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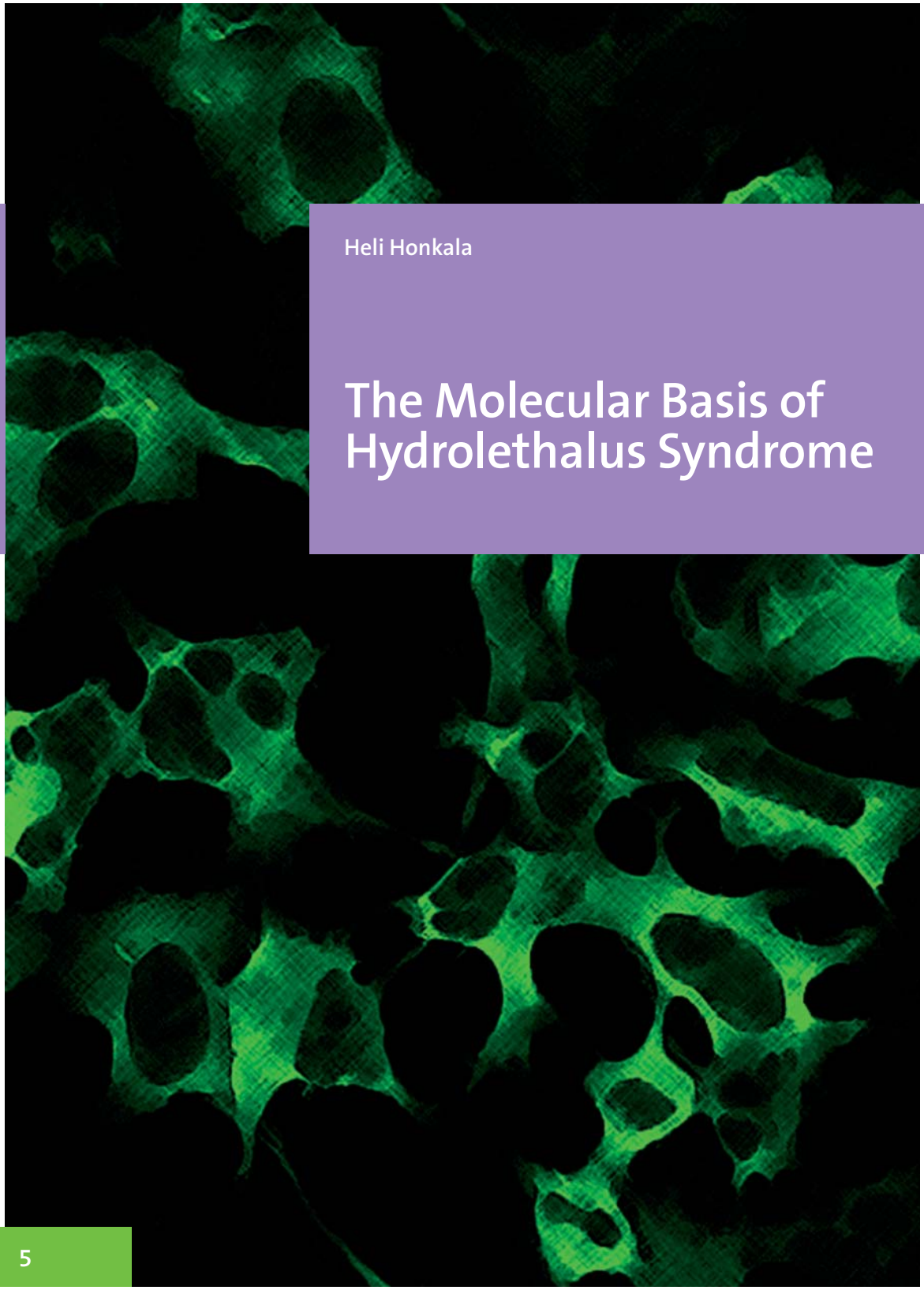

RESEARCH

Heli Honkala

The Molecular Basis of Hydrolethalus Syndrome

Heli Honkala

THE MOLECULAR BASIS OF HYDROLETHALUS SYNDROME

A C A D E M I C D I S S E R T A T I O N

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“Don't fear change – embrace it.”

Anthony J. D'Angelo

To my family

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ABSTRACT

Hydrolethalus syndrome (HLS) is a severe fetal malformation syndrome that is inherited by an autosomal recessive manner. HLS belongs to the Finnish disease heritage, an entity of rare diseases that are more prevalent in Finland than in other parts of the world. The phenotypic spectrum of the syndrome is wide and it is characterized by several developmental abnormalities, including hydrocephalus and absent midline structures in the brain, abnormal lobation of the lungs, polydactyly as well as micrognathia and other craniofacial anomalies. Polyhydramnios are relatively frequent during pregnancy. HLS can nowadays be effectively identified by ultrasound scan already at the end of the first trimester of pregnancy. Usually, pregnancy is terminated due to the severe developmental defects of the fetus.

One of the main goals in this thesis was to identify and characterize the gene defect underlying HLS. The defect, an A to G point mutation, was found from a previously unknown gene that was named *HYLS1*. Since *HYLS1* was an unknown gene with no relatives in the known gene families, many functional studies were performed in order to unravel the function of the gene and of the protein it codes for. Studies with overexpression cell models showed that the subcellular localization of the HYLS1 protein was different when the wild type and its mutant forms were compared. Wild type protein was mainly localized diffusely in the cytoplasm whereas the mutant form was observed largely in the nucleus in punctate nuclear inclusions. The wild type form was seen to be completely localized in the nucleus when the function of the putative nuclear export signal in HYLS1 was blocked. In addition, HYLS1 was shown to possess transactivation potential which was significantly diminished in the mutant form. When studying possible differences in gene expression in fibroblast cells obtained from HLS cases and healthy controls using the microarray method, several genes belonging to many different cellular pathways (e.g. lipid metabolism, cell cycle regulation, signal transduction) were seen to be differentially expressed. Studies done with neuronal progenitor cells revealed that HLS cells had a higher proliferation rate and a lower apoptosis rate than control cells. Essential novel information was also gathered from studies performed at the tissue level. For example, cholesterol level was shown to be significantly elevated in HLS liver samples.

Neuropathological findings of mutation confirmed HLS cases were described for the first time in detail in this study. In addition to neuropathological features described earlier, such as absent midline structures and a keyhole-like opening in the base of the skull, new analyses revealed for example hypothalamic hamartoma as a frequent finding. Microscopically, many developmental defects of the central nervous system were found, these including cortical dysplasia, rosette structures in the cerebral cortex and irregular radial glial cell structures. Also, general pathological findings were described which showed an interestingly wide variation in the HLS phenotype.

At the beginning of this thesis work, HYLS1 was an unknown gene with an unknown function. Lots of new information about the function of the gene and the protein was obtained in this study although the precise function of HYLS1 remains to be elucidated. HYLS1 most likely participates in transcriptional regulation and also in the regulation of cholesterol metabolism and the function of HYLS1 is critical for normal fetal development. Identification of the gene defect made it possible to confirm the HLS diagnosis genetically, an aspect that provides valuable information for the families in which a fetus is suspected to have HLS. Studying developmental malformation syndromes such as HLS offers essential new information also for the understanding of molecular and cellular events required for normal fetal development.

Keywords: hydrolethalus syndrome, HYLS1, fetal development, developmental disorder, central nervous system

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TIIVISTELMÄ

Hydroletalus-oireyhtymä (HLS) on peittyvästi periytyvä, suomalaiseen tauti-perintöön kuuluva vakava oireyhtymä, joka ilmenee jo sikiönkehityksen aikana ja johtaa kuolemaan viimeistään ensimmäisten elinpäivien aikana. Oireyhtymän ilmiasun kirjo on laaja ja tyypillisiä piirteitä on useita, näistä yleisimpinä keskushermoston epänormaalit rakenteet kuten vesipäisyys ja aivojen keskiviivan rakenteiden puutos, keuhkojen epänormaali lohkojako, polydaktylia eli ylimääräiset sormet ja varpaat sekä pienileukaisuus ja muut kasvojen rakenteiden poikkeavuudet. Lapsiveden määrä loppuraskaudessa on usein moninkertainen normaaliin raskauteen verrattuna. Nykyään HLS pystytään tunnistamaan luotettavasti ultraäänitutkimuksen avulla jopa jo ensimmäisen raskauskolmanneksen lopulla ja useimmiten päädytään raskauden keskeytykseen sikiön vakavien kehityshäiriöiden vuoksi.

Tämän tutkimuksen yhtenä päätavoitteena oli tunnistaa ja karakterisoida geeni, jonka mutaatio johtaa hydroletalus-oireyhtymän syntyyn. Geenivirhe, A-G -pistemutaatio, löydettiin aiemmin tuntemattomasta geenistä, jolle annettiin nimi *HYLS1* oireyhtymän mukaan. *HYLS1* oli aiemmin tuntematon geeni eikä se kuulunut mihinkään aiemmin tunnettuun geeniperheeseen, joten väitöstyössä tehtiin monia toiminnallisia tutkimuksia geenin ja sen koodaaman proteiinin toiminnan selvittämiseksi. Solutason tutkimuksissa saatiin selville, että HYLS1-proteiinin normaalimuodon ja mutanttimuodon solunsisäinen sijainti on erilainen yliekspressiomallissa. Normaali proteiini sijaitsi lähinnä diffuusisti solulimassa, kun taas mutanttimuotoa havaittiin laajalti tumassa pistemäisinä rakenteina. Normaalin HYLS1-proteiinin nähtiin sijoittuvan tumaan silloin, kun sen mahdollisen tumastavientisignaalin toiminta estettiin. Lisäksi HYLS1:lla havaittiin olevan transaktivaatio-ominaisuus, joka oli merkittävästi pienentynyt silloin, kun proteiini on mutaation seurauksena viallinen. Kun HLS- ja kontrollisikiöiden ihon fibroblastisolujen geenieksprессiota tutkittiin mikrosirumenetelmän avulla, ilmentymiseroja nähtiin moneen eri signaalireittiin (mm. lipidimetabolia, solusyklin säätely, signaalitransduktio) kuuluvissa geeneissä. Solutason jatkotutkimuksissa HLS-tapausten hermoston prekursorisolujen havaittiin jakaantuvan kontrollisoluja nopeammin, kun taas niiden apoptoosivauhti oli hidastunut. Näiden tulosten lisäksi myös kudostasolla tehdyt tutkimukset toivat arvokasta tietoa. Esimerkiksi maksan

kolesterolipitoisuuden havaittiin olevan HLS-tapauksilla huomattavasti kohonnut kontrollitapauksiin verrattuna.

Tämän tutkimuksen aikana kuvattiin myös ensimmäistä kertaa yksityiskohtaiset neuropatologiset löydökset HLS-tapauksista, joiden *HYLS1*-mutaatio oli varmennettu. Makroskooppisissa tutkimuksissa aiemmin kuvattujen ja raportoitujen löydösten (mm. keskiviivan rakenteiden puutos aivoissa ja HLS:lle tyypillinen avaimenreikädefekti kallonpohjassa) lisäksi yleisenä löydöksenä kuvattiin nyt myös mm. hypotalaminen hamartooma. Mikroskooppisessa tarkastelussa löydettiin monia keskushermoston kehityshäiriöitä, kuten vaillinaisesti kehittynyt aivokuori, rosettirakenteet aivokuoressa sekä epänormaalisti järjestyneet radiaaligliasolut. Neuropatologisten löydösten lisäksi kuvattiin myös yleiset patologiset löydökset yksityiskohtaisesti. Löydösten perusteella voitiin todeta, että HLS:n ilmiasu vaihtelee suuresti.

Väitöstutkimuksen alkaessa *HYLS1* oli geeni, jonka toimintaa ei ollut aiemmin kartoitettu. Tutkimuksessa saatiin paljon uutta tietoa geenin ja proteiinin toimintaan liittyen, vaikka HYLS1:n tarkkaa toimintaa ei vielä tiedetä. Mitä todennäköisimmin HYLS1 on solun transkription säätelyyn ja ehkä myös kolesterolimetabolian säätelyyn osallistuva proteiini, jolla on hyvin keskeinen tehtävä sikiönkehityksen aikana. Geenivirheen tunnistaminen toi tärkeän mahdollisuuden HLS-diagnoosin geneettiseen varmistukseen. Tämän myötä voidaan tarjota hyödyllistä geneettistä tietoa perheille, joissa epäillään sikiöllä olevan hydroletalus-oireyhtymä. Nyt tutkitun oireyhtymän sekä lisäksi muiden harvinaisten oireyhtymien tutkimus tarjoavat osaltaan arvokasta uutta tietoa myös normaalin sikiönkehityksen tapahtumien tutkimukseen molekyyli- ja solutasolla.

Avainsanat: hydroletalus-oireyhtymä, HYLS1, sikiönkehitys, kehityshäiriö, keskushermosto

CONTENTS

ABBREVIATIONS

ACAT2	acetyl-Coenzyme A acetyltransferase 2
ACLS	acrocallosal syndrome
BrdU	bromodeoxyuridine
CCND1	cyclin D1
cDNA	complementary deoxyribonucleic acid
cM	centiMorgan
CNF	congenital nephrosis of the Finnish type
CNS	central nervous system
COS-1	African green monkey kidney cell line
CP	choroid plexus
CSF	cerebrospinal fluid
DHCR7	7-dehydrocholesterol reductase
DNA	deoxyribonucleic acid
EST	expressed sequence tag
FDH	Finnish disease heritage
FGF	fibroblast growth factor
GCPS	Greig cephalopolysyndactyly syndrome
GLI3	GLI-Kruppel family member 3
HEK-293	human embryonic kidney cell line
HH	hedgehog
HLS	hydrolethalus syndrome
HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
HYLS1	hydrolethalus syndrome 1
kb	kilobase
kDa	kilodalton
LD	linkage disequilibrium
LDLR	low density lipoprotein receptor
LMB	leptomycin B
MIM	Mendelian inheritance in man
mRNA	messenger DNA
NCBI	National Center for Biotechnology Information
NES	nuclear export signal
NLS	nuclear localization signal
NPC	nuclear pore complex
OFD	orofacio-digital
ORF	open reading frame
PBS	phosphate buffer solution
PCR	polymerase chain reaction

PHS	Pallister-Hall syndrome
pI	isoelectric point
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse-transcriptase PCR
SHH	Sonic hedgehog
SH-SY5Y	human neuroblastoma cell line
SLOS	Smith-Lemli-Opitz syndrome
SNP	single nucleotide polymorphism
SUMO	small ubiquitin-like modifier
TGF	transforming growth factor
THBS1	thrombospondin 1
UCSC	University of California, Santa Cruz
WNT	Wingless-type
wt	wild type

In addition, the standard abbreviations for nucleotides and amino acids are used. The abbreviations of the gene names are written in italics and the protein names in regular letters. Human gene names are capitalized, mouse gene names are written in lower case letters. Protein names are capitalized.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals (I-III):

I Mee Lisa*, **Honkala Heli***, Kopra Outi, Vesa Jouni, Finnilä Saara, Visapää Ilona, Sang Tzu-Kang, Jackson George, Salonen Riitta, Kestilä Marjo, Peltonen Leena (2005) Hydrolethalus syndrome is caused by a missense mutation in a novel gene *HYLS1*. Human Molecular Genetics 14:1475-88.

II Paetau Anders*, **Honkala Heli***, Salonen Riitta, Ignatius Jaakko, Kestilä Marjo, Herva Riitta (2008) Hydrolethalus syndrome: Neuropathology of 21 cases confirmed by *HYLS1* gene mutation analysis. Journal of Neuropathology and Experimental Neurology 67:750-62.

III **Honkala Heli**, Lahtela Jenni, Fox Heli, Gentile Massimiliano, Pakkasjärvi Niklas, Salonen Riitta, Wartiovaara Kirmo, Jauhiainen Matti, Kestilä Marjo. Unraveling the disease pathogenesis behind lethal hydrolethalus syndrome revealed multiple changes in molecular and cellular level. Submitted.

* Authors have contributed equally to the work.

Some unpublished data are also presented. Publication I has appeared in the thesis of Lisa Mee (USA 2005).

Author´s contribution to publications

I: HH contributed to the DNA, RNA and protein studies. HH also participated in drafting the manuscript.

II: HH participated in the general study design. HH also designed the PCR and sequencing processes of the samples and performed all the genetic analyses. HH drafted the manuscript with other authors.

III: HH contributed to the study design and sample collection. HH participated in all experiments and fully performed part of them, as well as gathered the final results and wrote the manuscript.

1 INTRODUCTION

Fetal development is an amazing event with a precisely orchestrated series of signaling cascades which control cells to become different tissues and finally, a living organism. Defects in these processes can lead to severe developmental malformations and to fetal death.

It is very important to identify any genetic defect behind an inherited disorder since even if the studied disease is rare, the results of the multiple functional studies performed in the course of research of these entities most probably will offer significant information also about the molecular and cellular events and pathways required for normal development in general. Also, identification of the defect provides a useful tool for confirming the diagnosis and thus, gives essential information for families at risk.

