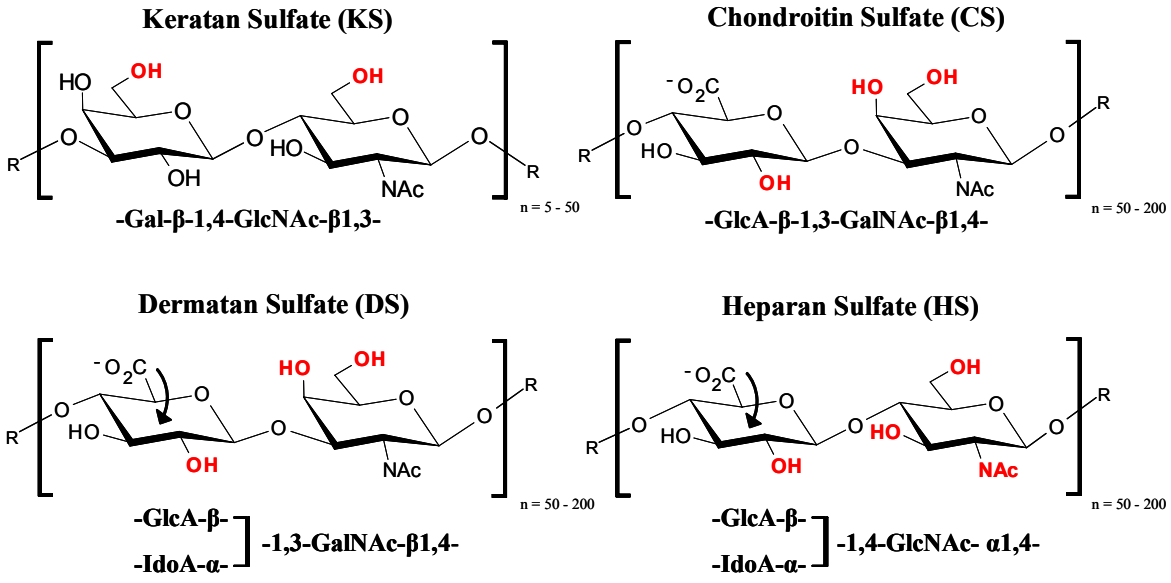


*Figure 2.2* **The disaccharide units of glycosaminoglycans**

Chemical structures of the disaccharide repeat units that make up the different GAG chains keratan sulfate (KS), chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS). Red functional groups indicate moieties which can be modified by the addition of sulfate. Arrow indicates that two C5 epimers are possible, generating GlcA or IdoA depending on the orientation.

The ability of HS to interact with a vast array of growth factors and signaling molecules sets this GAG sub-type apart from the other GAG family members and conveys heparan sulfate proteoglycans (HSPGs) with unique functional properties which are critical for regulating mammalian development (Bernfield *et al*., 1999). HSPGs can be divided into three primary categories based on protein back bone type and sub-cellular localization: the transmembrane syndecans (four isoforms), the glycosyl phosphatidyl inositol (GPI) anchored glypicans (six isoforms) and the ECM associated HSPGs agrin, collagen XVIII and perlecan (Bülow and Hobert, 2006) (Fig. 2.3). These HSPG families play distinct roles in regulating cell signaling, cellular adhesion, morphogen gradients, endocytosis and membrane trafficking (Kirkpatrick and Selleck, 2007).

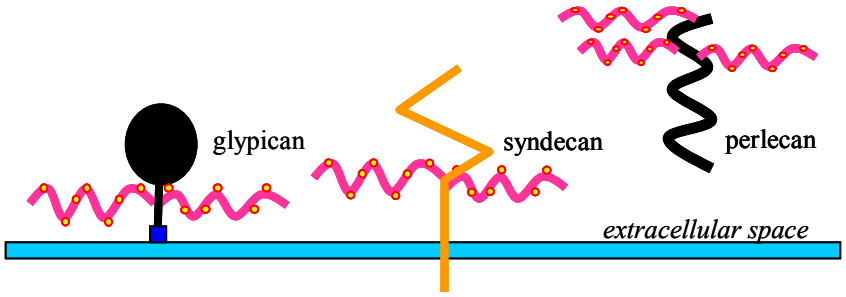


*Figure 2.3* **The heparan sulfate proteoglycan family**
Representation of the three primary heparan sulfate proteoglycan family members: glypican, syndecan and perlecan. Glypicans are attached to the cell membrane by a GPI-anchor while syndecans are transmembrane HSPGs. The ECM is composed of multiple HSPGs including collagen XVIII, agrin and perlecan. For simplicity, only perlecan is shown due to its high abundance and near ubiquitous nature.

### 2.2.1 Heparan sulfate proteoglycan biosynthesis

HSPG biosynthesis is a non-template driven process, relying on multi-enzyme regulation to generate a dynamically sulfated HS polymer attached to a protein core. As displayed in figure 2.4, HS biosynthesis begins in the Golgi with the sequential attachment of four monosaccharides (xylose, two galactose and glucuronate) to a target serine residue of the proteoglycan protein backbone. The addition of the initial *N*-acetylglucosamine (GlcNAc) to this tetrasaccharide linker by the enzyme EXTL3 initiates HS polymer synthesis. Subsequently, alternating glucuronate (GlcA) and GlcNAc residues are added by the co-polymerase enzyme complex EXT1 and EXT2, extending the HS chain up to 200 disaccharide units in length. Once synthesized, the diversity of HS structure is generated by a variety of HS biosynthetic enzymes in the Golgi (Esko and Selleck; 2002). The initial sulfate modification to the nascent HS chain is made by the enzyme *N*-deacetylase/*N*-sulfotransferase (NDST) which can modify up to 50% of the GlcNAc residues to *N*-sulfoglucosamine (GlcNS). Following *N*-sulfation, epimerization by the enzyme *C*-5 epimerase (C5epi) converts a portion of GlcA residues to iduronate (IdoA). Lastly, sulfation reactions at the 2*O*- position of uronic acid (UA) or the 3*O*- and/or 6*O*- position of GlcNAc/GlcNS are carried out by specific