Role of Heparan Sulfate N-sulfation in Mouse Embryonic Development

ANDERS DAGÄLV
Dissertation presented at Uppsala University to be publicly examined in C10:305, BMC, Husaragatan 3, Uppsala, Friday, June 11, 2010 at 13:15 for the degree of Doctor of Philosophy. The examination will be conducted in Swedish.

Abstract

Heparan sulfate (HS) is a sulfated glycosaminoglycan expressed by all cells in the body. It is found at the cell surface and in the extracellular matrix where it binds a large amount of various ligands including growth factors and morphogens. HS is important for building up morphogen gradients during embryonic development and to act as coreceptors for signaling molecules. Many different Golgi enzymes are involved in the biosynthesis of HS. It is known that some of these enzymes interact with each other but not how the whole biosynthesis machinery works or how the cell regulates the structure of the HS that it produces.

In this thesis, cells and mice deficient in two of these biosynthetic enzymes, glucosaminyl N-deacetylase/N-sulfotransferase-1 (NDST1) and the isoform NDST2 have been studied. NDSTs perform the first modifications during biosynthesis where they replace N-acetyl groups on N-acetyl-glucosamine units with sulfate groups. It is known that deficiency of NDST1 is lethal, while lack of NDST2 only results in abnormal connective tissue type mast cells. Here it is shown that deficiency of both NDST1 and NDST2 is embryonically lethal. The embryonic stem (ES) cells extracted from the inner cell mass of double knockout blastocysts show in addition an impaired differentiation capacity compared to wild-type ES cells and fail completely to differentiate into cardiac muscle cells which NDST1−/−, NDST2−/− and wild-type ES cells all do.

Cultured mast cells that lack NDST2 produce heparin that is low-sulfated compared to wild-type HS. To our surprise, we could show that mast cells deficient in NDST1 instead produce a more highly sulfated heparin than wild-type cells. We use a model that predicts that the biosynthesis enzymes work together in a multienzyme complex, the GAGosome, to explain our results. We hypothesize that NDST1 has a higher affinity for the GAGosome than NDST2 which only in the absence of NDST1 gets incorporated into the enzyme complex. When all GAGosomes contain NDST2, a more highly sulfated glycosaminoglycan chain will be synthesized.

A splice variant of NDST1, NDST1S, has also been studied. We could show that NDST1S lacks enzyme activity but that it probably has the capacity to incorporate into GAGosomes. Overexpression of NDST1S results in altered structure of the HS produced by the cells. We speculate that expression of the splice variant during development may be one way to regulate HS structure.

Keywords: heparan sulfate, heparin, proteoglycan, NDST1, NDST2, coreprotein, syndecan

Anders Dagälv, Department of Medical Biochemistry and Microbiology, Box 582, Uppsala University, SE-75123 Uppsala, Sweden

© Anders Dagälv 2010

ISSN 1651-6206
ISBN 978-91-554-7825-4
urn:nbn:se:uu:diva-123474 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-123474)
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I  Dagälv, A., Holmborn, K., Eriksson, I., Ringvall, M., Kjellén, L. Lack of both $N$-deacetylase/$N$-sulfotransferase 1 and 2 causes early embryonic lethality and defective in vitro differentiation of embryonic stem cells. *Manuscript*

II Holmborn, K., Dagälv, A., Kjellén, L., Ábrink, M. Divergent effects of heparan sulfate/heparin biosynthesis enzymes $N$-deacetylase/$N$-sulfotransferase 1 and 2 on mast cell development. *Manuscript*


Reprints were made with permission from the respective publishers.
# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>9</td>
</tr>
<tr>
<td>Glycosaminoglycans and proteoglycans</td>
<td>9</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>9</td>
</tr>
<tr>
<td>Heparin</td>
<td>10</td>
</tr>
<tr>
<td>Biosynthesis of heparan sulfate</td>
<td>10</td>
</tr>
<tr>
<td>GAGosome</td>
<td>11</td>
</tr>
<tr>
<td>Heparan sulfate proteoglycan (HSPG) core proteins</td>
<td>11</td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>13</td>
</tr>
<tr>
<td>Biosynthesis of CS</td>
<td>13</td>
</tr>
<tr>
<td>The N-deacetylase/ N-sulfotransferase enzymes</td>
<td>14</td>
</tr>
<tr>
<td>Mice with genetically modified NDST</td>
<td>14</td>
</tr>
<tr>
<td>Stem cells</td>
<td>15</td>
</tr>
<tr>
<td>Generation of new embryonic stem cells</td>
<td>16</td>
</tr>
<tr>
<td>Embryonic stem cell bodies</td>
<td>16</td>
</tr>
<tr>
<td>Mast cells</td>
<td>17</td>
</tr>
<tr>
<td>Present investigation</td>
<td>21</td>
</tr>
<tr>
<td>Results and discussion paper 1 - Lack of both N-deacetylase/N-sulfotransferase 1 and 2 causes early embryonic lethality and defective \textit{in vitro} differentiation of embryonic stem cells</td>
<td>21</td>
</tr>
<tr>
<td>Results and discussion paper 2 - Divergent effects of heparan sulfate/heparin biosynthesis enzymes N-deacetylase/N-sulfotransferase 1 and 2 on mast cell development</td>
<td>22</td>
</tr>
<tr>
<td>Results and discussion paper 3 - Heparan sulfate biosynthesis:characterization of an NDST1 splice variant</td>
<td>23</td>
</tr>
<tr>
<td>Future perspectives</td>
<td>25</td>
</tr>
<tr>
<td>Populärvetenskaplig sammanfattning</td>
<td>27</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>30</td>
</tr>
<tr>
<td>References</td>
<td>32</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA</td>
<td>Carboxypeptidase A</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>CTMC</td>
<td>Connective tissue type mast cells</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulfate</td>
</tr>
<tr>
<td>ECB</td>
<td>Embryonic stem cell body</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem</td>
</tr>
<tr>
<td>EXT</td>
<td>Exostosin</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GalNAc</td>
<td>$N$-acetylgalactosamine</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>$N$-acetyl-glucosamine</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>IdoA</td>
<td>Iduronic acid</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>MC</td>
<td>Mast cell</td>
</tr>
<tr>
<td>MCP</td>
<td>Mast cell protease</td>
</tr>
<tr>
<td>NDST</td>
<td>$N$-deacetylase/$N$-sulfotransferase</td>
</tr>
<tr>
<td>MMC</td>
<td>Mucosal mast cells</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan</td>
</tr>
</tbody>
</table>
Background

Glycosaminoglycans and proteoglycans

Glycosaminoglycan (GAG) chains are polysaccharides with a backbone of repeating disaccharide units consisting of an N-acetyl-hexosamine combined with a hexose or a hexuronic acid. The backbone units in the completed GAG chains are modified in various ways. Several different types of GAGs exist, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, heparin and hyaluronan. Covalently attached to a core protein, GAG chains form proteoglycans (PG). Hyaluronan is an exception being unmodified and not attached to any core protein. This work will mostly deal with heparan sulfate and chondroitin sulfate.