Hydrolethalus syndrome (HLS, MIM 236680) is a lethal malformation syndrome of the fetal stage belonging to the group of disorders called the Finnish disease heritage. HLS is inherited autosomally recessively and it is enriched in the Finnish population with an incidence of at least 1:20,000. HLS is characterized by several malformations of the developing organs. In the central nervous system, prominent features include absent midline structures and the hydrocephalus. Other hallmark features consist of micrognathia, polydactyly of hands and feet, defective lobation of the lungs, craniofacial anomalies as well as polyhydramnios during pregnancy. HLS was first described in the 1980´s when it was separated from the Meckel syndrome (Salonen et al. 1981) and thus, HLS became its own syndrome entity. In addition to Finnish cases, some non-Finnish cases resembling HLS have been reported abroad.

Since the molecular background of HLS had not been characterized before this study, the main goal in this study was first to identify the gene and the mutation(s) underlying HLS. As the disease-causing mutation was found, subsequent analyses consisted of determination of the protein function as well as unraveling the disease pathogenesis of HLS with studies both at the cellular and tissue levels. Also, the neuropathological picture of this severe syndrome was described in detail for the first time. The general pathological findings reported simultaneously with the neuropathological results revealed a highly interesting wide phenotypic spectrum in HLS.

2 REVIEW OF THE LITERATURE

2.1 The Finnish disease heritage

An entity called the Finnish disease heritage (FDH) is a set of inherited conditions that are more common in Finland than elsewhere in the world when compared to population size (Norio et al. 1973; Perheentupa 1972). Nowadays, 36 disorders are included under this definition. All of these disorders are monogenic and relatively rare, 32 of them being autosomal recessive, two autosomal dominant and two X-linked (Norio 2003c). At the same time, some diseases like cystic fibrosis and phenylketonuria that are common elsewhere have very low incidence in Finland (Guldberg et al. 1995; Kere et al. 1994; Pastinen et al. 2001).

Almost one third of the conditions in FDH cause some form of mental retardation and half of the diseases are lethal at some point of affected´s life. In most cases, incidences of the diseases vary from 1:10,000 to 1:100,000 in Finland (Norio 2003a). As the Finnish gene pool is relatively homogeneous it has proven to be a useful tool in finding the disease-causing mutations in the genome (Peltonen et al. 1999) and in fact, the underlying causative genetic defect are currently reported for all of the disorders except for PEHO syndrome (www.findis.org). It has been shown that one founder mutation was the main contributor to all these diseases. In most diseases, more than 90% of the Finnish patients have the same Fin_{major} mutation causing the disease (Norio 2003c). However, there are also some disorders where the frequency of the Fin_{major} mutation is between 70 and 80% and in addition, other mutations have been found at varying frequencies, see e.g. (Huopaniemi et al. 1999; Kestilä et al. 1998; Sankila et al. 1992).

The reason why this unique set of diseases has been enriched in Finland is largely due to the population history of this isolated area with its sparseness of population, vast forest areas, numerous lakes and partly to pure chance, also, since the ancient inhabitants of Finland brought with them a random assortment of possible disease mutations (Norio 2003a, 2003b). This assortment stayed largely unchanged because the great population migrations did not happen in the northern part of Europe as they did in central and southern Europe. Close consanguinities between parents is not part of the cause because the rate of consanguinity and inbreeding coefficients are very small in Finland. Instead, remote consanguinities are important factors in FDH (Norio 2003a).

Early and late settlement areas in Finland are often mentioned when discussing disorders belonging to FDH. The early population in Finland was mostly

concentrated in the coastal region whereas the late settlement area in the inland was effectively populated starting in the 1500s mainly from the Savo area, the national movement strongly influenced by King Gustavus Vasa (Norio 2003a). A map describing the areas of early and late settlement is presented in Figure 3 in section 2.3.1 (page 23).

There are different kinds of spreading patterns of disorders belonging to FDH and thus, the autosomally recessively inherited disorders have been divided into five groups (Norio 2003a). In the largest group of 16 disorders (for example congenital lactase deficiency), most of the birthplaces of the affecteds´ grandparents are located at the late settlement area (Figure 1A). This kind of a spreading pattern suggests that the disease mutation had appeared in the Finnish population before the population started to spread to late settlement area. The second group of distribution includes the six most common disorders (for example congenital nephrosis of the Finnish type, CNF) and they have been determined to have spread to most parts of the country (Figure 1B). Based on this fact, these mutations are probably the oldest FDH mutations in the population. The third group is formed only by diastrophic dysplasia and the Meckel syndrome. They are mostly distributed in the western part of the country and the distribution follows the population density in Finland. These disorders are seen worldwide and there is a possibility that these mutations have been brought to Finland with the western Indo-European immigrants (Norio 2003b). The fourth group comprised of northern epilepsy and the Finnish variant of Jansky-Bielschowsky disease are strictly regional, thus suggesting that their mutation is very young (Figure 1C). The remaining four disorders (for example RAPADILINO syndrome) are grouped as their own entity because their maps are atypical with no similarities with the other four groups. Also, only a small number of families are affected by these disorders.

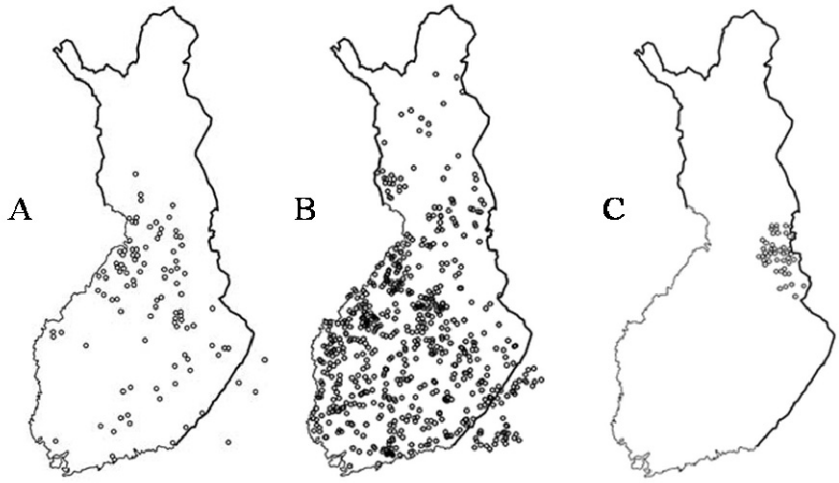

Figure 1. Distribution patterns of congenital lactase deficiency (A), CNF (B) and northern epilepsy (C). Modified from Norio 2003c.

2.2 Identification of disease genes

The important step in the era of DNA research was made when the helical structure of DNA was first published in the 1950´s (Watson and Crick 1953). Identification of disease genes has experienced an enormous leap in the recent years due to the development of new study methods. The human genome project was initiated in 1990 in order to determine the nucleotide sequence of the human genome and at the same time, identify the genes that this sequence contains. The initial draft version of the genome was published by the International Human Genome Sequencing Consortium (Lander et al. 2001) simultaneously with a private company Celera Genomics (Venter et al. 2001). The nearly complete version of the sequence was published a couple of years later by the International Human Genome Sequencing Consortium (IHGSC 2004).

2.2.1 Positional cloning

When searching for the disease-causing gene in a population, usually there is no information of the possible causative gene readily available. A method called positional cloning has been widely used in these kinds of situations in the research of monogenic diseases (Collins 1992, 1995). A work-flow for positional cloning is

presented in Figure 2. In this method, disease gene identification is based on its genomic location instead of its known function. When studying isolated populations, there is a great possibility that a common genetic factor may be found behind the same phenotype shared with the patients. In order to achieve reliable results, the phenotypic criteria for the disease should be strict enough and the diagnoses should be done as accurately as possible.

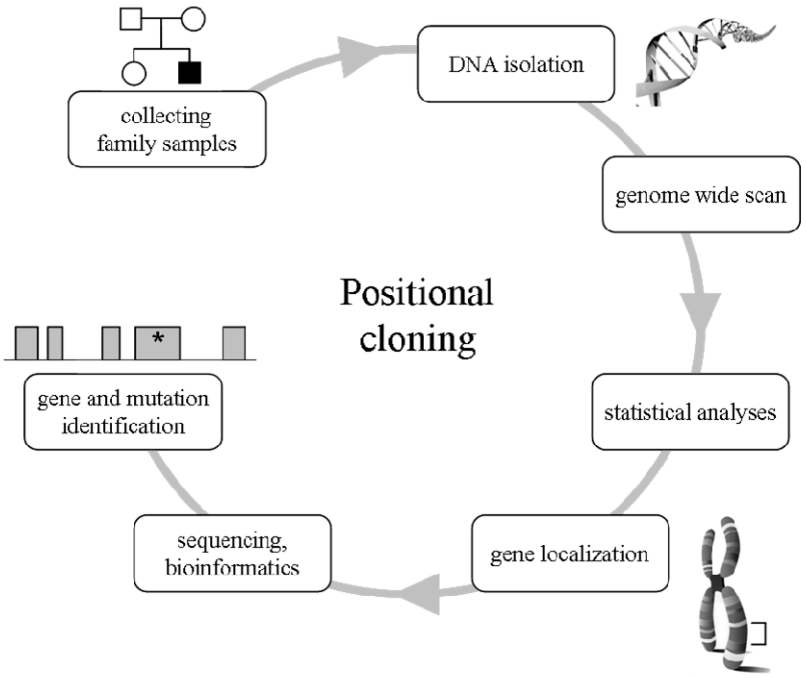

Figure 2. Main steps of the positional cloning method.

In the positional cloning method, DNA material is first collected from the affecteds, siblings and parents as widely as possible and the samples are genotyped using a genome-wide marker genotyping (see section 2.2.2). After the genotyping step, linkage analysis (see section 2.2.2) is performed to find a genomic region where the possible genetic defect is situated.

2.2.2 Genetic mapping

Microsatellites and SNPs as genetic markers

The most used methods for genotyping are based on the microsatellite markers (Dubovsky et al. 1995) and single nucleotide polymorphisms (SNPs) (Frazer et al. 2007), which are naturally occurring variations in the genome between individuals. Microsatellites are stretches of DNA where a certain nucleotide combination most often comprised of 2-4 nucleotides is repeated several times. The best microsatellite markers are the ones which have several different alleles in the population. Microsatellites are spread over the human genome at the average of once in every 2 kilobases (kb) (Lander et al. 2001). Initial screening of the genome is usually done using these variable markers, but additional markers are often needed for further studies. SNPs are less polymorphic when compared to microsatellites because they usually contain only two alleles, but lower mutation rates make them also more stable (Gray et al. 2000). The appearance of SNPs is quite dense, approximately 10 million of them can be found in the human genome. The microsatellite scan was a widely used technique earlier, but nowadays SNP scans are more commonly used since this method has proven to be more efficient with the developed and the time-saving array-based technique.

Linkage and haplotype analyses

Before it is possible to find the specific disease-causing gene, the genomic candidate region has to be identified by a statistical approach called linkage analysis (Dubovsky et al. 1995). The purpose of the analysis is to find a specific chromosomal region for a disease locus using polymorphic markers. The closer a marker locus is to a disease locus the more rarely recombinations are able to separate these two loci from each other. Thus, two loci are said to be genetically linked. In the linkage analysis method, the proportion of recombinations observed is used as a measure of genetic distance between two loci. To determine possible linkage, the recombination fraction between the two loci must be resolved. Also, it has to be determined whether the recombination fraction is significantly different from 0.5, the value that is expected with no linkage (Teare and Barrett 2005). Depending on the data available, there are different forms of linkage analysis that can be used, including two-point, multipoint, parametric and non-parametric analyses.

When the initial identification of the candidate region is made, regions are often found to be broad and to contain a large number of genes. Thus, haplotype analyses

and linkage disequilibrium (LD) studies are used for fine mapping in order to make the critical region as short as possible. Haplotypes are combinations of alleles of adjacent markers in the genome that tend to be transmitted together from one generation to the next. Haplotypes get shorter with time because of the recombinations between homologous chromosomes in meiosis. In general, the older the formed haplotype is, the shorter is its length. Haplotypes become shorter rapidly if a phenomenon called recombination hot spot is located at the genomic region (Nishant and Rao 2006). LD in turn refers to the non-random association of particular alleles at loci close to each other that occur more frequently than would be expected. When studying monogenic diseases in the isolated population such as Finns, genomic areas where the affecteds share the same haplotype are often searched for. This is applicable when it can be assumed that the disorder is caused by one ancestral founder mutation. In the course of identifying the disease genes, it has been noted that in the Finnish population, the size of the shared region can vary significantly between different syndromes. The reason for this can be for example the age of the mutation (Peltonen et al. 1999).

2.2.3 Mutation analysis

After the genetic mapping procedure, individual candidate genes in the critical region have to be tested to determine if they are causing the disease phenotype or not. Nowadays, mutation screening to find the disease-causing DNA change is commonly performed by the direct sequencing method. If the function of the genes in the critical region is known and their function can be linked to the disease, the best candidate genes for the sequencing can be easily found. But even if information from all genes is nowadays easy to find using different databases, the screening project can still be complicated and might need a lot of time to be successful.

When screening for mutations in the DNA sequence, several different kinds of them can be found. In a point mutation, one of the nucleotides in the sequence is changed to another. This change can lead to either nonsense, missense or splice site mutation. The nonsense mutation causes a premature stop codon in the coding region of the gene, this causing either protein degradation or a truncated protein. In a missense mutation, one of the amino acids in the polypeptide is changed to an other, this change possibly affecting the structure and/or function of the protein. In splicing mutation, mutations affecting the consensus sequences at splice donor or acceptor sites or at splice branch sites may make them less effective and eliminate the splicing partially or completely. It is also possible that nucleotide change might introduce a new splice site (Cartegni et al. 2002). A point mutation can also be

translationally silent, thus not affecting the amino acid composition of the produced polypeptide.

Other types of mutations include deletions, insertions and duplications that can range from one nucleotide to large genomic segments. If deletions and insertions do not occur in the group of three nucleotides or multiples of this, they cause frameshift mutations leading to a premature stop codon or defective splicing. In an inversion mutation, an entire section of DNA is reversed. A small inversion may involve only a few bases in a gene, while longer inversions can include large regions of a chromosome containing several genes. Translocation in turn is a type of unnatural genome rearrangement, where two non-homologous chromosomes exchange parts of their arms and this can lead, for example, to the disruption of a gene.

To confirm a particular change in DNA to be disease-causing, several criteria have to be met. Mutation screening in patients has to reveal at least one change in the DNA sequence that segregate with the disease in the patients´ families and this segregation has to be consistent with the proposed mode of inheritance. Possible disease-causing DNA variants are also important to differentiate from neutral polymorphisms. For this, a large enough number of unaffected individuals representing the respective population have to be screened. Also, functional analyses are usually needed to perform to determine the role of the sequence variation in the cellular and tissue levels (see below).

Functional analyses

Numerous functional analyses are possible to be performed to study the nature of the DNA variants and their consequences. *In silico*, this can be made by analyzing the gene and/or the protein sequence by exploring various internet databases and using computer-assisted bioinformatic sequence analysis tools. Analyses can be made for instance to predict the physical and chemical characteristics of the proteins as well as to search orthologies with other species. One should bear in mind, however, that these programs might give contradictory results and that the predictions are based solely on the sequence provided in the analysis. Thus, further laboratory research is usually needed to confirm the proposed function. Just to mention a few, genomewide gene expression profiling using microarray-based techniques, analysis of spatial and temporal gene expression patterns by reverse transcription polymerase chain reaction (RT-PCR) analysis, determination of the intracellular localization of the protein using an overexpression system in cell line cultures and immunoblotting are methods that can be used in gene and protein characterization. A laborous procedure for creating an animal model based on the sequence variation in question can at best give definitive verification for the pathogenic role of the altered gene form.

2.3 Hydrolethalus syndrome

2.3.1 History

Hydrolethalus syndrome (HLS, MIM 236680) is a rare lethal malformation syndrome of the fetal stage characterized by several developmental malformations. This syndrome is enriched in the Finnish population and is part of the Finnish disease heritage. HLS has an autosomal recessive heritability with an incidence of at least 1:20,000 in Finland. HLS was first described by Riitta Salonen and her colleagues in 1981 during a nationwide study of Meckel syndrome (MIM 249000). Both of these syndromes are characterized by severe central nervous system (CNS) malformations as well as polydactyly (ie. extra fingers and toes) as their main features. Although these two syndromes share similar defects, there are also clear differences. For example, in HLS there have not been cases with the cysts in the kidneys or other parenchymatous organs, this being perhaps the single most characteristic anomaly of Meckel syndrome (Salonen et al. 1981). In addition, in HLS, the frequent finding is hydrocephalus, that is an uncharacteristic feature in Meckel syndrome.

Figure 3 shows the areas of early and late settlement areas as well as the distribution of the birthplaces of the great grandparents of Finnish HLS cases. This kind of map is typical for several other diseases of FDH, too (Norio 2003a). It has been estimated that the HLS mutation was most probably introduced to the Finnish population about 30-40 generations ago (Visapää et al. 1999).

