Charles University in Prague
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Abstract booklet of Ph.D. Thesis

IS *Caenorhabditis elegans* A SUITABLE MODEL ORGANISM FOR THE STUDY OF HUMAN LYSOSOMAL ENZYMOPATHIES? A STUDY OF FABRY, SCHINDLER, POMPE AND MUCOPOLYSACCHARIDOSIS IIIC DISEASES IN *C. elegans* AND HUMANS, RESPECTIVELY.

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Prague 2008

Postgraduate Programmes in Biomedicine

Charles University in Prague
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Field of study: Cell Biology and Pathology

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Ph.D. thesis sent:

Defence of Ph.D. thesis:

Place of defence:

Ph.D. thesis is available at dean´s office of 1st Faculty of Medicine, Charles University in Prague.

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1. Abstract:

The Ph.D. thesis is focused on lysosomal storage disorders (LSD) with impaired lysosomal metabolism of glycans, namely on Fabry, Schindler, Pompe diseases and muccopolysaccharidosis (MPS) type IIIC. We hypothesized that the worm, which has active glycan metabolism, will have orthologs corresponding to human enzymes deficient in the above disorders and could be a suitable model for study of cellular pathology of these LSDs. The protein families of glycosidases which are deficient in the first three disorders are well characterised while the protein deficient in MPS IIIC was unknown and its enzymatic action was only partially characterized. The gene deficient in MPS IIIC was recently identified in our lab (publication 2) and, as is shown below, it belongs to the lysosomal proteins that have no apparent ortholog in the worm.

The main task of the thesis was to find and characterize the *C. elegans* orthologs of human α-galactosidase (α-GAL), α-N-acetylgalactosaminidase (α-NAGA), acid α-glucosidase (GAA) and heparin acetyl-coenzyme A:α-glucosaminide N-acetyltransferase (HGSNAT) whose deficit leads to Fabry, Schindler, Pompe and MPS IIIC diseases, respectively, and to study their biological functions in *C. elegans* by RNA-mediated interference (RNAi). Additional aims were to perform basic bioinformatic analysis and characterize tissue and cellular distribution of HGSNAT mainly at microscopical level.

We found a single *C. elegans* ortholog GANA-1 of both human α-GAL and α-NAGA proteins which are presumed to share a common ancestor. Biochemical, RNAi, GFP expression and bioinformatic analyses confirmed our supposition that GANA-1 has dual enzymatic activity and is localized in the acidic cellular compartment and showed that *gana-1* probably developed from the hypothetical ancestral gene before the duplication event which led to formation of two homologous animal genes: α-Gal and α-Naga.

We found four potential *C. elegans* orthologs (AAGR-1-4) of human GAA. We analysed these orthologs by combination of biochemical, cell biological and bioinformatic approaches and determined that AAGR-1 and -2 have predominantly acidic and AAGR-3 and -4 neutral pH optimum and that AAGR-2 is prevalent contributor to the total acidic and AAGR-3 to the total neutral GAA activity. We found out that AAGR-1 and -2 are localised in membrane-bound cellular compartments and that AAGR-2 is inhibited by acarbose, in contrary to AAGR-1 which is not inhibited. These results were further supported by the analysis of *aagr-1* deletion mutant. Based on these finding we consider AAGR-1 the most probable true ortholog of human GAA.

We found out that RNAi did not provide sufficient decrease of glycosidases activities which is necessary for development of lysosomal storage and we assumed that relevant deletion mutant could be suitable models.

We verified the sequence of HGSNAT and found that cDNA is 4.5 kb long, is composed of 18 exons and is expressed in various human tissues. Basic bioinformatic analyses showed that HGSNAT contains N-terminal signal peptide, 11 predicted transmembrane domains and 4 potencial N-glycosylation sites. We found out that HGSNAT does not share sequence homology with any known prokaryotic or eukaryotic N-acetyltransferase except its mammalian orthologs. Analysis of the cellular distribution of HGSNAT revealed that HGSNAT is present only in a subpopulation of lysosomes and is localised in microdomains of lysosomal membranes. Based on partially co-lokalisation of HGSNAT and sortilin we hypothesize that lysosomal sorting of HGSNAT could be sortilin dependent.