Heparan sulfate

Heparan sulfate (HS) contains repeating N-acetyl-glucosamine (GlcNAc) and glucuronic acid (GlcA) units that are modified by epimerization of GlcA to iduronic acid (IdoA), N-deacetylation and re-N-sulfation of GlcNAc residues and O-sulfation at various positions. A fully modified disaccharide consists of an N-sulfated glucosamine residue carrying a with 3-O-sulfate and a 6-O-sulfate groups and an IdoA residue which is 2-O-sulfated (Fig. 1A; (Kjellen, 2003). The large number of sulfate groups makes the GAG chain densely negatively charged. Most of the disaccharides in the HS chain are however modified to a lesser extent (Esko and Selleck, 2002). The mature HS chain can be divided into different domains where the N-sulfated disaccharides with modified by O-sulfation and epimerization are present in NS-domains. These are interrupted by unmodified regions so called NA-domains. NA/NS domains with alternating N-sulfated and N-acetylated disaccharides will sometimes surround the NS-domains (Fig. 1B; (Maccarana et al., 1996). The only modification that can take place without an initial N-sulfation is 6-O-sulfation (Holmborn et al., 2004). The HS chains contain binding sites for a very broad range of different molecules from growth factors and anti-thrombin to viruses and cellular adhesion molecules (Lindahl et al., 1998; Gray-Owen, 2003).
Figure 1A, An unmodified HS disaccharide to the left and its fully modified counterpart to the right. The GlcNAc has been converted to a GlcNS residue and the GlcA into an IdoA residue. All potential sulfation sites are occupied. B. The HS chain can be divided into NS, NA and NS/NA-domains.

Heparin

Heparin is a highly sulfated variant of HS with almost all of its sugar residues modified. This results in a very high charge density. Therefore, heparin binds a lot of positively charged substances by electrostatic interaction. Heparin is as far as known only synthesized by mast cells (MC) where it is found attached to serglycin core proteins. In the secretory granules it binds and stabilizes proteases, histamine and other positively charged inflammatory mediators (Pejler et al., 2007). Heparin may also be found in the surroundings of MCs after MC activation leading to granulation. Heparin has long been used in the clinic to prevent blood coagulation and lately also other applications have been suggested. (Lever and Page, 2002).

Biosynthesis of heparan sulfate

The formation of HS starts in ER and continuous in the Golgi compartment. First a tetrasaccharide consisting of one xylose, two galactoses and one GlcA is synthesized. The xylose is added by a xylosyl-transferase, the galactoses by galactosyltransferases 1 and 2, respectively, and finally the GlcA is transferred by glucuronyltransferase I. Then an EXTL enzyme adds a GlcNAc followed by the addition of alternating GlcA and GlcNAc residues by the exostosins (EXT) -1 and -2 enzymes. An average HS chain is about 50 disaccharides long when completed.

As the polymerization continues, the N-deacetylase/N-sulfotransferase (NDST) enzymes start modifying the chain by removing acetyl groups from the GlcNAc residues replacing them with sulfate groups and thus forming GlcNS. These N-sulfated units make the GAG-chain a substrate for other modification enzymes. The next modification is epimerization of GlcA into
iduronic acid by 5′-uronosyl epimerase followed by O-sulfation at C2 of the IdoA residue by the 2-O-sulfotransferase. 6-O-sulfotransferases then transfer sulfate groups to C6 of GlcN units. Finally, 3-O-sulfation which is less abundant, occurs. Also, a few glucosamine residues may be unsubstituted. However, it is not known how these residues are formed. (Esko and Selleck, 2002; Lindahl and Li, 2009).

GAGosome

Little is known about the organization of the biosynthetic enzymes in the Golgi compartment. It has been speculated that the enzymes are assembled into large complexes, GAGosomes, responsible for both elongation and modification of the HS chain (Esko and Selleck, 2002).

In the end of the 90’s it was shown that overexpression of NDST2 in HEK293 cells resulted in a highly sulfated heparin like structure of the HS synthesized, but overexpression of NDST1 did not increase the sulfation of HS to the same extent (Cheung et al., 1996; Pikas et al., 2000).

In 2006 Johan Ledin in our lab suggested that NDST1 is preferred before NDST2 as a GAGosome component (Ledin et al., 2006). Later, Presto et al could show that NDST1 binds to EXT2 (Presto et al., 2008). While more NDST1 protein and enzyme activity was present in EXT2 overexpressing cells, EXT1 expression instead decreased NDST1 levels and activity, probably due to competition between EXT1 and NDST1 for binding to EXT2. It was hypothesized that binding to EXT2 is important for NDST1 to be transported to its site of action in the Golgi. In the absence of EXT2, NDST1 will be degraded. It is not known whether also NDST2 is influenced by the presence of EXT2 and/or EXT1 (Presto et al., 2008).

Other interactions described between enzymes of the HS/heparin biosynthetic machinery include that between EXT1 and EXT2 in the HS-polymerase (McCormick et al., 2000), the interaction between C5-epimerase and 2-O-sulfotransferase (Pinhal et al., 2001) and that between the linkage synthesizing enzymes xylosyltransferase and galactosyltransferase-I (Schwartz et al., 1974), respectively.

Heparan sulfate proteoglycan (HSPG) core proteins

The HS chains are attached to serine residues of the core proteins. These are often found in a Ser-Gly-X-Gly sequence close to acidic and hydrophobic amino acids that make up a proper environment for HS formation (Esko and Zhang, 1996).