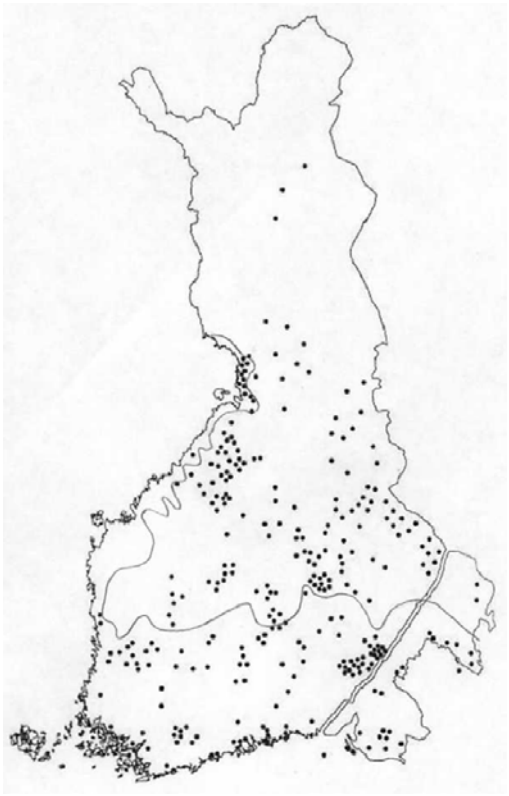

Figure 3. Distribution pattern of hydrolethalus syndrome in Finland. The line represents the division between early and late settlement areas, early settlement concentrating in the coastal area and late settlement on the mainland. Modified from Visapää et al. 1999 and Norio 2003a.

2.3.2 Clinical picture

HLS is characterized by several developmental malformations of the CNS and many other organs. Some of the malformations are very frequent but some of them are not seen in every case. The phenotypic appearance of the syndrome can thus be decribed as a wide spectrum of features. A large hydrocephalus, micrognathia (small mandible) and polydactyly can be named as typical manifestations of HLS. The main features and their observed frequencies in HLS cases (Salonen and Herva 1990) have been listed in Table 1. Most prominent clinical features are shown in figure 4 on page 26.

Table 1. Main clinical features and percentage of the observed frequencies in 56 individuals with hydrolethalus syndrome. Modified from Salonen and Herva 1990.

Clinical feature	Percentage
Micrognathia	100
Polyhydramnios	91
Anomalous nose	89
Small / deep set eyes	87
Hydrocephalus	85
Gestational age < 38 weeks	78
Polydactyly	78
Occipital bone defect	75
Stillborn	73
Anomalous / low set ears	64
Defective lobulation of the lungs	62
Abnormal genitalia	59
Abnormal larynx / trachea	58
Cleft lip / palate	52
Club feet	48
Congenital heart disease	46
Short limbs	20
Urinary tract anomalies	16

Polyhydramnios is one of the typical HLS features with the amount of the amniotic fluid found to be up to 8 litres (Salonen et al. 1981) while normally, the amount of the amniotic fluid varies from 0.5 to 1 litre (Sariola et al. 2003). In the past, many of the HLS fetuses were stillborn, but some of the liveborn infants lived from a few minutes to a few days (R. Herva, personal communication; (Salonen and Herva 1990). HLS can nowadays be diagnosed already *in utero* by ultrasound scan (Hartikainen-Sorri et al. 1983) already at the end of the first trimester of pregnancy (Ämmälä and Salonen 1995). Due to the severe malformations most of the families decide to terminate the pregnancy.

The most frequent CNS abnormality in HLS is a large hydrocephalus, with an extreme case of 2100 ml of fluid reported (Salonen et al. 1981). A few cases with anencephaly have also been seen (Salonen and Herva 1990; Salonen et al. 1981). No cases with holoprosencephaly with only one brain hemisphere have been described.

In the typical cases with hydrocephalus, the hemispheres are separated and they lie at the bottom of the skull. The upper midline structures, including the corpus callosum and septum pellucidum, are absent. A special kind of occipitoschisis is found in many times, forming a cleft in the base of the skull in the midline of the occipital bone and forming a keyhole-shaped opening. In some cases the pituitary or olfactory nerves are absent. Frequently, the gyration of the brain is grossly abnormal. It has been suggested that a rapidly expanding hydrocephalus may cause the defects in the craniofacial development (Krassikoff et al. 1987).

Craniofacially, the most frequent finding is micrognathia, i.e. a small, sometimes rudimentary, mandible. Cleft palate or lip has also been reported as well as malformations in the tongue, nose, ears and eyes (Salonen and Herva 1990; Salonen et al. 1981). In the eyes, a coboloma and hypoplasia of the optic nerve has been reported (Kivelä et al. 1996). In the limbs, polydactyly is frequently documented. Polydactyly, which means extra toes and/or fingers, is usually postaxial in the hands and preaxial in the feet. Hallux duplex, a duplicated big toe, is a hallmark feature in the feet. Also other abnormalities in the limbs, for example club-foot-like deformity and abnormally short arms and legs have been found (Herva and Seppänen 1984; Salonen et al. 1981).

About half of the reported cases have had a congenital heart defect (Salonen and Herva 1990), the main finding being a large ventricular septal defect. Also respiratory organs, including the larynx and the trachea, have sometimes been reported to be abnormal. In some cases, the lobulation of the lungs has been incomplete or completely absent. There have been cases with mild abnormalities of the genitalia, but the sex has always been distinguishable (Salonen et al. 1981).

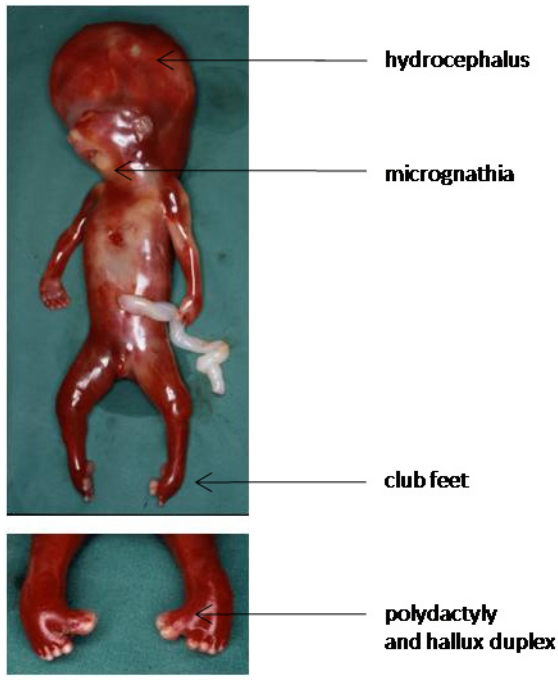

Figure 4. An HLS fetus of 19th gestation week with some of the most prominent clinical features shown.

There have been several reports about non-Finnish cases having overlapping features with the Finnish HLS cases, see e.g. (Adetoro et al. 1984; Anyane-Yeboa et al. 1987; Aughton and Cassidy 1987; Bachman et al. 1990; Chan et al. 2004; de Ravel et al. 1999; Krassikoff et al. 1987; Morava et al. 1996; Rakheja et al. 2004; Shotelersuk et al. 2001; Toriello and Bauserman 1985). Some of the cases were either stillborn (Rakheja et al. 2004) or were detected by ultrasound and because of the severe findings, the pregnancy was terminated (Bachman et al. 1990; Chan et al. 2004). Also a "milder" form of HLS has been reported. For example, Shotelersuk et al. (2001) reported a case that survived for over a month and Aughton and Cassidy (1987) and de Ravel et al. (1999) reported cases that lived for several months. It has been suggested that the less severe cases are also true HLS cases and that the underlying cause of the inconsistency between severe and mild cases is allelic variability (de Ravel et al. 1999; Shotelersuk et al. 2001). Also, Pryde et al. suggested allelic variability for HLS between Finnish and foreign cases (Pryde et al.

1993). In addition, Christensen et al. proposed that acrocallosal syndrome and HLS could be allelic syndromes (Christensen et al. 2000).

2.3.3 Genetics

Visapää and her colleagues assigned the hydrolethalus syndrome locus to chromosome 11q23-25 in Finnish families (Visapää et al. 1999). The initial genome scan study was performed using DNA material from 15 affected individuals and 20 healthy family members from eight HLS families. The critical locus was first assigned to an 8.5 cM interval by linkage analysis and the locus was finally further restricted to a 0.5-1 cM region using linkage disequilibrium and haplotype analyses. In addition, seven HLS cases all from different families and the parents of four of these families were included in the linkage disequilibrium studies. The parents of HLS cases were generally non-consanguineous. Six parents were found to be related, the earliest identified link between ancestors being seven generations old and the most recent link was found in the family where the parents were first cousins. Genealogical studies from 40 families affected by HLS revealed that HLS does not have any specific regional clustering in Finland and that the majority of the great-grandparents´ birthplaces are situated in the late settlement area (Figure 3). According to the genealogical studies, one major founding mutation for HLS could be predicted (Visapää et al. 1999), as in several other Finnish syndromes (Peltonen et al. 1995). To assure the strict qualification of HLS cases in this study, all the diagnoses were confirmed by one clinician and all the affecteds in the families represented very typical cases of HLS with the prominent features of the syndrome (Visapää et al. 1999).

2.3.4 Differential diagnostics

There are some syndromes and conditions that have a partially overlapping spectrum of features with HLS but there are also some fundamental differences that separate these syndromes from each other. Since there can be a quite significant phenotypic spectrum inside multiple developmental anomaly syndromes, it can sometimes be difficult to categorize affected patients to some distinct syndrome. The difficulties of differential diagnostics in multiple severe malformation syndromes containing also midline development disturbances have been discussed in several publications, see e.g. (Hennekam et al. 1991; Pryde et al. 1993).

In addition to Meckel syndrome, from which HLS was initially separated, other resembling syndromes include for example orofaciodigital syndrome (OFD) type IV

(Mohr-Majewski or Baraitser-Burn syndrome; MIM 258860) (Toriello et al. 1997), OFD type VI (Varadi-Papp syndrome, MIM 277170) (Doss et al. 1998; Varadi et al. 1980), and acrocallosal syndrome (ACLS, MIM 200990) (Koenig et al. 2002; Nelson and Thomson 1982). In OFD type IV, symptoms include brain abnormalities, minor facial and oral anomalies and variable digital defects such as polydactyly. OFD type IV is suggested to represent genetic heterogeneity among the patients (Toriello et al. 1997). OFD type VI is distinguished from other oral-facial-digital syndromes by metacarpal abnormalities with central polydactyly and by cerebellar abnormalities (Doss et al. 1998). ACLS is characterized by agenesis of the corpus callosum, minor craniofacial anomalies, pre- and postaxial polydactyly and psychomotor retardation (Koenig et al. 2002). In addition to these syndromes, Pallister-Hall syndrome (PHS, MIM 146510) and Smith-Lemli-Opitz syndrome (SLOS, MIM 270400) also resemble HLS. These two syndromes are discussed in more detail below.

In 1995, Verloes used a mathematical approach to compare syndromes with complex phenotype and with partially overlapping symptoms by ranking the phenotypic features and using statistical methods to analyze the outcome (Verloes 1995). For this approach, Verloes used a term “numerical syndromology”. The syndromes used in the comparisons included hydrolethalus, Smith-Lemli-Opitz, Pallister-Hall, OFD type VI and holoprosencephaly-polydactyly syndromes. He concluded that these syndromes are clearly independent phenotypic entities. Still, as the phenotypic spectrum of these syndromes can vary significantly, the diagnosis of some uncertain cases can be quite difficult.

Pallister-Hall syndrome

PHS is a disorder with the most resemblance to HLS. PHS was first described in the 1980s with several malformations (Clarren et al. 1980; Hall et al. 1980). As in HLS, also in PHS malformation of the brain can be seen, the hallmark feature being hypothalamic hamartoblastomas. Other frequently occurring features are craniofacial anomalies in general as well as micrognathia, polydactyly, defective lobation of the lungs, congenital heart defects and cleft lip or palate (Hall et al. 1980). Skeletal malformations have also been reported (Roscioli et al. 2005). Most of these features can be seen in HLS fetuses, too. In contrast, for example an imperforate anus and renal abnormalities are the features often reported from PHS patients but not seen in HLS. Life expectancy is also higher in PHS than in HLS. The symptoms in PHS can vary from very mild to severe, the patients´ life span varying from few months to adulthood. The variance in the phenotypic spectrum is

true for HLS cases, too, but the symptoms are more severe in HLS generally leading to death at the very early stages of life.

The causative gene for PHS is *GLI3* at chromosome 7p13, the disease-causing mutations being nonsense and splicing mutations (Johnston et al. 2005; Kang et al. 1997a; Kang et al. 1997b). The mode of inheritance in PHS is autosomal dominant. *GLI3* is homologous to the *Drosophila* cubitus interruptus (ci) gene product (Ci) and acts as a transcription factor in the Sonic hedgehog (SHH) signaling pathway. This pathway contributes to several essential cellular events during fetal development, including development of the neural tube, craniofacial structures, the lung and the limb, among others (Biesecker 2006). Mutations in *GLI3* also cause Greig cephalopolysyndactyly syndrome (GCPS, MIM 175700) and thus, PHS and GCPS are allelic syndromes. However, the mutations causing GCPS are partially of different kinds (deletions, chromosomal translocations) and located in a different region than in PHS (Johnston et al. 2005). Johnston et al. also suggested that a truncated functional repressor protein of GLI3 causes PHS, whereas haploinsufficiency of GLI3 would in turn be the cause for GCPS. In addition to PHS and GCPS, also in acrocallosal syndrome at least one case with a mutation in the *GLI3* gene has been reported (Elson et al. 2002). As GLI3 has been excluded as the site of mutation in some cases of ACLS, the authors suggested that ACLS may be a heterogeneous group of disorders where the phenotype, in some cases, results from a mutation in GLI3 and represents a severe, allelic form of GCPS (Elson et al. 2002).

Smith-Lemli-Opitz syndrome

SLOS is an autosomally recessively inherited congenital syndrome with multiple developmental abnormalities first reported in 1964 (Smith et al. 1964). SLOS has a wide spectrum of phenotypic features that vary from mild symptoms with learning and behavioral problems to a lethal malformation syndrome (Hennekam 2005; Porter 2008). Prominent features of the syndrome include microcephaly, micrognathia, growth retardation, holoprosencephaly, ptosis, cleft palate and polydactyly. Because of the variable phenotype, division into a mild (SLOS I) and severe (SLOS II) forms of the syndrome have been suggested (Curry et al. 1987).

SLOS is caused by mutations in the sterol 7-dehydrocholesterol reductase gene (*DHCR7*), which is the last step in the cholesterol metabolism pathway. Over 130 different mutations of *DHCR7* have been so far identified in SLOS patients (Correa-Cerro and Porter 2005; Porter 2008). The mutations cause increased serum levels of 7-dehydrocholesterol and decreased serum levels of cholesterol in patients (Irons et al. 1993; Tint et al. 1994). The relation between the genotype and severity of the symptoms in SLOS is controversial, since reports demonstrating both quite poor

(Correa-Cerro et al. 2005) and quite good (Witsch-Baumgartner et al. 2000) genotype-phenotype correlation in patients have been published. SLOS diagnosis can nowadays be made prenatally by measuring 7-dehydrocholeserol levels in amniotic fluid or chorionic villus samples or alternatively by direct mutation analysis of *DHCR7* (Waye et al. 2007). The possibility for non-invasive diagnosis by maternal urinary steroid level measurement has also been suggested (Jezela-Stanek et al. 2006). Based on genetic studies, SLOS is very rare in Finland (Witsch-Baumgartner et al. 2008).

2.4 Fetal development

2.4.1 General aspects

Human fetal development from a single cell to a living organism is a complex and remarkable phenomenon, guided both by genes and the environment. Fetal development can be divided into three main stages called blastogenesis, organogenesis and fetogenesis. Blastogenesis is the interval of the first four weeks after fertilization, until the neural tube closure. Organogenesis is the stage from the end of blastogenesis to nine weeks of gestation. This is the main stage of organ development. Fetogenesis lasts from week nine until birth, the stage when the fetus has the major organ patterning mainly developed and when the fetus grows in size. Growth of the fetus is fastest in the early stages of fetogenesis (Carlson 2004; Sariola et al. 2003).

Organs start to develop at an early stage of fetal development. For example the brain, eye, heart and gastrointestinal tract start their development in a three week old embryo, the spinal cord and auditory system at three or four weeks, the olfactory, renal and limbs at 4-5 weeks and the respiratory tract and genitalia at five weeks. Based on their complexity, organs take different times to complete their development. For example, the heart is formed at 6 weeks, the face and limbs at eight weeks whereas the structural development of the brain continues until 28 weeks. Organs of course grow in size and mature further *in utero* and also postnatally after their structural development is completed in the fetal period (Carlson 2004; Sariola et al. 2003).

Inductive signaling between tissues is a critical regulator of the development of different organs. For example in the developmental processes of the lung and the kidneys, interaction between the mesenchyme and the epithelium of the tissue is critical for appropriate formation of the organ (Sariola et al. 2003). It is typical of

mesenchyme-epithelium signaling that both tissues participate actively in the signaling events. Signaling continues through the whole organ development and it is mediated through soluble molecules. In order to be able to react correctly to every signal, cells must have the right receptors in their surface to receive the messages. Also, molecules for intracellular signal transduction and target gene expression machinery must be available.

2.4.2 Key molecules and signaling pathways

Normal human development requires the precise functioning and coordination of many complex pathways and signaling cascades. Interestingly, a large proportion of the genes regulating the fetal development are basically the same in different groups of animals. These key molecules are used not only in the early embryonal stage but later in the organ development, as well. Studies of these key molecules in relatively simple organisms such as *Drosophila melanogaster* (fruit fly) and *Caenorhabditis elegans* (ground worm) have given us a massive amount of essential knowledge about the molecular cascades required during the developmental processes. Many signals that are essential in the development of vertebrates are the members of the following four families: Hedgehog (HH), Wingless (WNT), fibroblast growth factor (FGF) and transforming growth factor beta (TGFβ) (Carlson 2004; Sariola et al. 2003). They can be found in every organ of the developing fetus. It is mostly quite hard to unravel the meaning of a single molecular signal during development since the same signals are used constantly and since the cells express and also receive several signals simultaneously. In addition, different signal pathways cross each other and also affect each other.

In the early development, some of these important molecules are distributed from certain signaling centrals (such as the isthmic organizer and the zone of polarizing activity), which are temporary structures that produce many different kinds of signaling molecules simultaneously and thus affect the formation and differentiation of the surrounding tissues. One crucial group of molecules are the ones that transmit the signaling between cells in the body. This usually leads to activation of the transcription factors that in turn affect the genes that they are targeted to.

2.4.3 Development of the central nervous system

Nervous system development can be divided into the following eight main subsequent stages: 1) induction of neuronal tissue in the ectoderm and patterning, 2) establishment of neuroblasts and their migration, 3) differentiation of neuronal cell

types, 4) axonal guidance, 5) establishment of synaptic connections, 6) regulation of the amount of neuronal cells by apoptosis, 7) further arrangement of synaptic connections, and 8) arrangement of synaptic connections by their activity and usefulness, i.e. synaptic plasticity (Sariola et al. 2003). The most important gene groups of the developmental toolbox used for CNS development include in large part the same groups as mentioned earlier, such as Hedgehog (HH), Wingless (WNT), fibroblast growth factor (FGF) and the Notch families as well as bone morphogenetic proteins, a subclass of TGFβ superfamily, see e.g. (Bertrand and Dahmane 2006; Ford-Perriss et al. 2001; Fuccillo et al. 2006; Mehler et al. 1997; Monuki 2007).

Development of CNS starts at the very beginning of embryonic stage when a group of ectodermal cells are induced by the underlying ectoderm (notochord). This event is called primary induction. Induction is a phenomenon where a group of cells coordinate adjacent cells changing their behavior and therefore making them change in some way. Induced neuroepithelial cells then form a thickened neural plate overlying the notochord. The neural plate develops further from the neural groove to the closed neural tube, an event that is assisted by several essential developmental molecules and their gradients (see above). Neural tube closure is complete already on the 28th day after fertilization (Sadler 2005). The brain itself is developed from the dilations processed by the cephalic portion of the neural tube, formed by the migrating neurons that establish different laminar compartments (Bystron et al. 2008).

Cortical lamination is a phenomenon where laminar compartments of the developing brain cerebral cortex with their own, special cell types are formed. Three main types of precursor cells in the early stages of the cortex development have been identified: radial glial cells and short neural precursors in the ventricular zone and intermediate progenitor cells in the subventricular zone (Dehay and Kennedy 2007). The ventricular zone cells are involved in the generation of lower layer neurons, while the cells situated in the subventricular zone generate upper layer neurons (Dehay and Kennedy 2007). Radial glia produced in the ventricular zone provide a ladder system to assist the neurons in their way to the correct compartment in the cortex (Campbell and Götz 2002). However, different neuronal types have been found to use distinct modes of migration in the developing cortex. The early-generated neurons use somal translocation, and pyramidal cells predominantly use glia-guided locomotion. A third mode of migration is used by cortical interneurons. They migrate tangentially into the cortex, then seek the ventricular zone before moving radially to take up their positions in the cortical anlage (Nadarajah and Parnavelas 2002). The mature cortex contains several different layers (Figure 5). The heterogeneous and confusing nomenclature used to describe the developing CNS led a special committee to clarify the sequence of events taking place during cortical

development and this also set the nomenclature used for different compartments (Bystron et al. 2008). Recent studies have however increased our knowledge of new cell types, patterns of genetic expression and additional cellular compartments and thus, some revisions for the committee nomenclature have been suggested (Bystron et al. 2008).

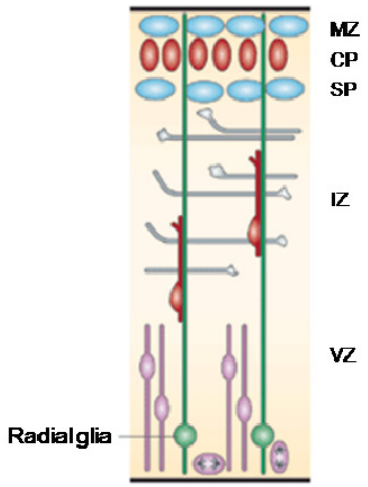

Figure 5. During the cortical lamination, several distinct cellular zones are formed. Modified from (Nadarajah and Parnavelas 2002). VZ, ventricular zone, IZ, intermediate zone, SP, subplate, CP, cortical plate, MZ, marginal zone.

The central nervous system contains more cell types than any other organ in the human body. The overall number of these cells is great, about 10^{11} neurons and 10^{12} glial cells (Diaz 2006). Although the basics of the cell types and their function in the brain is currently known, there still remains the possibility that all of the possible cell types of the brain are not yet identified (Diaz 2006).

The main classes of the cell types in CNS include neurons and glial cells. Neurons and glial cells can be further divided into many subgroups. Neurons can be classified by their structure and function or, for example, based on which neurotransmitter they produce. In addition to neurons, much of the total volume of the brain is made up of cells that support neurons in various ways but which do not carry information themselves. The collective name for these support cells is glial cells and they can be

divided into the groups of oligodendrocytes, astrocytes, and microglia. The word "glia" comes from the Greek word for glue. Glial cells have several different functions in the brain. In addition to support, their functions include transporting nutrients to nerve cells, cleaning up debris and digesting parts of dead neurons. Glial cells also provide insulation to neurons through the production of myelin. These cells are called oligodendrocytes in the CNS. For a general review of glial cells, see e.g. (Ndubaku and de Bellard 2008). Radial glial cells have an important role in key developmental processes of the CNS as precursors during neurogenesis and as mentioned earlier, in the lamination events of cerebral cortex (Campbell and Götz 2002) (Figure 6). Glial cells produced later in CNS development are mainly astrocytes: type 1 in the white matter and type 2 in the grey matter. Microglial cells in the CNS are resident immune cells that regulate and participate in immune responses in CNS tissue (Garden and Möller 2006). They also appear to play an important role during the normal function of the mature nervous system. One special glial cell type is also ependymal cells which give rise to the choroid plexus, a structure located inside the brain ventricles that produces cerebrospinal fluid (CSF) (Sariola et al. 2003).

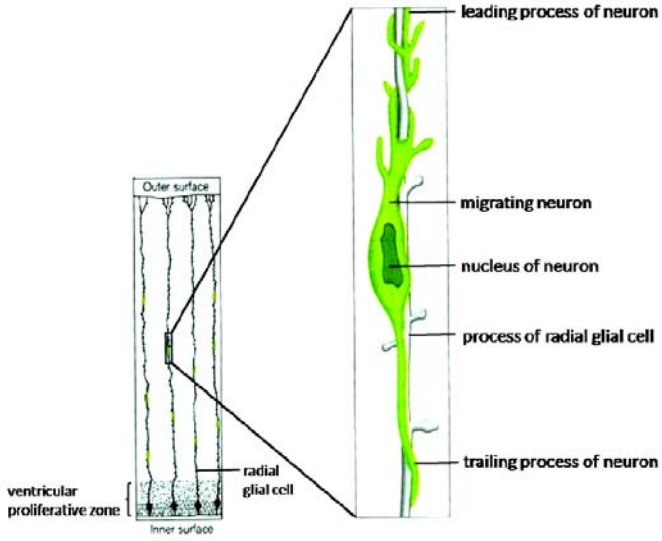

Figure 6. A radial glial cell and a migrating neuron. Modified from www.dls.ym.edu.tw/lesson3/nerv1.htm

2.4.4 Congenital malformations and malformation syndromes

Approximately 2-3% of the living newborns can be diagnosed with at least one congenital malformation. These malformations can range from very mild symptoms to very complex and gross anatomical abnormalities. Only about half of the malformations can be explained by genetic or environmental factors. Estimations of genetic causes cover about 20-30% of the cases from which about 3-10% are due to chromosomal aberrations and 4-8% are of monogenic background. Environmental factors such as teratogens explain roughly 7 to 10% and multifactorial causes 20 to 25% of the malformations (Carlson 2004; Sariola et al. 2003).

The period for most likely susceptibility to abnormal development caused by external factors such as teratogens (e.g. alcohol, drugs, ionizing radiation) is between three and eight weeks of gestation. As mentioned earlier, this is the period when most of the body regions and major organs begin to develop. Developmental abnormalities arising after eight weeks are likely to affect the function of the organ or interaction between them (for example mental retardation) or be related to the growth of already formed body parts. It should be noted, though, that different external factors can influence the fetus in the different developmental stages. Also, different organs have different periods of susceptibility during embryonic development and moreover, the most complex organs such as the brain, show prolonged periods during which they have a high probability of being influenced by these agents (Carlson 2004).

Disorders caused by a genetic factor are naturally inborn, but the onset of the recognizable symptoms can vary significantly from fetal period to adulthood. If we use monogenic disorders of Finnish disease heritage as examples, hydrolethalus, Meckel syndrome and lethal congenital contracture syndrome (LCCS) for instance lead to stillbirth or death already *in utero* or shortly after birth whereas the onset in neuronal ceroid lipofuscinoses (NCLs) can vary from infancy to adulthood depending on the form of the disease (Norio 2003c). In PLOSL (polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy), the patients are usually symptomless until 20 years of age (Norio 2003c).

3 AIMS OF THE STUDY

The aims of this study were the following:

- To identify the gene and the mutation(s) causing HLS (I).

- To define the general pathological and detailed neuropathological status associated with a defect in the *HYLS1* gene (II).

- To characterize the function of the HYLS1 protein and to gain insight into the pathogenesis of HLS (I, III).

4 SUBJECTS, MATERIALS AND METHODS

4.1 Families, DNA and tissue samples

The study consisted altogether of 54 Finnish families with 73 suspected HLS cases. The HLS diagnosis was based on ultrasound and/or autopsy findings. We had DNA samples from all the cases and also tissue samples from some of the cases. We also had an opportunity to study 11 foreign families with at least one individual diagnosed or suspected to be an HLS case. In five of these families, only the affected´s sample was available. In addition, we had an opportunity to use DNA and tissue samples from fetal control cases aborted for social reasons.

4.2 Ethical aspects

Studies on DNA and tissues of HLS cases and their family members as well as fetal control material were approved by the Ethical Committees of the Joint Authority for the Hospital District of Helsinki and Uusimaa.

4.3 Methods presented in original publications

The methods used in the present study are listed below and described in detail in the original publications (Roman numerals I-III).

Method	Original publication
Antibody production	I
Bioinformatic analyses	I, II
Cell cultures, primary and stable cell lines	I, III
Cell transfections	I, III
Cloning of cDNA	I
Confocal microscopy	I
DNA extraction	I, II
DNA sequencing	I, II
Flow cytometry	III
Fluorescence microscopy	I, II, III
Genotyping	I
Haplotype and LD analysis	I
In situ hybridization	I
Microarray analysis	III
Light microscopy	I, II
Lipid extraction from tissue	III
Polymerase chain reaction (PCR)	I, II
Production of transgenic HYLS1 *Drosophila melanogaster* lines	I
Proliferation assay	III
Protein detection by immunofluorescence staining	I, III
Protein detection by immunohistochemical staining	II
Protein detection by western blot analysis	I
Pulse-chase analysis	I
Reverse-transcriptase PCR	I
RNA extraction	I, III
Transactivation assay	III

4.4 Methods used in unpublished studies

Sumoylation assay (section 5.1.3)

For studying possible interaction between HYLS1 and SUMO-1 (small ubiquitin-like modifier 1), the overexpression cell model was used. COS-1 cells were seeded onto 10 cm Petri dishes at 1.8 x 10^6 cells per dish and transfected the next day with either wt or mutant human HYLS1 construct alone or together with His-tagged SUMO-1 construct. In addition, HYLS1, SUMO-1 and either FLAG-tagged PIAS1 construct or FLAG-tagged ARIP3 construct were transfected simultaneously. Transfections were performed using the Fugene HD transfection reagent according the manufacturer´s instructions. The cells were collected 48 h after transfections in phosphate buffer solution (PBS) containing 20 mM N-ethylmaleimide (NEM). Cell extracts were prepared in buffer containing 2% SDS, 10 mM Tris–HCl pH 8.0, and 150 mM NaCl, then incubated for 10 min at 95 °C, and diluted 1:9 in buffer containing 1× TBS, 10 mM NEM and 1% Triton X-100. The cell lysate was homogenized using a syringe and needle and centrifuged at 16,000 × g for 20 min at 4 °C. A total of 20 µl of the lysate was analyzed using 11% SDS-PAGE and western blotting followed by ECL detection.

Neuropathological study of *D. melanogaster* (section 5.2.3)

The fly strains created in publication I were used to study the neuropathological effects of wt and mutant HYLS1 in *D. melanogaster* model. A new generation of each strain was allowed to hatch after the parents were removed and a subgroup of each population was collected at time points of 0, 15, 30, 35 and 45 days. The flies were kept in +25°C and the population in each growth tube was transferred to a new one every two or three days to ensure fresh food supplies. The collected flies were terminated with CO_2, formaline-fixed and cast in paraffine blocks. The blocks were cut into thin slices with a microtome and stained with hematoxylin-eosin. The head sections were examined using an Axioplan 2 imaging microscope.

Cilia staining (section 5.3.3)

For studying the cilia structure of fibroblast cells from HLS fetuses and healthy controls, immunofluorescence staining with acetylated tubulin was used. The cells were seeded onto coverslips in 6-well plates at 1×10^6 cells per well. For immunofluorescence studies, the cells were fixed with 4% paraformaldehyde in PBS

(pH 7.3) at room temperature (RT) for 10 min and blocked and permeabilized with 0.2% saponin/0.5% bovine serum albumin (BSA) in PBS. The cells were then incubated with acetylated tubulin as primary antibody for 1 hr in room temperature (RT). After washes, the cells were incubated with secondary antibody for 40 min in RT. After the staining procedure and the last washes, the cells were mounted onto microscope slides and the data was acquired using a Leica TCS SP confocal microscope.

5 RESULTS AND DISCUSSION

5.1 Identification and characterization of the gene underlying HLS

5.1.1 Defining of the critical genomic region for HLS locus (I)

Before this study, the initial critical genomic region for HLS was mapped to chromosome 11q23-25, the size of the region being 8.5 cM, after which the region could be restricted to a 1 cM region between markers D11S933 and D11S934 (Visapää et al. 1999). In this study, the region could be further restricted to a 904 kb interval using information of the ancestral haplotype and the observed high LD between markers D11S4158 and D11S975. This was done by analyzing 16 Finnish HLS families and four unrelated Finnish HLS cases and using eight markers (D11S1752, D11S933, D11S4158, D11S1896, D11S934, D11S4110, D11S990 and D11S975) over the critical region. In this 904 kb region, the following nine genes with known function were identified: *PKNOX2, FEZ1, EI24, ITM1, CHEK1, ACRV1, PATE, PUS3* and *DDX25* (publication I and Figure 7 on page 44). In addition, one hypothetical transcript, *FLJ32915*, was found. Mutation analyses were performed for all of these genes by sequencing all coding regions and flanking intronic regions using two controls and two affected individuals that were homozygotes for the characteristic haplotype of disease alleles.

Sequencing revealed several SNPs, and these were analyzed in the whole family material. SNPs from eight candidate genes in this critical region were monitored for LD in disease alleles and one major haplotype could be found in all the analyzed affected chromosomes. Based on this analysis the critical region could be further restricted to 476 kb between genes *PKNOX2* and *DDX25* when ancestral recombinations were observed in five families. One of the SNPs in the predicted transcript FLJ32915 showed a highly significant P-value of $10^{-\infty}$ for LD between allele G and the disease phenotype. All HLS cases were homozygous for the G allele at this SNP, whereas all parental control alleles carried the normal A allele. The four cases without family members were also homozygous for this G allele. This SNP was the only nucleotide change to be completely co-segregating with the disease in the families, thus implying that it actually is the disease-causing mutation for HLS. Additional confirmation for the assumption that this SNP was the disease-causing mutation was provided by the fact that in one of the families, the father of the family was homozygous for all informative markers over the whole critical genomic region except the A to G transition in *FLJ32915* for which he was heterozygous.

The nucleotide change found to be causative for HLS was found from a predicted transcript *FLJ32915* and since the transcript was nameless at that time, we named the gene and the gene product *HYLS1* ("hydrolethalus syndrome 1").