Glypicans and syndecans are cell surface HSPGs while perlecan, agrin and collagen XVIII are found in the extracellular matrix (Lindahl et al., 1998; Bernfield et al., 1999). One intracellular HSPG, serglycin, is also
found, mostly in cells of hematopoetic origin (Fig. 2; (Kolset and Tveit, 2008)

The functions of HSPGs at the cell surface are e.g. to act as coreceptors (Fig.3B; (Yayon et al., 1991) and support cell matrix adhesion. They can also act as recognition sites for viruses (Esko and Selleck, 2002). The extracellular matrix PGs make up a reservoir of different growth factors and together with cell surface PGs, the extracellular matrix PGs are important for generating and maintaining morphogenic gradients (Fig. 3B; (Iozzo, 2005).

Figure 2. Syndecans and glypicans can be found on the cell surface. Perlecan and agrin is secreted from the cell and found in the extracellular matrix. Serglycin is the only intracellular heparin PG (modified from (Ringvall, 2004)).

Figure 3A. the HSPG coreceptor binds both to the ligand and to the receptor as shown here for the FGF-2/FGFR complex. B. HS chains contribute to form morphogen gradients. The red cells are secreting a growth factor, which diffuses along the cells and are captured by HS chains stabilizing the gradient.

The **glypican** family of proteoglycans consists of six members (Glypican 1-6) which are attached to the cell surface via GPI-anchors. In all glypicans, the attachment sites for the HS-chains are restricted to the last 50 amino acids in the C-terminal end, locating the HS-chains close to the membrane (Filmus and Selleck, 2001).
Four different syndecans have been described. They have short cytoplasmic tails that can interact with cytoplasmic molecules through their PDZ-domains. The intracellular parts as well as the attachment sites for HS are highly conserved indicating that these regions are the physiologically most important. The same syndecan molecule can carry both chondroitin sulfate (CS) and HS (Couchman, 2003; Tkachenko et al., 2005).

Chondroitin sulfate

CS and dermatan sulfate (DS) are close relatives to HS. They differ from HS in the composition of the carbohydrate backbone where CS and DS contain N-acetylgalactosamine (GalNAc) instead of GlcNAc. In DS some of the GlcA residues have been converted to IdoA, the level of epimerization varying between tissues and cell types. CS/DS are important in many of the processes during development such as cartilage and matrix formation (Prabhakar and Sasisekharan, 2006), brain development (Purushothaman et al., 2007), cell adhesion and anticoagulation (Liaw et al., 2001). PGs carrying CS are the most abundant PGs in the CNS (Schwartz and Domowicz, 2004). CS has attracted less attention than HS and its role during development may have been underestimated (Sugahara et al., 2003).

Biosynthesis of CS

CS biosynthesis starts with the formation of the same tetrasaccharide linkage region as in HS (GlcA-Gal-Gal-Xyl), attached to a serine residue of the core protein. Accordingly, lack of xylosyl transferase or galactosyl transferase-1 results in a failure to initiate synthesis of both HS and CS chains (Esko and Zhang, 1996). The GalNAc I transferase which adds the first GalNAc residue decides that a CS and not a HS chain will be synthesized. This enzyme has also been shown to have polymerization capacity (Sugahara et al., 2003). The chain is extended by the addition of alternating GlcA and GalNAc monosaccharides in the Golgi compartment (Silbert and Sugumaran, 2002).

The galactose residues in the linkage region are to some extent sulfated in CS but never in HS (Sugahara et al., 1988; Ueno et al., 2001). The sulfation of the linkage region galactose residues may be performed by the same sulfotransferases that add sulfate to the GalNAc in the CS chain. This would imply that sulfation of the linkage region is a late event during biosynthesis. If this is the case, it is unlikely that galactose sulfation directs the biosynthesis machinery to synthesize CS rather than HS (Silbert and Sugumaran, 2002). Further sulfation of the CS chain includes 6-O-sulfation and 4-O-sulfation of the GalNAc residues. Some CS chains will also contain 2-O-sulfated GlcA. Epimerization of GlcA into IdoA which occurs during DS biosynthesis is, often accompanied by 4-O-sulfation of the adjacent GalNAc.
residue. The IdoA can be further modified by addition of 2-\(O\)-sulfate groups (Hiraoka et al., 2000; Evers et al., 2001; Kang et al., 2002; Silbert and Sugumaran, 2002).

The \(N\)-deacetylase/ \(N\)-sulfotransferase enzymes.

The NDST enzymes make up a family of four isoforms, NDST1-4. NDST1 and 2 are both found in almost all cells (Kjellen, 2003). NDST 3 is expressed during embryogenesis and is present in the brain, testis and kidney of adult mammals (Pallerla et al., 2008). NDST4 is also present during embryonic development and has been detected in the brain of adult animals (Aikawa et al., 2001).

The initial step in the modification of the HS chain is carried out by the NDSTs and is crucial for the subsequent reactions. Only two of the five possible modifications of the HS chain were found in HS completely devoid of \(N\)-sulfate groups, synthesized by embryonic stem cells lacking NDST1 and NDST2, the formation of \(N\)-unsubstituted glucosamine residues (GlcNH\(_2\)) and 6-\(O\)-sulfation (Holmborn et al., 2004).

The NDSTs are bifunctional enzymes with two catalytically active domains, one catalyzing \(N\)-deacetylation and one \(N\)-sulfation. The specific activity of the \(N\)-deacetylase and the \(N\)-sulfotransferase, respectively, varies between the isoforms (Pikas et al., 2000; Aikawa et al., 2001). An interesting feature of the biosynthesis is that not all of the GlcNAc molecules in the HS chain will be modified. Instead, the HS chain will have a block structure where completely \(N\)-sulfated stretches (NS-domains) will alternate with unmodified regions. The length of the NS domains probably depends both on the total NDST concentration and the relative amount of the different isoforms. Recently, work in our lab could also show that the concentration of the sulfate donor PAPS greatly influences the formation of the NS-domains (Carlsson et al., 2008).

The amount and patterns of modifications of the HS chain is different in different tissues and cell types (Ledin et al., 2004). The modification density and structure also change during differentiation (Johnson et al., 2007). However, a certain cell at a defined time point only appears to produce one kind of HS attached to all HS proteoglycan core proteins that it produces (Zako et al., 2003). This means that in this case a HS chain of a glypican will have the same structure as a HS-chain of a syndecan.

Mice with genetically modified NDST

NDST1, NDST2 and NDST3 deficient mice have previously been generated (Forsberg et al., 1999; Humphries et al., 1999; Fan et al., 2000; Ringvall
et al., 2000; Pallerla et al., 2008). The NDST2\textsuperscript{-/-} mouse is viable, fertile and shows no obvious phenotype other than abnormal connective tissue type mast cells that contain less sulfated heparin than normal MCs (Forsberg et al., 1999; Humphries et al., 1999). A NDST3 deficient mouse line was established by Pallerla et al (Pallerla et al., 2008). These mice develop normally but show regional undersulfation of HS in the brain. However, NDST1/3 knockout embryos showed a more severe phenotype than the NDST1\textsuperscript{-/-} embryos.

NDST1\textsuperscript{-/-} mice have a much more severe phenotype. The prenatal mortality rate is higher and the pups that survive until birth die shortly thereafter due to lung failure. They also show more or less immense skeletal and craniofacial defects, including lack of eyes and the lower jaw (Fig (Ringvall et al., 2000). From other labs it has been reported a wide variety of effects such as ocular defects, neural tube closure malfunction, impaired FGF or FGF-receptor HS binding (Grobe et al., 2005). Lack of certain HS binding growth factors also results in the phenotypes mentioned above (Grobe et al., 2005; Pan et al., 2006; Pallerla et al., 2007).

The phenotypes of the NDST1 and NDST2 deficient mice indicate that NDST 2 is responsible for the N-sulfation of heparin in the mast cells while NDST1 is responsible for the sulfation of HS in all other cells.

Figure 4. An NDST1 deficient mouse embryo to the left and a control embryo (E18.5) to the right. Note the lack of the right eye, the malformed skull and jaw.

Stem cells

Mouse stem cells are attractive tools because they are relatively easy to grow and are cheaper and more ethical to work with than living animals. They were first isolated and maintained in 1981 (Evans and Kaufman, 1981; Martin, 1981). Since pluripotent embryonic stem cells from mice as the only species together with chicken are able to generate most kinds of cells including gametes (Gossler et al., 1986; Pain et al., 1996) they are very useful for developmental studies. For early embryonic lethal mutations like the NDST1/2 double knockout (Holmborn et al., 2004), stem cells are the only option for developmental studies.
A stem cell is defined as a cell that has not taken on the specific properties of a mature and specialized cell. In addition it is able to diverge into a specified cell and to self renew. There are four main types of stem cells, the totipotent, the pluripotent, the multipotent and the unipotent or progenitor cells. The totipotent cell can differentiate into any cell type including the placenta. The fertilized egg and the cells derived from the first divisions are totipotent. Pluripotent cells was first characterized in teratocarcinomas (Stevens, 1970). Pluripotent cells can give rise to all cells but the placenta and the totipotent cells. The embryonic stem (ES) cells are pluripotent. Multipotent cells descend from pluripotent cells and can give rise to specialized cells. The hematopoietic and the neural stem cells are multipotent cells giving rise to blood and neural cells, respectively. Progenitor cells only produce one type of cell. Examples are the erythroid progenitor that differentiates into red blood cells or the satellite cell that is a progenitor of muscle cells.

**Generation of new embryonic stem cells**

ES cells are derived from blastocysts of a fertilized mouse. The blastocyst is made up by an outer layer of cells and the inner cell mass (ICM), containing the embryonic stem cells. The blastocyst will implant 3.5 days after fertilization. However, if the mouse is suckling, hormones are secreted that delay implantation. This is to prevent a new litter to be born too close to the previous one. The blastocyst will during this time grow bigger and in this way contain more cells, which increases the chance of isolating stem cells. To achieve this delay, the mouse is injected during the first days after fertilization with Depo-Provera and Tamoxifen, substances which mimic the “delay” hormones (Evans and Kaufman, 1981; Hunter, 1999).

**Embryonic stem cell bodies**

An embryonic stem cell body (ECB) is a lump of cells used as a model for cell differentiation during embryogenesis. It is possible to push the cells to form different cell types by adding various growth factors to the medium in which the EBCs are grown.

The hanging drop technique is to prefer if the experiment is to be repeated (Wobus et al., 1991). In this technique, the cells are loosened from the surface of the culture dish in which they are grown and placed in 25 µl droplets containing 600-2000 cells on the bottom of a bacteriologic petri dish. The dish is inverted and the drops will end up hanging from the bottom. By the help of gravity and adhesion a round lump of cells will be formed. After two days, the dish is inverted and differentiation medium is added. This medium lacks leukemia inhibition factor (LIF), but contains appropriate growth factors for differentiation into the cell type wanted (Fig. 5). It is also possible to form ECBs by removing LIF from the medium of undifferentiated cells. The
drawback with this method is that the number of cells in the bodies may vary which makes the experiments more difficult to reproduce.

Since embryos lacking both the NDST1 and NDST2 enzymes die shortly after implantation, in vitro differentiation of the corresponding ES cells is the only way to study the impact of these mutations on development. HS synthesized by these cells is devoid of N-sulfate groups but contains a low amount of 6-O-sulfate groups and contain a few N-unsubstituted glucosamine residues (Holmborn et al., 2004).

Figure 5. Formation of ECBs. ES cells are grown in the presence of LIF to avoid differentiation. When the cells have reached an appropriate number, the cells are cultured in drops hanging from the lid in a Petri dish put upside down. 600-2000 cells are placed in each drop and LIF is removed from the medium. After 2 days of incubation in this state, the dish is inverted and the cells will from now on be grown in medium with appropriate growth factors. The bodies may after a few days either be placed in tissue culture chambers or still be held in suspension.