5.1.2 The disease-causing mutation in HLS (I)

The disease-causing nucleotide change in *FLJ32915*, now named *HYLS1*, was found to be an A to G transition (c:1166 in cDNA, NCBI code NM_145014) in exon 6 (Figure 7). This point mutation causes one amino acid change in the HYLS1 polypeptide where aspartic acid 211 is changed to glycine (D211G). As is described in section 5.1.3, several studies led to the confirmation that this change is a true mutation.

The homozygous form of this nucleotide change has been found altogether in 64 diagnosed Finnish cases, these cases belonging to 47 families. Of these cases, 36 also had samples of their parents available (25 families), the parents being the heterozygous carriers of the mutation. In addition, we found one suspected HLS case with typical symptoms that was heterozygous for the *HYLS1* mutation. No additional mutations were found in the coding region of *HYLS1*. Because this fetus represents quite a typical HLS case, the possibility exists that this fetus indeed is a true HLS case, the other mutation located outside the *HYLS1* exonic regions. In eight suspected Finnish HLS cases no mutation was found. Since we currently classify only cases with D211G mutation as true HLS cases, these remaining cases might represent some other syndrome resembling HLS or they might have the mutation for example in the regulatory region of *HYLS1* or for example in the intronic region of the genomic DNA. One possibility for the mutation is also a genomic deletion. Unfortunately, since fresh tissue material for these cases was not available, screening the cDNA for the mutations was not possible.

We also had an opportunity to sequence DNA samples from 11 non-Finnish fetuses and newborns with a suspected diagnosis of HLS, but no mutations in the *HYLS1* gene could be found. These cases have been pathologically similar to HLS to some extent, but also differing features have been found. Usually, the cases have had hydrocephalus and polydactyly as frequent findings. Still, it should be noted that these two features are quite common malformations and can be found in several other syndromes in addition to HLS (R. Salonen, personal communication). Also, in many cases, the affected having a foreign background had lived even for several months, whereas all the mutation-confirmed Finnish HLS cases had died at the latest during in the very first hours or days of their lives. This would suggest that the Finnish cases suffer from ultimately severe defects in their vital organs. It has been

suggested that the underlying cause of the variability of the phenotype between Finnish and especially milder foreign cases is allelic variability (de Ravel et al. 1999; Pryde et al. 1993; Shotelersuk et al. 2001). In our opinion, though, the foreign cases (especially with the "mild" form of HLS) with no *HYLS1* confirmed mutation(s) might actually represent syndromes resembling HLS but that are in fact genetically different. Of course, this does not exclude the possibility of allelic variability by yet unknown mutations in the regions surrounding *HYLS1*, but getting the answer would require not only thorough sequencing analysis of the identified areas of *HYLS1* but extensive determination and study of the regulatory areas of the gene, as well.

The population frequency of the A to G change was determined using 454 control individuals collected from various regions of Finland and in addition, a control panel of 49 individuals of mixed European descent was used. As a result, 1.1% (2/176) of the individuals originating from the early settlement area in western Finland carried the change, whereas 2.5% (7/278) of the individuals collected from the late settlement area in eastern and central Finland were found to carry this change. No carriers were identified in the European panel of control individuals. The observed carrier frequency in the late settlement area is a bit higher than the expected frequency (2%). This might be explained by the fact that as for most early malformation syndromes, some (maybe uncertain) cases could have been overlooked and remain non-diagnosed as HLS.

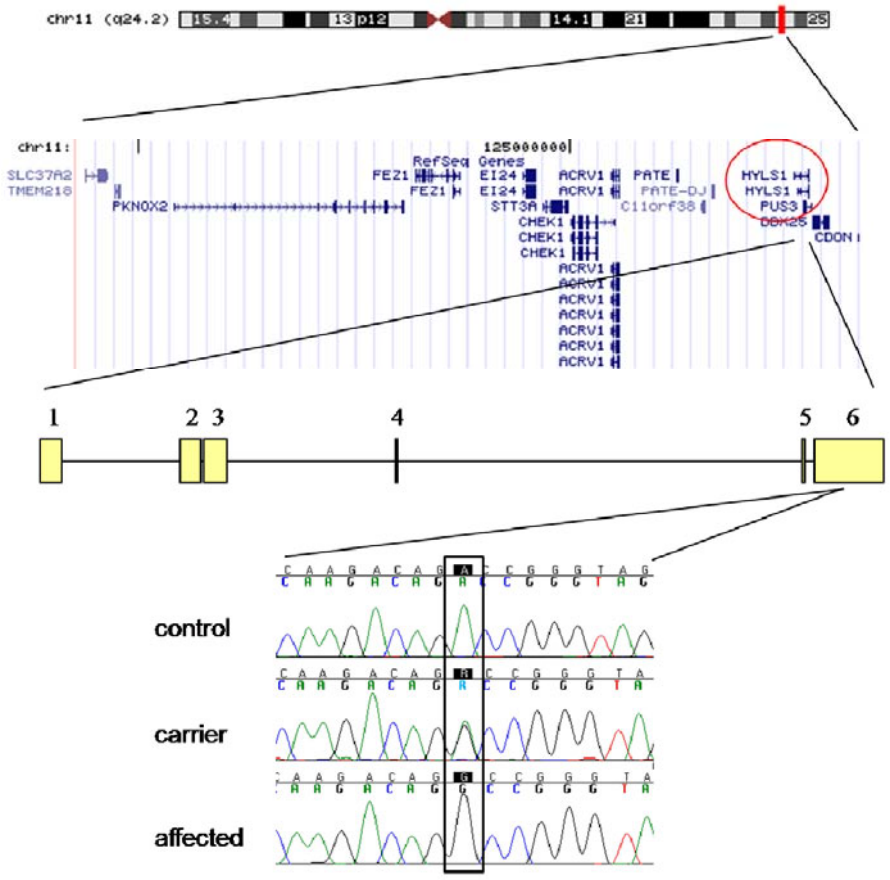

Figure 7. Schematic presentation of chromosome 11 and the 904 kb critical region for disease causing mutation in HLS between markers D11S4158 and D11S975. Also, the structure of the *HYLS1* gene with six exons and the causative A to G point mutation in the last exon are shown.

5.1.3 Basic characteristics of the *HYLS1* gene and protein (I, unpublished)

The *HYLS1* gene

Since the A to G transition in *FLJ32915*, now named *HYLS1*, was found to be a disease-causing mutation in HLS, we wanted to characterize the basic properties of this gene and the gene product.

HYLS1 covers a genomic region of approximately 17 kb with six exons in chromosome 11 (UCSC assembly 2006: chr11:125,258,719-125,275,750). The region of the gene coding for protein is composed of an open reading frame (ORF) of 897 nucleotides and situated entirely in exon 6, resulting in a polypeptide of 299 amino acids. *HYLS1* has several alternative transcripts, all of them having the same conserved translated region coded by exon 6. The most common transcripts are named *HYLS1a* (NCBI: AK127394), containing exons 2, 4 and 6 and *HYLS1b* (NCBI: BQ686872 and CB141176), containing exons 2 and 6. For a detailed picture of the sequence, see Figure 2 in publication I. The most possible promoter sites for HYLS1 were predicted using Web Promoter Scan Service (PROSCAN). According to the results, the two most likely promoter sites could be found from a region 3.4 kb upstream from the first *HYLS1* exon (nucleotides -3382-3133) and inside *HYLS1* in intron 3 (nucleotides 4626-4876).

Although exon 6 is the only protein coding exon in *HYLS1* and alternative splicing most likely does not affect the structure of the produced protein, the other exons might contain essential regulatory information that could have an effect on the expression level of HYLS1 in different tissues and/or developmental stages. For example, a 5´ untranslated region can act as an essential regulatory region for transcription or translation in genes with only one protein coding exon, see e.g. (Fukada et al. 2003; Ji et al. 2004). Since the amount of the genes identified in the human genome has turned out to be unexpectedly low, proteome complexity has to be explained through other mechanisms than merely the number of genes. Alternative splicing has been suggested as one of the main sources of the complexity, since half of the human genes have been approximated to be subjected to alternative splicing (Lander et al. 2001; Venter et al. 2001) and it has even been suggested that the majority of human transcripts are alternatively spliced (Graveley 2001). In addition to regulation at the DNA or RNA levels, over 200 different post-translational modifications have been identified (Banks et al. 2000), these most probably creating variability, too.

When comparing the observed EST (expressed sequence tag) homologies, *HYLS1a* has been identified in several tissues, including the brain, lung, liver, kidney, uterus, breast, placenta, colon and in addition, in pooled germ cell tumors. *HYLS1b* can be found at least in pancreatic carcinoma and liver. We could identify these two most common isoforms when studying the skin fibroblast cell lines´ cDNA obtained both from HLS cases and healthy controls. The occurrence of these isoforms were confirmed from a fetal cDNA panel as well as from placental tissue obtained from a healthy control and an affected individual by RT-PCR analyses using primers crossing various *HYLS1* exon boundaries. The observed fragments from PCR analyses corresponded to the expected sizes of *HYLS1a* and *HYLS1b*. In addition,

three other isoforms representing alternative splicing can be found in the NCBI and Celera databases. These three isoforms were not found from our study material. Although these isoforms were not found, it does not exclude the possibility that these isoforms are expressed, but maybe only in restricted stages during fetal development. On the contrary, *HYLS1a* and *HYLS1b* could be expressed through the whole fetal period. In addition to the A to G change, sequencing analysis of the placental RT-PCR products did not reveal any other DNA changes in an affected individual.

Also, northern blot analysis was done of control and affected mRNA in order to monitor the possible expression level changes or splice variants of the *HYLS1* gene in affected individuals. A transcript of approximately 1.7 kb was observed both in samples obtained from control and HLS cases´ fibroblasts and no significant difference in the steady state expression level was seen. When studying a commercial human fetal multiple tissue blot, the expression levels of the transcript were found to be highest in the brain, lung, kidney and liver.

The HYLS1 protein

After we had gathered the basic information of the *HYLS1* gene, we also performed several *in silico* analyses for the amino acid sequence of HYLS1. Interestingly, according to the Pfam database, HYLS1 does not possess any other recognizable functional regions than a low complexity region between amino acids 112 and 120. These regions are typical for proteins that are structurally less complex and they have been shown to be functionally important in some proteins (Wan and Wootton 2000; Wootton 1994). Still, they are generally not well understood (Wan and Wootton 2000; Wootton 1994). HYLS1 also lacks any secretory signal or signal peptide. Nevertheless, some differences can be found between wild type (wt) and mutant protein properties *in silico*. When comparing the isoelectric point (pI) values using the Expasy tool, the pI for wt protein was 6.98, whereas for the mutant protein the value was 7.67, this change most probably affecting the solubility of the protein. In addition, the PeptideCutter program estimated an Asp-N endopeptidase cleavage site at polypeptide position 211 for wt HYLS1, whereas this site was altered in the mutant form of the protein.

When comparing amino acid sequence orthologs of HYLS1, we found that the aspartic acid mutated in HLS is conserved across the species from human to *C. elegans* (see Figure 3 in publication I). Surrounding the mutated site, amino acid sequences in general show high conservation. These findings most probably indicate an important role for this mutated site and for the area surrounding it for also protein´s stucture and/or function. Aspartic acid is a bulky amino acid with a

negative charge, whereas glycine is the smallest of the amino acids with a neutral charge. Thus, glycine usually enables the polypeptide backbone to make different kinds of turns when folding. Therefore, although the mutation does not cause the protein to be truncated it may cause a severe defect in the correct structure and therefore affect the proper function of the protein.

The D211G change in HYLS1 was predicted to be probably damaging for the protein when the effect of the mutation was analyzed in the PolyPhen program (Polymorphism Phenotyping, http://genetics.bwh.harvard.edu/pph). The classification steps for PolyPhen are benign, possibly damaging and probably damaging, the latter representing the most probability for pathogenicity of the amino acid change. We also analyzed the D211G change with the SIFT program (Sorting intolerant from tolerant, http://blocks.fhcrc.org/sift/SIFT.html) (unpublished data). In this program, substituted amino acids with probabilities <0.05 are predicted to affect protein function and thus, the substitution is considered non-tolerated. As a result of this analysis, the HYLS1 substitution was predicted to affect the protein´s function.

When using the SUMOplot program (http://www.abgent.com/tools/sumoplot), wt polypeptide sequence was found to have a high probability for a sumoylation site containing lysine residue two amino acids upstream of aspartic acid 211 in the HYLS1 polypeptide sequence. In the mutated form of the polypeptide, this high probability of the site was lost (unpublished data). Thus, the mutation in HYLS1 might affect the possible sumoylation event. The small ubiquitin-like modifier, SUMO, has been a target of extensive study in the recent past. Although SUMO is related to ubiquitin which primarily targets a substrate for degradation, sumoylation regulates a substrate's functions mainly by post-translational modifications like altering the intracellular localization or protein-protein interactions. These changes in turn affect gene expression, signal transduction as well as genomic and chromosomal stability and integrity. For reviews, see e.g. (Gill 2004; Müller et al. 2001; Zhao 2007). We studied the binding of SUMO with HYLS1 in several experiments using overexpressed cell lines, both with SUMO and HYLS1 alone as well as with its interaction partners PIAS1 or ARIP3 added in the reaction. As a result, no interaction between HYLS1 and SUMO could be seen (unpublished, data not shown).

As HYLS1 was an unknown gene and protein, the *in silico* studies provided essential tools for studying the DNA and polypeptide sequence further. When using computer-assisted programs to predict different aspects of a DNA or polypeptide sequence, one should still note that the results do not provide the definitive truth of the DNA/protein properties and functions. Instead, the *in silico* results give an initial estimation of the feature in question that offers possibilities to perform further functional studies for example in cell or tissue model to verify the results.

As described above, many combined facts support the assumption that the A to G change in *HYLS1* is a disease-causing mutation. Among other *in silico* results, this point mutation was found to cause an amino acid to be replaced by another, a change that is probably damaging to the protein. Also, the high conservation level of the amino acid composition at the mutation site between orthologs most probably indicates an important role for the mutated area in protein´s stucture and/or function. To further identify the consequences of the mutation, several functional analyses were performed, the results of which are presented in the following sections.

5.1.4 Developmental expression of *Hyls1* in mouse tissues (I)

Mouse *Hyls1* gene

Mouse orthologs of *HYLS1* were found as ESTs both from the NCBI and Celera databases. Mouse *Hyls1* transcript was confirmed by sequencing of RT-PCR products using post-embryonic cDNA from mouse brain and aligning the resulted sequence with the human *HYLS1* sequence. The predicted size for the mouse transcript was 2.1 kb and this was confirmed by northern blot analysis from the embryonic mouse tissue, where a transcript of approximately 2 kb in size was observed. The expression was observed at each embryonic stage in the mouse, the highest expression level was seen at E11. Post-embryonically, the transcript was seen to be expressed from P1 to six months using RT-PCR analysis. Also, the chromosomal position where the mouse *Hyls1* can be found is syntenic to the human 11q24 region, where human *HYLS1* is situated. Mouse *Hyls1* contains four exons, the ORF being 984 nucleotides long, this frame coding for a predicted polypeptide of 328 amino acids. The similarity of mouse and human ORFs was over 80% both at the mRNA and at the amino acid levels. Exon 4 in *Hyls1* is the only translated one with a high similarity in the sequence with exon 6 in human *HYLS1*. Other *Hyls1* exons did not show significant similarity with human *HYLS1* exons.

In situ hybridization of *Hyls1*

To characterize the spatial expression pattern of *Hyls1* during mouse development, *in situ* hybridizations were performed. Embryonic sections of E12.5 and E15.5 mice as well as brain sections of three months old mice were used. At E15.5, expression could be seen in several tissues and structures, expression being particularly high in the developing CNS. In the cephalic region, *Hyls1* was detected in the telencephalon, the midbrain and the medulla. Also the ganglionic eminence and the choroid plexus expressed *Hyls1* at E15.5. In addition to these structures, expression

could be seen in the spinal cord and in the dorsal root ganglia in the nervous system area. Expression could also be seen in the neural layer of the retina. Other structures with *Hyls1* signal were the pancreas, kidney, stomach, intestine, testis and the cartilage tissue of developing limbs. Expression was also strong in the cardiovascular and respiratory organs, especially in the epithelium of the lungs. We also studied E12.5 mouse embryonic sections and as a result, we could see that the expression pattern covered the developing brain, neural tube, olfactory and oral epithelium, lung epithelium, heart, cloaca, urethra and cartilage tissue. In the adult mouse brain, the expression could be detected in the CA1 and CA3 fields of the hippocampus and in addition, in the cortex. For a detailed picture of this expression pattern, see Figure 4 in publication I.

Although there is a possibility that human and mouse HYLS1 proteins may not function identically, the observed broad expression pattern in the mouse correlates well with the phenotype of human HLS cases, the pattern reflecting the affected tissues in HLS. The expression of *Hyls1* in many embryonic mouse tissues further strengthens the initial theory of the essential role of HYLS1 in fetal developmental processes in many developing organs, including the CNS.