Mast cells

Paul Erlich first described mast cells (MCs) in his thesis published in 1878 “Beiträge zur Theorie und Praxis der histologischen Färbung”. The granules he observed in these cells led him to believe that they nourished the surrounding tissue and named them “mastzellen” ≈ feeder cells. Now we know that the granules Erlich noticed are filled with histamine and other inflammatory mediators, proteases and PGs (Metcalf et al., 1997).
The source of MCs are pluripotent hematopoietic cells in the bone marrow (Kitamura et al., 1977). From the bone marrow they travel through the vascular system to their site of action where they mature into mast cells under the influence of growth factors of which stem cell factor is the most important (Gurish and Boyce, 2002). The main function of MCs is to participate in the defense against pathogens through both the innate and the adaptive immune system. MCs can be activated through different ways, but the most well known are activation by antigen crosslinking of IgE bound to FcεRI on the MCs (Blank and Rivera, 2004). This is also what happens during an allergic shock (Puxeddu et al., 2003).

Figure 6. **Activation of mast cells via IgE antibodies** (picture taken from wikimedia).

When MCs become activated they release a lot of preformed components into their surroundings (Stevens and Austen, 1989). These compounds include the MC specific proteases (MCP) chymases, tryptases and MC-CPA. Chymases and tryptases are serine proteases, chymases possessing chymotrypsin like activity and tryptases trypsin like activity. MC-CPA is a zinc dependent metalloexoprotease, cleaving its targets from the C-terminal end (Pejler et al., 2007). MCs also contain preformed non-MC specific proteases like matrix metalloproteinases and cathepsin C, D, E (Dragonetti et al., 2000; Wolters et al., 2000; Baram et al., 2001; Hennigsson et al., 2005). Since
long it has been known that MCs in addition store large amounts of histamine and later it was shown that their granules also contain preformed cytokines like TNF-α (Gordon and Galli, 1990). Upon activation, MCs start producing and releasing other compounds like cytokines, growth factors, chemokines and free radicals (Metcalf et al., 1997; Galli et al., 2005).

In addition to activation by crosslinking of the FcεR1 receptor, MCs can be activated by other stimuli, including snake venom (Metz 2006). In response to activation, various specific cocktails of mediators will be released depending on which stimuli the MCs received (Galli et al., 2005).

In mouse and rat as well as in humans two major types of MCs can be distinguished. In rodents they are classified as connective tissue type (CTMC) and mucosal MCs (MMC), which in addition can be divided into subtypes (Metcalf et al., 1997). The CTMCs are found in connective tissue, e.g. in the skin, peritoneum and in the submucosa, the connective tissue underlying the mucosa. The MMCs are, as evident from their name mostly found in the mucosa. The CTMCs and the MMCs are morphologically quite similar, the CTMCs being slightly bigger than the MMCs, but the content of their granules are more diverse (Kitamura, 1989; Gurish and Austen, 2001). The MC-specific proteases in CTMCs are of both of the tryptase and chymase type, but MMCs only contain chymases see table 1 (Metcalf et al., 1997). They differ also at PG level where MMCs contain serglycin with highly sulfated chondroitin sulfate chains whereas CTMCs contain heparin instead (Kolset et al., 2004).

Table 1. MC proteases

<table>
<thead>
<tr>
<th></th>
<th>Tryptase</th>
<th>Chymase</th>
<th>MC-CPA</th>
<th>Serglycin side chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCTMC</td>
<td>mMCP-6, mMCP-7</td>
<td>mMCP-4, mMCP-5</td>
<td>Yes</td>
<td>Heparin</td>
</tr>
<tr>
<td>mMMC</td>
<td>mC-1, mC-2</td>
<td></td>
<td>No</td>
<td>CS</td>
</tr>
<tr>
<td>hMC_T</td>
<td>α, β</td>
<td>α</td>
<td>Yes</td>
<td>Heparin: CS ratio 1:1</td>
</tr>
<tr>
<td>hMC_T</td>
<td>α, β</td>
<td></td>
<td>No</td>
<td>CS</td>
</tr>
</tbody>
</table>

Mast cell contents of mouse MCs (mCTMC and mMMC) and human mast cells (hMC_TC and hMC_T).

In humans the two types of are named after the contents of proteases instead of their location. MC_Ts contain only tryptases whereas MC_TC contain both tryptases and chymase (Metcalf et al., 1997). In humans CS is synthesized by both types MCs but the MC_TC also contain heparin in a 1:1 ratio to CS (Pejler et al., 2007).

The protease content of the MCs is very high, amounting to up to 16μg proteases/ 10^6 cells (Schwartz et al., 1987). The release of this huge amount of mediators will affect the organism and can lead to anaphylactic shock. To learn more about how the MCs evolve, stem cells from bone marrow of
mice have been cultured under the influence of different growth factors that are known to be important for MC maturation. Through these studies, SCF has been shown to be important for MC maturation. IL-3 is often used in the cultures, but MCs differentiated in the presence of IL-3 have been shown to lack mMCP-4, a marker for terminally differentiated mCTMC (Gurish et al., 1992). The presence of IL-3 will on the other hand result in high expression of mMCP-5 and CPA (Pejler et al., 2007). Activated CPA is known to be important for the formation of mMCP-5 and in the absence of heparin, CPA will not be activated. Instead, the cells will be arrested in their undifferentiated state and no mMCP-5 will be formed (Forsberg et al., 1999). In vitro studies have also demonstrated that different interleukins, expressed at various maturation spots, will direct the MC into specific subpopulations with varying granule contents (Pejler et al., 2007). The mMCPs and CPA are stored in activated forms and can act as soon as they are released. More commonly, proteases are stored as zymogens in cells (Galli et al., 2005). The heparin and CS chains attached to serglycin in the granules have been thought to be important for directing the MC proteases into the granules. However, this has been questioned lately since the MC proteases seem to be guided into the granules also in serglycin defective MCs (Henningsson et al., 2006).

When released, the tryptases will diffuse away from the heparin because of the higher pH outside of the cells than in the slightly acidic granules. Histidines in the proteases will no longer be protonated and therefore the proteases will lose their affinity for heparin. However, the tryptases will reside in tetrameric complexes. Chymases will remain attached to heparin/CS also after degranulation and form complexes with their substrates which also bind to the GAG chain and is presented for the chymase in this way. Proteins without affinity for the PGs will escape degradation since they are not presented for the chymases (Pejler et al., 2007).
Present investigation

Results and discussion paper 1 - Lack of both \( N \)-deacetylase/\( N \)-sulfotransferase 1 and 2 causes early embryonic lethality and defective \textit{in vitro} differentiation of embryonic stem cells.