5.2 Characterization of the HYLS1 protein

5.2.1 Expression and stability (I)

Using computer assisted programs, *HYLS1* was predicted to code for a 299 amino acid with a molecular weight of 34.4 kilodaltons (kDa). To characterize the relative molecular weight of HYLS1 protein, *in vitro* transcription and translation of an *in vitro* rabbit reticulocyte lysate system was used. As a result, both wt and D211G mutant constructs produced polypeptide chains of approximately 39 and 40 kDa. The 40 kDa polypeptide most likely represents the complete HYLS1 polypeptide, whereas the 39 kDa polypeptide most probably results from the use of an alternative translation initiation site 14 amino acids downstream from the first methionine.

Both constructs were also expressed in COS-1 cells after which a western blot analysis was performed. Synthesized polypeptides were detected with the rabbit polyclonal anti-HYLS1 antibody, a peptide antibody that was designed against amino acids 147-161 of the HYLS1 polypeptide. Both wt and mutant forms of HYLS1 showed identical expression of HYLS1 with immunopositive bands of approximately 40 kDa. This result well agrees with the *in vitro* translated polypeptides. The corresponding protein levels between the wt and the mutant HYLS1 form were similar. In addition to

these studies, a pulse-chase analysis was performed in order to study the stability of both polypeptides. The half-lives of both forms were about three hours and this analysis did not reveal any significant difference in the amount of immunoprecipitated polypeptides from wt and mutant constructs.

These analyses show that both wt and mutant forms of HYLS1 are of the same size and that their expression levels and half-lives are identical. Thus, it can be assumed that the mutation in the gene does not lead to degradation of the mutated protein after it has been translated suggesting that the deleterious effect of the mutation is due to altered functional properties of the protein.

5.2.2 Subcellular localization of HYLS1 in a cell model (I)

As HYLS1 was a novel protein, there was no previous information on its subcellular localization in the cells. When the localization was determined with computer assisted programs, SignalP did not predict a nuclear localization signal, but PSORTII analysis predicted a possible nuclear localization for the polypeptide with a probability of 52%.

To elucidate the localization of HYLS1 in a cell model and possible differences between wt and mutant forms of the protein, we transiently expressed HYLS1 in COS-1 cells. Wt and mutant HYLS1 constructs were also tagged with FLAG and Myc tags to confirm the specificity of anti-HYLS1 antibody. COS-1 cells were transfected either with FLAG-tagged, Myc-tagged or untagged wt or mutant HYLS1 constructs. The immunostaining was performed with specific antibodies for the constructs (anti-FLAG-, anti-Myc- and rabbit anti-HYLS1 antibodies, respectively). The synthesized polypeptides were detected with the same rabbit polyclonal anti-HYLS1 antibody as described in section 5.2.1. Localization of the tagged proteins showed the peptide antibody to be specific for HYLS1.

As a result, cells transfected with wt form of the protein showed mostly diffuse cytoplasmic staining, whereas mutant polypeptides largely localized to punctate nuclear inclusions (Table 2, Figure 6 in publication I). The proportion of cells containing solely nuclear inclusions was over 40% in cells containing the mutant form of the protein, compared to under 2% in cells containing the wt form. Cells with both staining patterns were observed approximately in half of the cells in both groups. The dual localization pattern observed here might at least partly explain the *in silico* localization results with no solid result of either nuclear or cytoplasmic compartment in the cell. Differential subcellular localization is also presented in Figure 8.

After observing the differential localization of wt and mutant HYLS1 forms, we were also interested to see whether HYLS1 co-localizes with some of the functional subcellular compartments. This was performed using antibodies having known subcellular localization. We did not see any significant co-localization when comparing the subcellular localization of HYLS1 with H4A3 (lysosomes), 58K (Golgi apparatus) or PDI (endoplasmic reticulum).

Table 2. Distribution of HYLS1 polypeptide in COS-1 cells.

construct	cytoplasmic %	cytoplasmic and nuclear %	nuclear %
wt	49.4	48.8	1.8
D211G mutant	6.2	53.0	40.7

5.2.3 Localization of HYLS1 in *D. melanogaster* model (I)

Subcellular localization of HYLS1 was also studied *in vivo* using a *Drosophila melanogaster* (fruit fly) model, an organism that does not naturally possess HYLS1 in its genome. Wt and mutant constructs encoding human *HYLS1* were overexpressed in the *D. melanogaster* third instar eye discs using a GMR-GAL4 driver. The antibody used here was the same peptide antibody that was also used in overexpression cell studies (described in section 5.2.2). As the antibody proved to be functioning in the overexpression cell model, we were convinced that it can be used also in the characterization of HYLS1 in the transgenic *in vivo* environment. First, western blot analyses were performed to protein extracted from adult fly heads expressing wt HYLS1 and also from control flies. A band of approximately 40 kDa was observed from the head lysates expressing HYLS1, which corresponds to the size of the HYLS1 protein. No band was observed in control head lysates, as expected.

The same cellular localization pattern observed in *in vitro* cell studies was also found when studying the immunostaining of larval eye discs with rabbit anti-HYLS1 antibody for both wt and mutant forms of HYLS1. Cells expressing wt HYLS1 largely showed cytoplasmic staining with some perinuclear inclusions and cells expressing the mutant form revealed a staining pattern of nuclear dot-like structures. Control cells containing no HYLS1 constructs showed no staining as was expected, as *D. melanogaster* does not naturally possess the *HYLS1* gene. These results

provided further evidence of differential localization between wt and D211G mutant HYLS1.

5.2.4 Analysis of nuclear export signal in HYLS1 (III)

In HYLS1, no putative nuclear export sequences could be identified in the amino acid sequence *in silico*. However, the dual subcellular localization of HYLS1 in the cytoplasm and nucleus studied both *in vitro* and *in vivo* would suggest that it might, in addition to most probably being transported into the nucleus, also be actively exported from it. To test this hypothesis, HEK-293 cells were transfected with constructs expressing either wt or D211G mutant HYLS1 and subjected to leptomycin B (LMB). LBM is known to be an inhibitor of the nuclear export mechanism that CRM1 (or exportin 1) mediates, and CRM1 is one of the best characterized nuclear export receptors. After immunofluorescence microscopy, the proportion of cells exhibiting either a nuclear, cytoplasmic or combined nuclear and cytoplasmic staining pattern was measured. As a result, we observed that the treatment of cells with LMB (15 ng/ml) for 6 hours led to exclusively nuclear localization of both wt and mutant HYLS1 that significantly differed from the untreated state (both wt and mutant treated state versus corresponding untreated state $p<0.001$ by student´s t-test). On the contrary, without LMB treatment most of the wt cells had cytoplasmic localization whereas cells transfected with mutant construct localized both in the nucleus and cytoplasm (student´s t-test between untreated wt and mutant $p<0.001$). An example result is presented in Figure 8.

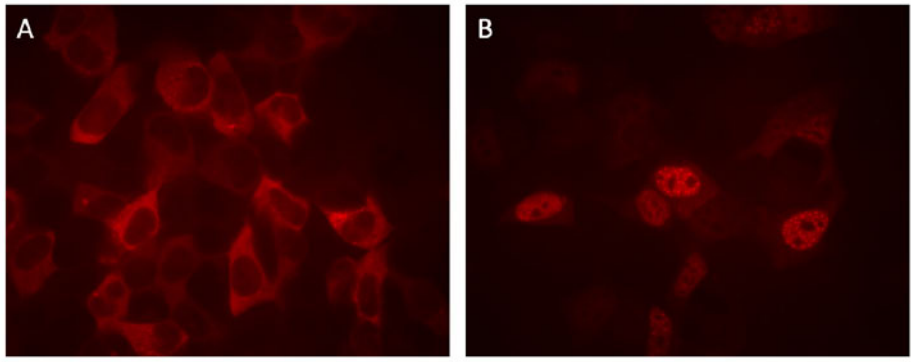

Figure 8. HEK-293 cells transfected with a construct expressing wt HYLS1. Before LMB treatment HYLS1 shows predominantly cytoplasmic localization (A), whereas after the treatment HYLS1 is localized in the nuclei (B).

5.2.5 Transactivation assay (III)

On the basis of the results from several functional studies presented in the previous sections, we assumed that HYLS1 might have a role as a transcriptional regulator. To study this hypothesis, we performed a transactivation assay with luciferase reporter to test whether the protein possesses transactivation capacity. For this, constructs expressing either wt or D211G mutant HYLS1 in fusion with the Gal4-DBD as well as the reporter vector pG5LUC were transfected to the SH-SY5Y neuroblastoma cells. As a result, the wt protein was shown to activate the transcription by 9.4-fold compared to blank vector, whereas the mutant HYLS1 was capable of increasing the transactivation only by 3.4-fold. The difference between vector and wt was statistically significant (p-value <0.01 with student´s t-test). The same was true between vector and mutant (p-value <0.05). Also the difference between the wild type and mutant form of HYLS1 was significant with a p-value of <0.01. Therefore, we could conclude that HYLS1 has transactivation capacity and this capacity is significantly decreased in cells containing the mutant form of the protein.

These results combined with the results of the *in vitro* and *in vivo* overexpression model of HYLS1 in cell studies (sections 5.2.2-5.2.4) lend support for the hypothesis that HYLS1 is shuttled between the nucleus and cytoplasm and that it is a protein that probably functions as a transcriptional regulator in cells. At present, it is known that many essential proteins are shuttled between the nucleus and cytoplasm to control their function, these including cell cycle regulators and RNA binding proteins, steroid hormone receptors as well as transport receptors and adaptors (Gama-Carvalho and Carmo-Fonseca 2001). Also, the activities of many transcription factors or cofactors have been reported to be controlled via nucleocytoplasmic shuttling (Gama-Carvalho and Carmo-Fonseca 2001; Smith and Koopman 2004). Abnormalities in the shuttling process may have a role for example in the development of neurodegenerative diseases (Chu et al. 2007). Shuttling proteins usually have both a nuclear localization signal (NLS) and a nuclear export signal (NES) that are used to guide the protein through channels formed by the nuclear pore complexes (NPCs). In some cases, the same amino acid sequence is used for both import and export purposes (Gama-Carvalho and Carmo-Fonseca 2001). Although conserved forms of these signals have been found, some of the signals have remained uncharacterized, see e.g. (Nakielny and Dreyfuss 1999). No known NLS or NES was found in HYLS1 when analyzing the polypeptide sequence *in silico*.

In addition to being transported between the nucleus and cytoplasm with the aid of carrier proteins, a protein can be passively diffused between these compartments without assisting proteins because of their small size, the size limit varying between 40 and 60 kDa (Terry et al. 2007; Yoneda 2000). In most cases, macromolecules

larger than this are actively transported across the NPC (Terry et al. 2007; Yoneda 2000). Thus, the molecular weight of HYLS1 is at the border of these two options. Nuclear export studies imply that HYLS1 contains a NES since LMB inhibits HYLS1 from getting out of nucleus. Since *in silico* programs did not identify any NES in HYLS1, the possibility exists that this signal could be either non-conservative or situated in a fragmented form in the polypeptide sequence. The mutated region in HYLS1 most probably is either directly part of the NES or the mutation changes the conformation of the protein in a way that the NES becomes inactive. Entrapment of the mutated protein in the nucleus and thus the lack of the normal regulatory mechanism of this transcriptional regulator in cells might explain the diminished transactivation potential of mutant HYLS1, as well.

Based on the results presented above, it can be hypothesized that HYLS1 is part of the transcription regulatory machinery. One explanation for the reduced transactivation potential in mutated HYLS1 could be that the mutated HYLS1 is unable to reach the activity level required for normal function and this leads to disturbed downstream pathway regulation. Another possibility is that the mutant HYLS1 form somehow disturbs normal developmental pathway(s) when it is mutated and for that reason the protein does not function normally. However, additional analyses would be needed to clarify the precise effect the mutation causes in the protein and also, whether HYLS1 directly binds to DNA and thus, acts as an actual transcription factor.

5.3 Unraveling the disease pathogenesis of HLS

5.3.1 Mutation confirmation of study samples (II)

The main focus in publication II was to produce a detailed neuropathological picture of HLS for the first time. Analyses were made from archival autopsy specimens of 21 HLS cases collected during 1981-2008. DNA from all of these cases was isolated and sequenced in order to verify the *HYLS1* mutation status. A special extraction method had to be used since the tissue samples were mostly embedded in paraffin blocks and in addition, the sequencing process had to be done with primer pairs flanking a region of no more than 300 bp in order to be able to get readable DNA stretches for sequencing analysis because the DNA was old and fragmented. In addition to the 21 samples verified to be homozygous for *HYLS1* mutation, one sample was found to be heterozygous for the mutation and in one sample, no mutation in *HYLS1* was found. These cases were excluded from further studies.

5.3.2 General autopsy findings in HLS (II)

The general autopsy findings of 21 mutation-confirmed HLS cases studied in publication II are listed in Table 3.

As it can be seen in the table, the phenotypic spectrum of HLS is notably wide. Some of the findings are very severe already at the early stage of fetal development whereas some cases show only mild symptoms (see Figures 1A and 1B in publication II). The reason for this variation is still uncertain, but it can be speculated whether the spectrum is caused by the mutated form of HYLS1 alone or if there are also additional factors behind the phenomenon. These can include for example possible modifier genes (Genin et al. 2008) and/or external factors, e.g. environmental conditions during fetal development.

All of the cases studied had micrognathia, i.e. a small mandible. Also a lung defect was a common finding with a frequency of 90% of the cases. Cleft lip or palate was found in about half of the cases. Other symptoms to be mentioned are hallux duplex that had been reported in nine cases (42%) and uterus duplex, a rare finding in healthy individuals that was found in over 20% of the cases.

Table 3. General pathological findings in HLS cases. Modified from Table 1 in publication II.

Case	1	2a	3	2b	4	5	6a	7	8	9	10	6b	11	12	13	14	15	16	17	18	19
Gestational age	12+1	13+3	15+6	17+6	18+0	18+1	18+2	18+2	19+0	19+5	19+5	19+6	20+5	24+0	33+5	33+5	34+0	34+0	36+4	36+6	40+6
Survival	ia	ia	ia	ia	ia	ia	ia	ia	ia	ia	ia	ia	ia	ia	6 d	20 min	sb	sb	5 h	sb	1 min
Sex	f	f	f	m	f	m	m	m	m	m	m	f	f	m	f	f	m	f	f	m	m
Body length, ch (cm)	8	10	17	20	19	22	17,5	20,5	20	22	25	22	25	19,5	42	35,5	45	47	48	45	49
Body weight (g)	9	31	103	210	160	315	149	209	323	245	250	170	360	245	2326	1162	2520	2520	2695	2050	3864
Cleft lip/palate	+	-	-	-	+	-	+	+	+	+	-	+	+	+	-	-	+	+	-	-	nr
Small mandible	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Polydactyly of fingers	-/-	-/-	-/+	-/-	+/+	+/+	+/+	-/-	- /-	-/-	-/-	+/+	+/+	-/-	-/+	+/-	+/+	+/+	-/-	-/-	-/-
Polydactyly of toes	-/-	+/+	+/+, hd	-/-	+/+, hd	+/++	+/-	-/-	+/+, hd	+/+, hd	+/+, hd	+/+, hd	-/+	+/+, hd	-/-	+/+, hd	++/+	+/+, hd	-/-	-/-	-/-
CHD	-	-	-	-	-	-	+	-	+	nr	-	nr	+	+	-	-	+	-	-	+	-
Anomalous tongue	-	+	-	+	-	+	+	-	-	+	-	+	+	+	+	-	-	+	-	+	-
Abnormal larynx/trachea	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Lung defect	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Uterus duplex	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-	-	-

Cases are arranged according to their gestational age; 2a & 2b and 6a & 6b are siblings; ia = induced abortion; sb = stillborn; ch = crown-heel; hd = hallux duplex; chd = congenital heart defect; + = abnormal finding positively identified; - = abnormal finding not present; ++ = multiple extra toes; nr = not reported

5.3.3 Macroscopic neuropathological picture of HLS (II)

Major CNS findings in HLS cases are presented in Table 4. The highly characteristic finding was a hydrocephalus with unique features in which the lateral ventricles opened into an interhemispheric space that was filled with cerebrospinal fluid. This kind of hydrocephalus was seen in 17 of 21 cases. A constant and a hallmark finding was a special kind of occipitoschisis, the cleft extending from the foramen magnum to form a keyhole-shaped opening in the skull base (Figure 9A). Sometimes, the cerebellum or the meninges bulged through the opening in the neck beneath the intact skin. An "open-book" phenomenon was a typical finding, formed by the separated cerebral hemispheres that were lying on the bottom of the skull (Figure 9B). A large amount of cerebrospinal fluid above was discovered especially in the cases born in the second or third trimesters of pregnancy. The upper midline structures, septum pellucidum, corpus callosum, and fornicles were absent.

Hydrocephalus is one of the most common malformations with an estimated incidence of 1 in 1500 births (Zhang et al. 2006) and there are many different possibilities for the cause. Most cerebrospinal fluid (CSF) is produced by a highly vascular in-growth through the ependymal lining of the brain ventricles known as the choroid plexus (CP) (for a review, see e.g. (Brodbelt and Stoodley 2007). In addition to its function as the producer of CSF, CP is an important interface between the peripheral blood and CSF. It has been suggested that CP is also involved in cell growth, axon guidance and migration of neurons in CNS via the polypeptides it produces (Emerich et al. 2005). It has also been shown that hydrocephalus in rats impairs the cortical development of the brain, the results suggesting that the circulating CSF and its associated factors are essential for development of the neural tube during embryonic development (Emerich et al. 2005). As hydrocephalus is one of the hallmark features in HLS, it would be interesting to study the possible role of excess CSF in the pathogenesis of the syndrome. Hydrocephalus can form, for example, due to an imbalance between the production and resorption of CSF or if its circulation is blocked (Carlson 2004). The precise mechanism of hydrocephalus in HLS or whether it is a primary or a secondary effect of HYLS1 malfunction is not currently known. In our *in situ* hybridizations performed using embryonic mouse sections (publication I), we could see a specific staining of CP in mouse brain. This result might indicate that HYLS1 expressed in CNS has a role in the production of CSF in the brain.

Table 4. Major CNS findings in HLS cases. Modified from Table 2 in publication II.

Case	1	2a	3	2b	4	5	6a	7	8	9	10	6b	11	12	13	14	15	16	17	18	19
Macroscopic findings																					
Brain weight (g)	nr	4	nr	28	16	nr	nr	40	nr	6	35	nr	40	nr	206	153	146	nr	249	265	286
Hydranencephaly/anencephaly	-	-	-	-	-	-	hyd	-	-	hyd	-	hyd	-	an	-	-	-	-	-	-	-
Hydrocephalus/"open-book"	+	+	+	+	+	+	nd	+	+	nd	+	nd	+	nd	+	+	+	+	+	+	+
Olfactory aplasia	+	+	+	+	+	+	nd	+	+	nd	+	nd	+	nd	+	+	+	nr	+	+	+
Callosal agenesia	+	+	+	+	+	+	nd	+	+	nd	+	nd	+	nd	+	+	+	+	+	+	+
Fused thalami	+	+	+	+	+	+	nd	+	+	+	+	nd	+	nd	+	+	+	+	nr	+	+
Hypothalamic hamartoma/bulging	-/-	-/-	-/+	-/+	-/+	-/-	nd	-/-	-/+	nd	-/-	nd	-/+	nd	-/+	+/-	+/-	+/-	-/+	+/-	+/-
Occipitotemporal hypoplasia	+	+	+	-	+	+	nd	+	+	nd	+	nd	+	nd	+	+	+	+	+	+	+
Infratentorial hypoplasia	+	+	+	-	+	+	nd	+	+	nd	+	nd	+	nd	+	+	+	+	+	+	+
Occipitoschisis/"key-hole"	+	+	+	+	+	+	+	+	+	+	+	+	+	nd	+	+	+	nr	+	+	+
Microscopic findings																					
Leptomeningeal heterotopia	-	-	+	-	-	+	nd	+	-	nd	+	nd	+	nd	+	+	+	nr	+	+	+
Polymicrogyria	+	+	+	+	+	+	nd	+	+	nd	+	nd	+	nd	+	+	+	nr	+	+	+
Lissencephaly II	-	-	-	+	-	+	nd	+	+	nd	+	nd	+	nd	+	+	+	+	+	+	+
Neuroepithelial rosettes	+	+	+	-	-	+	nd	-	+	nd	+	nd	-	nd	+	+	+	nr	+	-	+
Periventricular heterotopia	-	-	-	+	+	-	nd	-	-	nd	-	nd	-	nd	+	-	+	nr	+	-	-

The case order is the same as in Table 3; + = abnormal finding positively identified; - = abnormal finding not present; nd = not detectable; nr = not reported

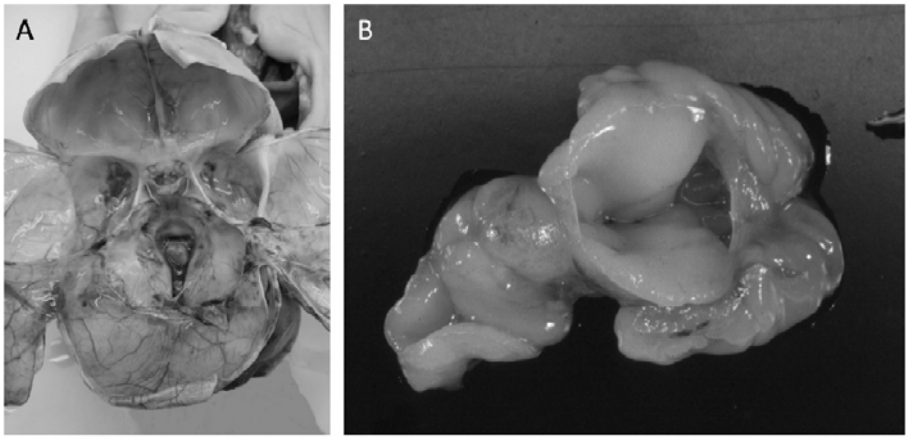

Figure 9. A) A key-hole like opening in the skull base of an HLS case of 33rd gestational week. B) Abnormal "open-book" brain structure of an HLS case of 13th gestational week.

Ependymal cells lining the brain ventricles contain motile cilia, but their biological function has remained obscure. Ibañez-Tallon et al. (2004) have reported that ependymal cilia generate a laminar flow of cerebrospinal fluid through the cerebral aqueduct. They proved that the axonemal dynein heavy chain gene (*Mdnah5*) was specifically expressed in ependymal cells and that it is essential for ultrastructural and functional integrity of ependymal cilia. In *Mdnah5*-mutant mice, a lack of ependymal flow caused closure of the aqueduct and formation of hydrocephalus during mouse brain development (Ibanez-Tallon et al. 2004). According to these findings, cilia might have an essential role in cerebrospinal fluid regulation.

The macroscopic brain architecture in HLS was also defective. The gyration of the brain was often grossly abnormal in the frontoparietal regions, and the temporal and occipital regions were found to be severely hypoplastic. Normally developed amygdala or hippocampi could not be found. A narrow cleft at the junction of thalami and rostral mesencephalon was identified, this probably representing a hypoplastic third ventricle. The brainstem and cerebellum were hypoplastic. The mesencephalon was the most normal-appearing region of the brainstem. The cerebellum was found to be abnormally small.

A very interesting finding was a polyploid hypothalamic hamartoma that was observed in several cases. These were of considerable size in some of the cases that were born in the last trimester of the pregnancy. In the early fetal cases, the

hamartoma was more difficult to observe and in some cases, only a bulging of the hypothalamic plate region could be seen. According to the numerical syndromology studies Verloes have reported (Verloes 1995), three non-Finnish cases (Anyane-Yeboa et al. 1987; Hingorani et al. 1991) classified as HLS all also had a hamartoblastoma finding in addition to other malformations similar to HLS. This would be an interesting small detail to be discussed when possible phenotypic borders are set for the foreign suspected HLS cases.

In four of the cases (cases 6a, 6b, 9, and 12 in publication II), the main macroscopic finding was anencephaly (one case) or hydranencephaly (three cases) instead of the usually found "open-book" hydrocephalus. Two of the fetuses with hydranencephaly were siblings (cases 6a and 6b). Alobar or semilobar holoprosencephaly was not found in this study; in the HLS cases a complete interhemispheric fissure could always be observed, indicating hemispheric cleavage. At the moment, it is not known why some of the HLS cases have anencephaly or hydranencephaly instead of the "open-book" appearance in the brain.

Cilia in HLS cells (unpublished)

Cilia are microtubule-based hair-like organelles extending from the cell surface of almost all polarized cell types of the human body, the amount of cilia ranging from one to hundreds per cell; for a review, see e.g. (Fliegauf et al. 2007). As mentioned in the previous section and as discussed in several recent reports, cilia are proven to be highly essential structures in several body functions, cerebrospinal fluid regulation as one example (discussed above). Evidence about cilia being also an important part of central signaling mechanisms, including the SHH signaling pathway, have been reported (Fliegauf et al. 2007). The SHH pathway will be discussed further in section 5.3.7. As the normal fetal developmental events, for example the formation of left-right asymmetry, is also highly dependent on the normal ciliary function and since HLS resembles Meckel syndrome, another lethal malformation syndrome of the fetal period in which the cilia formation is severely disturbed in mutant cells (Kyttälä et al. 2006; Tallila et al. 2008), we performed immunofluorescence staining using skin fibroblasts from HLS cases and healthy controls in order to compare the formation of the cilia between the cell groups. The initial results showed that in contrast to Meckel syndrome, both the wt and HLS mutant cells contain cilia (Figure 10) (unpublished data). However, we cannot exclude the possibility that there is a defect of cilia to some degree in HLS cells. For example, in the *C. elegans* animal model of another ciliary disease called the Bardet-Biedl syndrome (MIM 209900), the cilia are somewhat visible both in control and mutant cells but their structure is abnormal in mutant cells (Li et al. 2008). Thus,

more studies would be needed to find out whether detailed cilia formation is disturbed also in HLS.

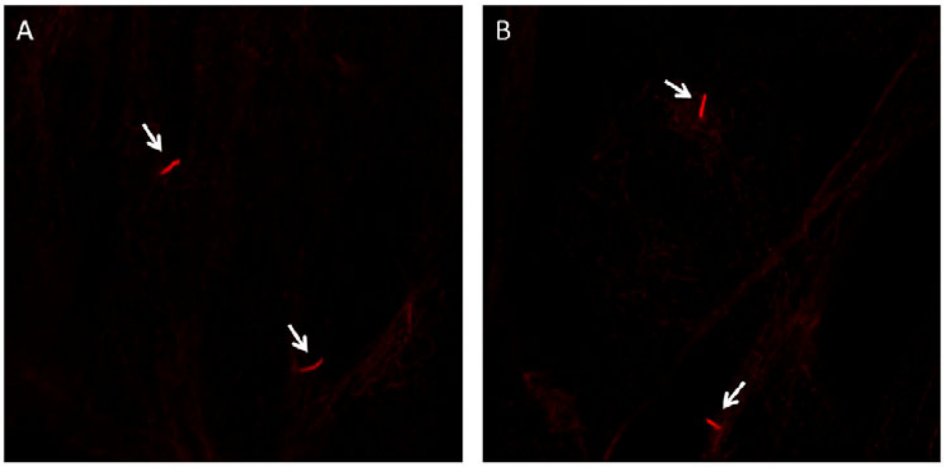

Figure 10. Skin fibroblast cells from both a healthy control (A) and an HLS fetus (B) show cilia staining (arrows).

5.3.4 Microscopic neuropathological picture of HLS (II, unpublished)

In addition to macroscopic examination of the HLS fetuses, a detailed microscopic study of the samples was performed using immunohistochemical stainings.

When studying the cerebral cortex histologically, it was found to be immature with polymicrogyric and lissencephaly type 2 –like areas. Disorganized neuroblastic rosettes were also observed (Figure 11A) as were irregular and disrupted bundles of radial glial structures (Figure 11B). Ectopic neuroblastic rosettes could also be frequently observed under the cortical region, with a quite high proliferation rate when using MIB-1 staining. As an explanation for these rosette structures, we hypothesized that they could represent aberrant miniature “neural tubes”, caused by an abnormal signal promoting the development of the dorsal neural tube in these immature and disorganized regions of the fetal brain.

Calretinin positive neuronal clusters were prominent in the superficial cortical areas. Because these positive cells also expressed reelin, they can be considered to be Cajal-Retzius cells. Reelin, a protein secreted by Cajal-Retzius cells, is essential for

completion of neuronal migration and cortical lamination. Abnormal amount, clustering and persistence of these cells have been reported in cases of polymicrogyria and this phenomenon is probably common in disorders related to defective neuronal migration (Eriksson et al. 2001). Another interesting aspect is that according to some studies with mouse models, the normal function of GLI3 protein has been demonstrated to be essential for correct cortical lamination (Friedrichs et al. 2008; Theil 2005). This fact combined with the disturbed cortical structure in HLS fetuses might suggest that HYLS1 functions using a similar mechanism as GLI3.

The hypothalamic hamartomas mostly consisted of neuronal cells, but also some glial cells were seen. In addition, some smaller, probably immature cells and neuropil were seen. Quite normal looking, although fragmented, choroid plexus structures were seen in many cases at the basal corners of the open interhemispheric space, and also often in the region of the fourth ventricle. The cerebellar cortex showed narrowing of all its layers. The basis of the pons was histologically severely atrophic and defects were also seen in the brain stem area.

The histology of the spinal cord was studied in four cases (cases 2b, 9, 15 and 17 in publication II). The central canal was slit-like in the cervical part and the posterior columns were hypoplastic. One spinal cord was bifid in the ventral part, one had a dorsal hamartomatous bulging in its cervical part and two spinal cords had double central canal in the lumbar region. The motor columns were found to be quite normally populated.

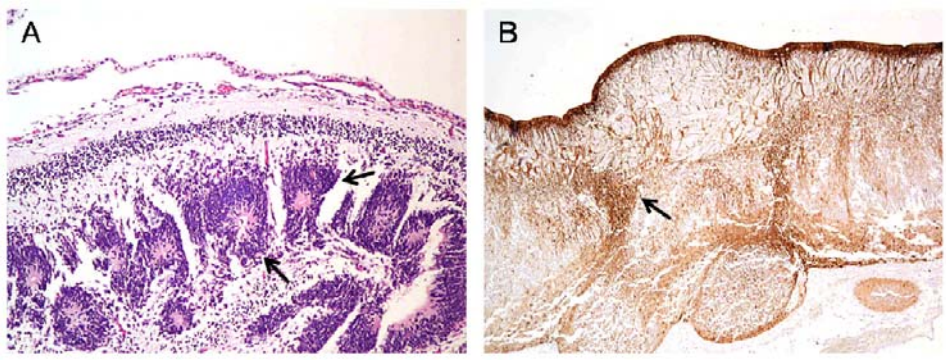

Figure 11. A) Immature and disorganized neuroblastic rosettes (arrows) were observed in the cerebral cortex of an HLS fetus at the 17th gestation week. B) Also irregular and disrupted bundles of radial glial structures (arrow) could be seen in the same fetal sample.

Interestingly, in microarray analysis (see section 5.3.5), the neuropilin 2 (*NRP2*) gene was found to be downregulated by over 3-fold in HLS cells when compared to control cells. Neuropilins are found to function as receptors for some of the semaphorins, whereas semaphorins act as chemorepulsive signals in interneuron migration. Marin et al. used a mouse model to demonstrate that interneurons expressing neuropilins avoid entering the striatum, a subcortical part of the telencephalon, because of this repulsive signal and are thus directed to the cortex (Marin et al. 2001). Loss of neuropilin function, in turn, increases the number of interneurons that migrate into the striatum. Marin et al. concluded that their observations reveal a mechanism by which neuropilins mediate sorting of distinct neuronal populations into different brain structures, and provide evidence that, in addition to guiding axons, neuropilins also control neuronal migration in the central nervous system. Therefore, in the absence of neuropilins, the correct migration pattern of interneurons could also be compromised in the brains of HLS cases. Thus, the expression array finding nicely binds with the disrupted cortical patterning observed in our studies of brain architecture.

In conclusion, the pattern of brain pathology found in the HLS cases described above clearly belongs to the midline patterning defects. The pattern seems to be unique for the hydrolethalus syndrome, combining the features of disturbed neurulation, forebrain patterning, and migration. As the SHH signaling pathway plays an essential part in fetal midline development, it is tempting to hypothesize that defects in HLS are caused by a defect in this pathway, too. Currently, additional analyses would be needed to confirm this hypothesis. The SHH pathway will be discussed further in section 5.3.7.

Fly model of HLS neuropathology (unpublished)

In addition to characterization of the human fetuses affected with HLS, a *Drosophila melanogaster* model of HLS generated earlier was used to determine whether any neuropathological changes similar to those seen in humans could be replicated when studying neural structures of flies expressing either wt or mutant forms of HYLS1 (unpublished, data not shown). HYLS1 was directed to be expressed in either eye retina with the gmr fly line or in CNS with the elav fly line using the GAL4-UAS system (St Johnston 2002). As a result, no significant differences between wt and mutant flies were found. Some vacuolar structures of the eye and the CNS were observed in mutant flies, but this could be due to the strong overexpression of HYLS1 in these structures in some of the fly lines, rather than a specific indication of altered HYLS1 function.

5.3.5 Genome-wide gene expression analysis (III)

Although *HYLS1* was suggested to encode a transcriptional regulator, the exact information of the gene and the protein function was unclear. To gain additional clues via cellular pathways, we performed a genome-wide gene expression analysis of fetal fibroblast cell lines (control N=4, HLS N=3) to obtain novel data on cellular pathways influencing the HLS pathogenesis. For the analysis, we used the genome-wide Affymetrix array system. After the chip data was initially analyzed, we ended up with altogether 802 transcripts and from this group we chose a subgroup of these as statistically significant to be analyzed further.

Several pathways were found to be significantly differentially expressed between the control and HLS cells (Figure 12). When studying the whole data set, the difference in the amount of expression of the transcripts varied from -8.02 to +13.79. For the whole list of regulated genes, see Supplementary Table 1 in publication III. After examination of the gene lists with the Webgestalt program, many of the upregulated transcripts were found to be associated with the cell cycle regulatory cascades and with specific signal transduction pathways. The downregulated transcripts fell into the general category of lipid metabolism.