Most NDST1 deficient embryos will make it until birth, but newborn pups will die due to immature lungs. They also suffer from other severe developmental defects like undeveloped jaws, missing right eyes and skull malformations (Ringvall et al., 2000).

NDST2 mice are viable and fertile and show no obvious defects, but when the mast cells were analyzed it became apparent that no sulfated heparin was produced and the protease content was altered (Forsberg et al., 1999; Humphries et al., 1999).

To study how lack of both NDST1 and NDST2 would affect development we generated NDST1\(^{-/-}\) NDST2\(^{-/-}\) mice. These mice show the same phenotype as the NDST2\(^{-/-}\) mice and were fertile. Therefore they could be used to generate NDST1/2\(^{-/-}\) embryos. However, genotyping of embryos demonstrated that no double knockout embryos were recovered after E5.5. The E5.5 embryos also showed a greatly disturbed morphology.

When blastocysts with the NDST1\(^{-/-}\)2\(^{-/-}\) genotype were grown in tissue culture, they were not able to expand their ICM, indicating a greatly disturbed differentiation capacity. This was confirmed when expression patterns of markers for the three germ layers were studied in ES cell cultures.

In addition to the NDST1/2\(^{-/-}\) ES cells we have also isolated ES cells from NDST1 and NDST2 single knockouts. When the HS composition was evaluated in these cells, we found that HS from NDST1\(^{-/-}\) ES cells had a lower N-sulfation degree, while NDST2\(^{-/-}\) ES cells produced a HS with almost normal sulfation. The NDST1/2\(^{-/-}\) ES cells synthesize HS that completely lacks N-sulfation, but where some GlcNAc residues are 6-O-sulfated.

The \( N \)-sulfotransferase activity was also measured and the levels were strikingly lower in NDST1\(^{-/-}\) ES cells, but almost normal in the cells lacking NDST2. After differentiation for 8 days the \( N \)-sulfotransferase activity had increased in all ES cells, except for the NDST1/2 knockouts where no enzyme activity was detected. The numbers of \( N \)-sulfate groups also increased during differentiation in all ES cells except for the NDST1/2 knockouts.
Judging from the results obtained for \( N \)-sulfotransferase activity as well as mRNA expression of the different NDST isoforms we could conclude that the loss of one isoform did not lead to increased expression of another.

The cardiac differentiation pathway was analyzed in the different cell types. We found that all ES cells except the NDST1/2 double knockouts were able to differentiate into cardiomyocytes. To our surprise, differentiation in cells lacking NDST2 was somewhat delayed. Previous investigations have shown that lack of either EXT1 or EXT2, the two HS polymerization enzymes will result in early embryonic lethality (Lin et al., 2000; Stickens et al., 2005).

However, the NDST1/2\(^{-/-}\) phenotype is even more severe since the embryos die at an earlier stage of development. Maybe, the HS synthesized in the early NDST1/2\(^{-/-}\) embryo will interact with factors that will prevent the cells from differentiating.

Results and discussion paper 2 - Divergent effects of heparan sulfate/heparin biosynthesis enzymes \( N \)-deacetylase/\( N \)-sulfotransferase 1 and 2 on mast cell development.

MCs derived from the peritoneal cavity of NDST2\(^{-/-}\) mice synthesize a low sulfated heparin compared to peritoneal MCs from wild type mice (Forsberg et al., 1999; Humphries et al., 1999). The NDST2 deficient MCs also contain reduced levels of mMCP-6, lack mMCP-5 and active CPA.

Deficiency in the other important NDST isoform, NDST1 is lethal (Fan et al., 2000; Ringvall et al., 2000), but the importance of NDST1 for MC development had not been investigated. We also wanted to know if deficiency in both NDST1 and NDST2 was compatible with MC development.

We used three differentiation protocols. In order to test the combined NDST1 and NDST2 deficiency, ES cells was the only option. To study differentiation of NDST1\(^{-/-}\) MCs, embryo-derived MCs were studied. For studies of NDST2\(^{-/-}\) MCs we could culture peritoneal cells from adult mice.

When wild-type and NDST1/2\(^{-/-}\) ES-cells were cultured in parallel in the presence of differentiation medium, the wild-type cells formed MCs containing heparin as well as MC specific proteases. In contrast, no MCs were formed in the NDST1/2\(^{-/-}\) cultures. The inability of these cells to differentiate may reflect a general defect in mesoderm differentiation.

When embryo derived MCs lacking NDST1 where characterized they were found to produce a heparin with an increased negative net charge compared to heparin from wild-type cultures. Also the amount of all analyzed MC proteases was increased. This was also the case for MCs with the NDST1\(^{+/-}\) genotype. NDST2\(^{-/-}\) embryo-derived MCs instead resembled MCs previously characterized from NDST2\(^{-/-}\) adult mice. The cells had an altered morphology, synthesized low sulfated heparin and lacked CPA as well as
mMCP-5. NDST2\textsuperscript{−} MCs from the peritoneal cavity and cultured \textit{in vitro}, also showed a similar phenotype.

The qPCR data show that MCs express very high levels of NDST2 compared to undifferentiated ES cells and whole embryo at E11.5. NDST1 on the other hand has a lower expression in MCs than in whole embryo.

How then can the surprising finding of “a better” MC without NDST1 be explained? We think that the GAGosome model can be used for this purpose. The “frozen out” model was previously presented in Ledin et al 2006. There it is predicted that when NDST1 and NDST2 are both present, NDST1 will be preferred as GAGosome component. We also know that overexpression of NDST2 in HEK293 cells results in a more highly sulfated heparin-like HS chain than overexpression of NDST1 (Pikas et al., 2000). Therefore, GAGosomes containing only NDST2 will synthesize a more highly sulfated heparin then a mixture of NDST1- and NDST2-containing GAGosomes.

Results and discussion paper 3 - Heparan sulfate biosynthesis: characterization of an NDST1 splice variant

One alternatively spliced enzyme in the HS biosynthesis machinery is known since before, the 6-O-sulfotransferase-2 where the two forms both have been shown to be functional (Habuchi et al., 2003). The NDST1 splice variant characterized here, NDST1S, is approximately half the size of the original enzyme. It has no N-sulfotransferase activity since the C-terminal end containing the N-sulfotransferase activity is missing and it also appears to lack N-deacetylase activity. This was concluded after experiments in cell lines overexpressing His-tagged NDST1 (NDST1H) with or without NDST1S. After affinity chromatography on a Talon resin to capture NDST1H, the binding and non-binding fractions as well as the cell extracts were analyzed for content of NDST1H, NDST1S as well as N-deacetylase activity. It was apparent that the presence of NDST1S did not influence enzyme activity. Interestingly, NDST1S was present in the eluate from the Talon resin indicating that NDST1S either directly or indirectly associates with NDST1H.