The most upregulated genes inside the groups cell cycle regulation and signal transduction included dual specificity phosphatase 6 (*DUSP6*), platelet-derived growth factor alpha polypeptide (*PDGFA*), and mitogen-activated protein kinase kinase kinase 5 (*MAP3K5*). The fold-changes were 7.06, 3.76 and 2.92, respectively. Other interesting upregulated genes were for example cyclin D1 (*CCND1*, fold-change 2.91), fibroblast growth factor 5 (*FGF5*, 2.81) and CASP8- and FADD-like apoptosis regulator (*CFLAR*, 2.63). The downregulated genes related to lipid metabolism included like-glycosyltransferase (*LARGE*, fold-change -3.54) stearoyl-CoA desaturase (*SCD*, -3.50), acetyl-Coenzyme A acetyltransferase 2 (*ACAT2*, -3.02), low density lipoprotein receptor (*LDLR*, -2.83), 7-dehydrocholesterol reductase (*DHCR7*, -2.53) and 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (*HMGCS1*, -2.20) among others. Downregulated genes related to axon guidance included spondin 2 (*SPON2*, -7.15) and neuropilin 2 (*NRP2*, -3.19). These findings led to subsequent analyses that are discussed in sections 5.3.6 and 5.3.7.

As the analyses were performed using skin fibroblast, they might not reveal all the essential pathways defective in the CNS and therefore, it would be interesting to use cell lines of neuronal cell populations, too. Unfortunately, an insufficient amount of the neuronal cell lines was available for these analyses. However, the findings acquired using the fibroblast cell lines provide us with useful additional clues for the process of unraveling the HYLS1 function.

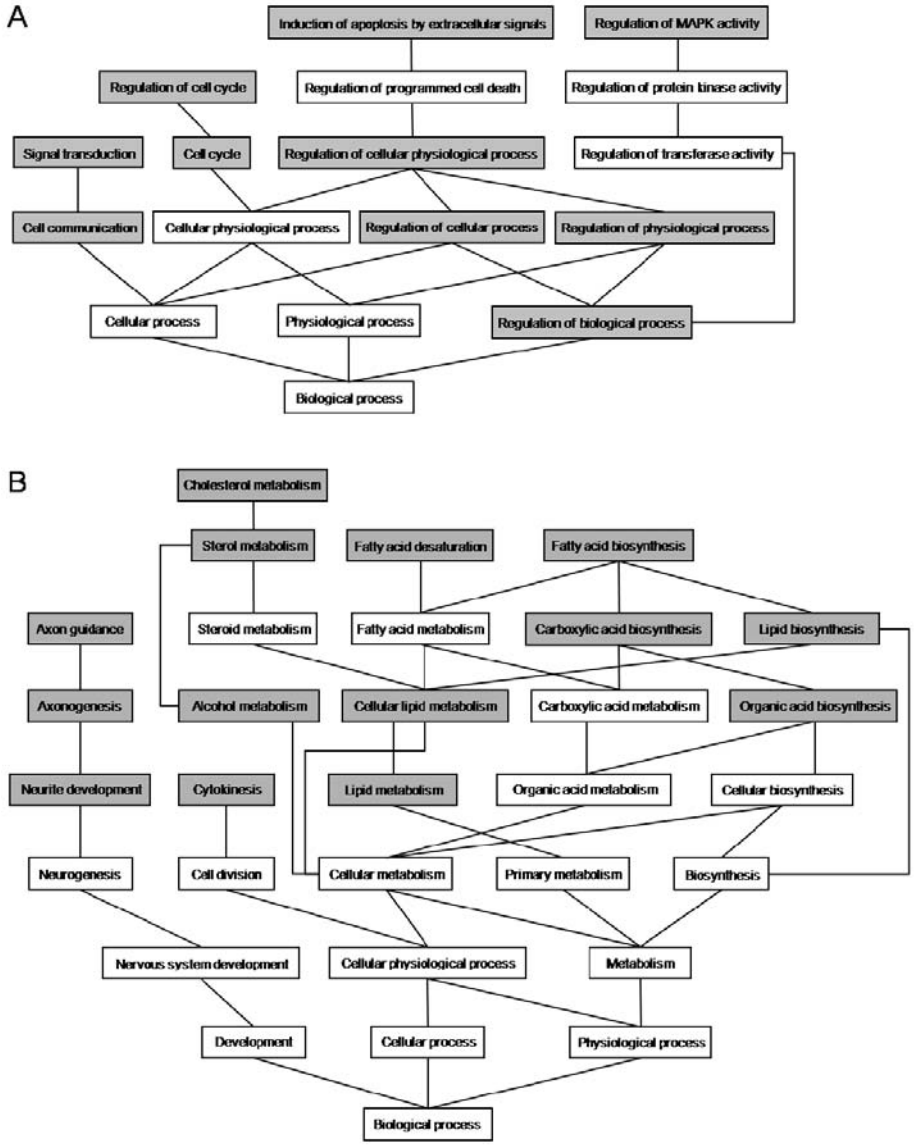

Figure 12. Most of the upregulated pathways (A) in HLS fibroblast cells are related to cell cycle regulation and signal transduction events while the downregulated pathways (B) are mostly involved in lipid metabolism. Significantly regulated pathways are marked with a gray background. Modified from the Webgestalt program output.

5.3.6 Cell proliferation rate and the amount of apoptosis (III)

In the gene expression analysis, we could see upregulation of several genes involved in cell cycle events and thus, we wanted to study the cell cycle rate in fetal cells. We had an opportunity to cultivate both HLS and control cells of fetal neuronal progenitor cell origin. The proliferative activity of HLS and control neuronal progenitor cells was detected by measuring the incorporation of bromodeoxyuridine (BrdU) and the proliferating cells were identified by immunocytochemistry. The result of this assay showed that the proliferation rate of the neural progenitor cells was significantly elevated in the HLS cells when compared with the control cells. The amount of the proliferation was 7.9% in HLS cells whereas only 1.7% in control cells. Student´s t-test showed a statistically significant result for this study with a p-value of <0.001.

In agreement with the elevated cell cycle rate in neuronal cells, the microarray analyses showed that for example the *CCND1* gene was upregulated almost by 3-fold in HLS fibroblasts. *CCND1* is an essential regulator of cell cycle events and when the expression level of *CCND1* is abnormal, it can be part of the cancer-causing events in tissues (Li et al. 2006b; Tashiro et al. 2007). Although tumour predisposition would be impossible to monitor in HLS cases, the possibility exists that the aspect of elevated cell cycle rate might at least partly explain the hamartoma findings in the brain of HLS fetuses. An interesting finding in the microarray study was also the upregulation of the thrombospondin 1 (*THBS1*) gene at the same time of *CCND1* upregulation, since CCND1 is known to act as an inhibitor of THBS1 (Li et al. 2006a). A possible explanation for this phenomenon could be that due to the initial upregulation of *THBS1* the level of *CCND1* is also elevated to counteract the inhibitory effect of THBS1 at the protein level, or on the other hand, that the cells are reacting to *CCND1* upregulation by also producing more *THBS1*.

In addition to the proliferation assay, apoptosis was measured from the progenitor cells by Annexin staining and using flow cytometry. As a result, the amount of apoptosis in control cells was on average 25.6% while in HLS cells the apoptosis amount was only 8.7%. In conclusion, the apoptosis rate in HLS patient neural progenitor cells was highly decreased, student´s t-test giving a statistically significant result ($p<0.05$). The difference in necrotic cells between the control and patient cell samples was not significant.

Remarkably, HLS neuronal progenitor cells showed a significantly decreased amount of apoptosis and increase in the proliferation rate. Finding of the reduced apoptosis rate is highly interesting because apoptosis is an essential and precisely controlled phenomenon of controlled cell death both in fetal development

(developmental programmed cell death) and in adulthood (homeostatic programmed cell death) (Buss et al. 2006). Apoptosis is used for example in sculpting the shapes of developing organs, like creating gaps between the digits in the limbs and also in the regulation of the amount of cells in the brain. According to an accepted theory, populations of neurons are produced in excess they then compete against each other for a limited supply of survival promoting trophic factors produced by their targets (Buss and Oppenheim 2004; Hua and Smith 2004). When creating animal models with a suppressed apoptosis rate, in many cases genetic reduction or elimination of cell death led to embryonic mortality or gross anatomical malformations, in some cases probably due to the continued proliferation of undead cells (Buss and Oppenheim 2004). This evidence nicely fits with the reduced amount of apoptosis in HLS cells and with the dramatic phenotype of the CNS and the other malformations observed in HLS. Still, the relationship between an elevated proliferation rate and the reduced rate of apoptosis needs to be studied further. It would be beneficial to clarify whether one of these phenomena is the primary and the other secondary symptom occurring during the abnormal brain development in HLS. In order to explain the interaction of these findings and to understand the mechanism causing the severe malformations in the brain, it is essential that the common denominator for all these findings could be found in the future.

5.3.7 Lipid metabolism (III)

Cholesterol level analysis

Since the gene expression analysis showed downregulation of genes involved in lipid metabolism, especially related to the cholesterol pathway, we wanted to see whether the lipid levels between the HLS and control samples differed from each other. We had an opportunity to use liver samples collected at the autopsies from two controls and from three HLS fetuses. After the lipids were extracted from the cells, the concentrations of cholesterol, phospholipids and triglycerides were determined. As a result, the cholesterol levels in the liver tissue in HLS fetuses were elevated by an average of 25.3% ($p<0.01$ by student´s t-test) when compared to control samples. There was no significant difference between the two groups in phospholipid or triglyceride levels ($p>0.05$). This result suggests that there exists a disturbance in the hepatic sterol balance in the liver cells of HLS fetuses. This result is also consistent with the microarray analysis findings where differential expression in lipid metabolism pathway between the controls and the HLS cases was observed.

In Smith-Lemli-Opitz syndrome (SLOS), mutations in *DHCR7* lead to increased levels of 7-dehydrocholesterol and decreased cholesterol levels in patient serum. In

our gene expression arrays (section 5.3.5), *DHCR7* was downregulated almost by 3-fold when compared to the control cells and in addition, increased cholesterol levels were observed in HLS liver samples. The microarray analysis also revealed other genes involved in the cholesterol metabolism such as *ACAT2*, *HMGCS1* and *LDLR* to be downregulated, in addition to *DHCR7*. These results lend support for the theory that in HLS fetuses, cholesterol is accumulated in the liver and this in turn would cause feed-back downregulation of these genes involved in the cholesterol metabolism. Another possibility would be receptor-mediated endocytosis of cholesterol in the form of low-density lipoprotein (LDL) particles from the circulation.

An interesting aspect is that the recent studies have shown that cellular lipid rafts have many essential functions and among others, they play a critical role in the signal transduction events of the cells (Korade and Kenworthy 2008). In our study, the group of genes representing the function in signal transduction were upregulated. This finding compared with lipid rafts and lipid metabolism nicely binds the up- and downregulated pathways together. Further studies would be needed to elucidate whether disturbed HYLS1 function and thus, also abnormal cholesterol metabolism, might have an effect in lipid raft composition and further, in signal transduction, too.

In the course of diagnosing SLOS, it has been reported that in addition to invasive methods to confirm the diagnosis of the fetus, also a non-invasive method which measured maternal urinary steroid levels (Jezela-Stanek et al. 2006) could be an option for initial analysis for the syndrome. It would be fascinating if the same kind of method could also be applicable in the initial analysis of HLS, especially if only mild symptoms can be observed in ultrasound scan.

Cholesterol, Hedgehog signaling pathway and developmental disorders

As HLS presents several defects in development, most of them belonging to the field of midline patterning defects, it would be interesting to consider a possible connection between the Hedgehog signaling pathway and HYLS1. The Hedgehog (HH) family of proteins is one of the key protein families in fetal development. This entity is currently widely studied; for reviews, see e.g. (Cohen 2003; Varjosalo and Taipale 2008). Pathways related to the members of this protein family pattern almost every aspect of the vertebrate body plan. The use of a single morphogen for such a wide variety of fuctions is possible because cellular responses to HH depend on the responding cell type, the dose of HH received, and the time cells are exposed to HH (Varjosalo and Taipale 2008). One of the pathways belonging to this signaling family is named after its critical protein Sonic hedgehog (SHH). Regulation of this pathway in mammals leads to the activation and repression of target genes by the glioma (GLI) transcription factors GLI1, GLI2 and GLI3. Abnormalities of the SHH

pathway lead to several human diseases, including birth defects and cancers. During embryonic development, the *SHH* gene is expressed in several places including the notochord, the floorplate of the neural tube, the brain, the zone of polarizing activity in the developing limbs, and the gut (Villavicencio et al. 2000). Mutations of *SHH* in human have been reported to cause holoprosencephaly (Monuki 2007; Roessler et al. 1996) and also to impair neural patterning activity (Schell-Apacik et al. 2003).

Comparing HLS with PHS and SLOS is of special interest since the pathways where the disease-causing genes are functioning are known in PHS and SLOS. PHS is caused by mutations in the *GLI3* gene, which is an essential transcription factor functioning in the SHH pathway. The defective gene in SLOS is *DHCR7*, and its gene product is an important part of cholesterol biosynthesis. Since the Hedgehog protein itself is dependent on a cholesterol residue in processing its final structure (Cooper et al. 2003), DHCR7 might indirectly take part in regulating the function of the SHH pathway. Interestingly, *DHCR7* and *SHH* are are reported to be coexpressed during midline development in *Xenopus* embryos (Koide et al. 2006).

Since PHS and SLOS share several similar features with HLS there is a possibility that HYLS1 is involved in similar kinds of molecular pathways as proteins behind these syndromes and thus, it is tempting to create a hypothesis where HYLS1 directly or indirectly affects the function of the SHH signaling pathway. Since HYLS1 has significant transactivational capacity it could, like GLI3, function as a transcriptional regulator in some stage of SHH pathway. On the other hand, as cells derived from the HLS fetuses show downregulation of several genes related to lipid metabolism and as cholesterol is needed for the proper function of the SHH protein, HYLS1 could have an indirect effect on the SHH pathway functioning via the cholesterol metabolism pathway instead of being directly part of the SHH pathway. This is an interesting matter to be studied further. However, there is a drastic diffence between these syndromes and HLS this being that HLS individuals confirmed to have the Fin_{major} mutation have lived at most for some days, whereas individuals affected with either PHS or SLOS can survive even to adulthood. This would be another big question which needs to be elucidated.

In addition to our hypothesis, also other groups have discussed about the possibility of some kind of relationship between HLS and the SHH pathway. Rakheja et al. published a case report where they had studied the sterol levels using a liver sample of a suspected HLS fetus and they concluded that HLS is not caused by a defect in post-squalene cholesterol biosynthesis (Rakheja et al. 2004). Unfortunately, at the time the study was published, the causative mutation for HLS was not yet known and thus, there is no proof whether this case truly represented HLS or not. Nevertheless, Rakheja et al. discuss the possibility that the gene underlying HLS does not affect normal post-squalene cholesterol biosynthesis but it is still part of the

SHH pathway and the mutated gene in HLS might be situated elsewhere in this pathway. After the HYLS1 mutation was published, Castori et al. suggested an involvement of a possible common signaling pathway between HLS and holoprosencephaly-diencephalic hamartoblastoma, an entity of the HLS-resembling symptoms where SHH signaling network could play a role in the pathogenesis (Castori et al. 2007).

6 CONCLUSIONS AND FUTURE PROSPECTS

During this study, the gene and the protein underlying HLS, a severe fetal malformation syndrome, were identified and characterized. Also several functional analyses were performed to further unravel the pathogenesis of HLS. As the point mutation in the *HYLS1* gene leading to one amino acid change in the protein can cause such a severe phenotype as in this syndrome, the outcome of the amino acid change itself must have drastic consequences in the protein structure and/or in the function of the protein. Further, HYLS1 must have an essential and central role in one or several pathways directing the correct embryonic and fetal development in humans. Most probably, the underlying cause for such a severe syndrome containing several malformations is a cascade phenomenon where the mutated protein causes abnormal events in one or several pathways; this further leads to defects in downstream pathways, as well. It should also be noted that HYLS1 might have different functions depending on the tissue and/or the developmental stage it is expressed in.

The results of this study have provided significant new information on the molecular and cellular defects that HYLS1 mutation causes during embryonic and fetal development. The study also unraveled important aspects of the HYLS1 function itself, and based on the results, the conclusion that HYLS1 is a transcriptional regulator, which is transported between the cell nucleus and cytoplasm could be drawn. These findings will form a basis for future studies of HLS. Although being a rare syndrome, the results of the multiple analyses reported in this thesis offer useful information about the molecular events and pathways, the proper function of which are required in normal embryonic and fetal development, too. The underlying mechanism and the pathway in which HYLS1 functions, as well as the interaction partners it functions with, would be important and interesting to study in the future.

It would be interesting to further delineate the effects of the abnormal cholesterol metabolism in HLS cases and the effect of this phenomenon on cellular events. Another intriguing aspect for future studies would be the characterization of even minor malformations possibly appearing in *HYLS1* mutation carriers. Although when the disease is transmitted autosomal recessively it is thought that the carriers are healthy, some subtle findings occurring among carriers have been discussed in the course of some syndromes. Since the phenotypic spectrum in HLS is so wide, it would be also fascinating to determine the external factors possibly affecting the phenotype of HLS during fetal development, such as the sterol levels of mothers during pregnancy.

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