When the structure of HS produced by NDST1S overexpressing cells was analyzed, it was shown that 6-O-sulfation was increased while N-sulfation was unaltered. We could also show that NDST1S showed affinity for EXT2, tentatively suggesting that it may take place in the GAGosome. Why this affects 6-O-sulfation, but not N-sulfation is less clear.

If NDST1S, a catalytically dead protein, would have a role to play we hypothesized that this would be during embryonic development, when rapid changes of HS structure would be important. We therefore investigated expression of NDST1S from E10 until E18. Preliminary results demonstrated that NDST1S was upregulated at E12 and E18.
Further work is obviously needed to investigate the physiological role of NDST1S.
Future perspectives

The generation of mice deficient in HS biosynthetic enzymes has made it possible to evaluate their importance during development *in vivo*. Sometimes, the deficiency leads to embryonic lethality, in these cases the knowledge of how to extract ES cells has been very useful to be able to further investigate the mechanisms of these enzymes. Some work has also been performed in order to understand how the enzymes cooperate and depend on each other, but more interesting work is yet to be done to straighten this out.

ES cells only expressing NDST2 produce a HS much less sulfated than wild-type ES cells, yet they are capable to differentiate into cardiomyocytes *in vitro*. The nonsulfated HS produced in the NDST1/2−/− ES cells on the other hand does not fulfill the requirements for cardiomyocyte differentiation *in vitro*. This indicates that it is not the fine structure of HS that is important for the growth factors that control cardiomyocyte differentiation, rather that the HS chains have a sufficient degree of sulfation. Is this also true for other differentiation pathways, and are some more growth factors more discriminating?

The hypothetic GAGosome is an interesting model to explain how the enzymes in the HS biosynthesis machinery cooperate and influence each other. However, a lot more work needs be done to further evaluate its existence in the cell. Questions like if there is one big complex involving all enzymes or small scattered complexes have to be answered. Are NDST1 and NDST2 competing for the same binding sites on other enzymes or maybe on the substrate or is some other mechanism more important for their regulation? NDST2 may not be used at all in HS biosynthesis in cells as long as NDST1 is present in fair amounts, but as soon as NDST2 reaches a threshold level it might have the chance to be incorporated into the GAGosome. This is supported by the fact that HS in NDST2−/− ES cells has the same N-sulfation profile as HS in wild-type cells, but when the expression of NDST2 increases such as in MCs and in NDST2 overexpressing cells, N-sulfation will increase.

We assume that the NDST1 splice variant has a function since it is translated into a protein, but what is the function? Is it simply expressed to steal binding sites for the functional NDST1 or is it there to regulate the fine structure in a more specific way? The increase in HS 6-O-sulfation in cells overexpressing NDST1S may indicate that this is the case.
At present we are performing experiments on another interesting and well-debated issue in the field, whether a cell, at a specified time point, can produce HS chains with different structure and modifications, or if all HS chains produced are overall homogenous. To answer this question we have decided to use MCs, since we know that they produce highly sulfated heparin stored intracellularly. There are also old unpublished results that indicate that the MCs synthesize at least one cell surface PG. In order to separate the surface proteoglycans from the cell we carefully trypsinize the cells to recover surface PGs and then lyse the cells to gain access to the intracellular PGs.

Ion exchange chromatography after removal of CS showed the cell surface heparin/HS chains behaved identically as the heparin chains recovered from serglycin inside the cells. This was also the case for heparin/HS recovered from NDT52-/- MCs; Cell surface as well as serglycin-derived polysaccharide chains had a lower but identical charge density.

We will now characterize the cell surface PG more closely and identify its core protein. It will also be interesting to determine the physiological role of cell surface heparin.
Populärvetenskaplig sammanfattning


Enzymerna som fäster sulfatgrupperna på HS-kedjan gör det i olika mönster som är viktiga för att en del av signalmolekylerna ska binda ordentligt. För andra molekyler räcker det att HS är tillräckligt negativt laddad. Mastcellernas heparin har en betydligt starkare laddning än de andra cellernas HS för att heparin ska kunna binda en stor mängd positivt laddade enzymer som lagras i mastcellerna.

Det jag studerat i den här avhandlingen är ett enzym som kallas N-deacetylas/N-sulfotransferas (NDST). Detta enzym sätter fast de första sulfatgrupperna på sockermolekylerna i den nyligen producerade kedjan. NDST-enzymet jobbar utefter kedjan där den först plockar bort några acetylgrupper och sätter fast sulfatgrupper där acetylgrupperna suttit, därefter hoppar det över några acetylgrupper för att sedan börja arbeta igen. På grund av att de efterföljande enzymerna i stort sett enbart kan fungera där NDST redan modifierat HS-kedjan kommer det att bildas negativt laddade mönster.

Det finns fyra olika former av NDST-enzymet som har döpts till NDST1-NDST4. De två viktigaste är NDST1 och NDST2. NDST1 har visats vara det som gör störst insats i alla celler förutom mast celler där NDST2 är viktigast. Detta har man kunnat visa genom att ta bort antingen NDST1 eller NDST2 från möss. Dessa möss saknar alltså det ena eller det andra enzymet. Möss som saknar NDST1 dör vid födseln, medan möss utan NDST2 ”bara” får onormal sulfatering av sitt heparin i mastcellerna.

I den första studien har vi visat att inget musembryo kan bildas om både NDST1 och NDST2 saknas.
Embryonala stamceller (ES) är celler som hör till de första cellerna i det nybildade embryot och som sedan ger upphov till alla celler som finns i den vuxna individen. För att enklare kunna studera hur frånvaron av NDST1 eller NDST2 påverkar utformningen av HS-kedjan och hur celler kan ta emot signaler via den har jag tagit fram ES som antingen saknar NDST1 eller NDST2. Sedan tidigare finns även ES som saknar båda enzymen.

I första studien visar vi att HS i ES som saknar NDST2 ser normalt ut, medan HS från ES som saknar NDST1 har färre sulfatgrupper. De ES som saknar båda enzymen har bara några få 6-O-sulfatgrupper som inte satts dit av NDST enzym. När vi sedan försöker förmå dessa celler att bilda hjärt-muskcelceller visar det sig att de ES som saknar ett av enzymen har den förmågan, medan de ES som saknar båda enzymen inte har den förmågan.

I vår andra studie har vi undersökt vad som händer i mastcellerna när man manipulerar med NDST uttrycket. Sedan tidigare är det känt att mastceller som saknar NDST2 har ett lågsulfaterat heparin som mer liknar HS. Det vi nu ville veta var hur avsaknaden av NDST1 påverkar heparinet i mastcellerna. Till vår förvåning såg vi att heparinet i dessa mastceller har en starkare laddning och dessutom binder de mer av de positivt laddade enzymerna som lagras i mastceller.

I vår sista studie har vi undersökt en kortare variant av NDST1 proteinet, som kallas NDST1S. Enligt våra resultat har NDST1S ingen enzymaktivitet, men det påverkar mängden av NDST1 enzym. Detta har vi sett genom att först modifiera celler att överproducera NDST1 och sedan modifiera hälften av dessa celler en gång till så att de även överproducerar NDST1S. När vi sedan jämför dessa celler kan vi se att de som överproducerar både NDST1 och NDST1S har lägre nivåer av NDST1 än de som bara överproducerar NDST1.

Vi förklarar våra resultat med att sulfateringen av HS och heparin sköts av ett komplex med enzym, med hjälp av vilket cellen kan sköta finjusteringar som behövs för att få rätt mängd sulfatgrupper på HS-kedjorna. I ES återfinns i stort sett bara NDST1 i detta komplex, därför ser HS i de celler som saknar NDST2 normalt ut. Plockar vi däremot bort NDST1 fungerar inte komplexet längre och det HS som bildas blir mindre sulfaterat än normalt, förmodligen för att NDST2 binder dåligt till komplexet.

I mastceller är koncentrationen av NDST2 så hög att NDST2 kommer att ockupera de flesta platserna i komplexet, trots sin dåliga förmåga att binda till det. Eftersom NDST2 är bättre på att sulfatera när det väl får vara med gör mastcellerna heparin. I studie två visade vi att om vi tar bort det NDST1 som mast cellerna trots allt innehåller kommer mastcellerna att producera heparin med ännu fler sulfatgrupper på. Vi tror att detta kommer sig av att det lilla NDST1 som finns i vanliga mastceller blockerar en del platser i komplexet och därmed minskar komplexets förmåga att sulfatera.

När det korta inaktiva NDST1S binder till detta komplex tar det upp platser som normalt används av NDST1. När dessa platser blir upptagna av

28
NDST1S förstörs de NDST1 som inte lyckats binda och då minskar den aktivitet som NDST1 kan utföra. På så vis kan cellen reglera mängden NDST1 på ett effektivt sätt.

Dessa tre studier tillsammans med tidigare upptäckter som inte beskrivits här stödjer teorin om att enzym som ingår i biosyntesen av HS/heparin sam- arbetar och påverkar varandra på olika sätt för att biosyntesen ska fungera på rätt sätt.
Acknowledgement

All the people taking part in the making of this thesis deserve my thankfulness.
I would especially like to thank the following people:

Lena, min handledare, som alltid hjälpt mig så mycket med mitt skrivande vid alla möjliga och omöjliga tidpunkter, för all hjälp och handledning och för stöd när resultaten varit omöjliga att upprepa. För att du alltid är så positiv även när det känns hopplöst.

Magnus, för all hjälp med mina mastceller och alla frågor jag haft runt dessa.

Maud och Karin, mina biträdande handledare för att ni hjälpt mig under den här tiden och Maud även för all hjälp och kunskap jag fått med och om mina ES celler.

Dorothe, min examinator, som hjälpt mig med forskningen och en hel del metoder.

Lenas group, Pernilla, Jenny, Katta, Beata, Audrey, Anh-Tri, Maria R, Mehdi, Håkan, Johan, Inger. You all make up a very nice and friendly environment to work in. Katta och Maria R för att ni lärde mig jobba med ES celler samt mössen. Inger för all hjälp och allt stöd jag fått under dessa år. Utan dig hade det varit mycket svårare att genomföra det här. Anh-Tri För alla timmar i gymmet och för ditt stöd när det varit jobbigt på olika sätt, särskilt nu på slutet.

Jimmy, för allt kul vi haft genom åren, hjälp med bilen och på labbet. Och för att du äntligen lät mig vinna förra året.

Birgitta och Anne, mina rumskompisar de första åren av min doktorandtid.

Imbims administration för all hjälp med lönen, stipendier, inlämningar, datorproblem och så vidare…

The Blodomlopp guys. For making blodomloppet to one of the big events during the year.
Everyone in the corridor, so many nice people have been around through these years.
All other IMBIM colleagues.

Micke som jag känt hela min doktorandtid och haft våra intressanta tvärvetenskapliga diskussioner.
alla mina innebandy och fotbollskompisar som jag har i IBS Nabla och Torpedo kamrat. Nabla, om två år spelar vi div. V!

Henrik, Erik och Linus för allt roligt vi haft under studietiden.

Johan, min gamla lägenhetskompis, du kommer alltid vara en del av familjen ;-) 

Anna, Jonas och lille Moses, vi får hoppas på fint väder till fisketuren i Venjan i sommar.

Finn och Soli, för ert intresse, jag har svarat så gott jag kan. Efter det här ska det bli skönt att få vila ut på Lund.

Moster Maja, du är alltid glad och positiv. Vi ses i sommar.

Övrig släkt och vänner, ingen är glömd.

Min bror Gunnar och Anna, för allt kul vi haft genom alla år, innebandy matcherna på 35BKs hemmaplan minns jag.

Mamma och Pappa, för allt ni gett mig och för att ni alltid finns där att prata med när det är jobbigt. Och för att Sörgården finns.

Lisa, för ditt stöd, din kärlek och för att du alltid finns där och tror på att jag klarar av allt trots mina egna tvivel. Tack så mycket.


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 569

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)