2. Introduction

2.1. *Caenorhabditis elegans* - a model organism

2.1.1. Main features

Caenorhabditis elegans (C. elegans) is a small (~1 mm long), free-living, soil nematode feeding primarily on bacteria. *C. elegans* has a constant number of somatic cells. Adult hermaphrodites and males have 959 and 1031 somatic nuclei, respectively. Full cell lineage is described in *C. elegans*.

Worms are reproducing with a life cycle of about 3 days under optimal environmental conditions. Mature adults are fertile for 4 days and their overall life span is about 17 days after reaching adulthood. *C. elegans* has five holocentric autosomes and sex chromosome (X). Sex is determinated chromosomally. Hermaphrodites are diploid for all autosomes and X chromosomes (XX). Males, on the contrary have only one copy of X chromosome (XO). Recombination occurs in males´ sperm and in both sperm and oocytes in hermaphrodites. Males arise by spontaneous non-disjunction of sex chromosomes during meiosis in hermaphrodite germ line with frequency of about 1:500 worms [1-3].

C. elegans can be easily cultivated under laboratory conditions and enables easy genetic manipulation. Self-fertilization of the hermaphrodites allows for homozygous worms to generate genetically identical progeny and male mating facilitates the isolation and maintenance of mutant strains as well as propagation of mutations between strains. Mutant animals can be obtained by chemical mutagenesis or exposure to ionizing radiation [4, 5]. The strains can be kept as frozen stocks for long periods of time. Due to these properties and its simplicity *C. elegans* became a useful model organism for concerted genetic, ultrastructural, behavioral and developmental studies in eukaryotic organisms [1, 2, 6]. It was the first of the multicellular eukaryotic organisms for which complete genomic sequence became known. About thirty-six percent of genes in *C. elegans* have homology to human genes, including those involved in different human pathology states [7].

RNA-mediated interference (RNAi) technique allows to inhibit *C. elegans* genes very easily [8, 9]. High throughput RNAi techniques were used for inhibition of virtually all *C. elegans* genes [10-14]. Besides RNAi, it is relatively easy to generate and observe the expression of transgenes, either with fluorescent or histochemical molecular tags, in the transparent body of the worm [15, 16].

2.1.2. The coelomocyte system

C. elegans has three pairs of coelomocytes located in the pseudocoelomic cavity. The coelomocytes were suggested to act as scavenger cells due to their ability to actively endocytose fluid from the pseudocoelom. Coelomocytes contain large distended rough endoplasmic reticulum and a number of vacuoles of various sizes and represent suitable cell type for studies of endosomal-lysosomal system [17, 18].

2.1.3. The lysosomal system and lysosomal proteins in *C. elegans*

C. elegans has a fully developed lysosomal system and the lysosomes take up exogenous macromolecules in the same way as lysosomes in mammalian cells [18-24]. *C. elegans* possesses a variety of proteases and peptidases. The representatives of lysosomal acid glycosidases and phosphatases in *C. elegans* are comparable to that found in the mammalian cells [25, 26]. They belong to the same or related protein families and share similar protein structure. The presence of simple and complex glycoconjugates (glycolipids and

glycoproteins) containing various sugar substrates has been reported [27, 28] as well as the presence of glycosaminoglycans (GAGs) [29-31]. All these compounds are degraded in the worm's endosomal – lysosomal system.

2.1.4. *C. elegans* as a model organism for human lysosomal storage disorders

C. elegans was repeatedly shown to be a suitable model for many human diseases[32, 33], including some lysosomal storage disorders such as Niemann-Pick type C disease [34] and juvenile neuronal ceroid lipofuscinosis [35]. It has been reported that *C. elegans* genome includes two distinct acid sphingomyelinase (ASM) genes [36], contrary to humans with only single ASM. Mutant strain of *C. elegans* with deficiency of the aspartyl protease cathepsin D has been described [37]. Number of works concentrated on *C. elegans* ortholog (CUP-5) of human mucolipin-1 [38-41]. Mucolipin-1 is a non-selective cation channel in the lysosomal membranes that is modulated by pH and its deficiency causes lysosomal storage disorder - mucolipidosis type IV. CUP-5 is essential for lysosomal biogenesis in *C. elegans*. In addition, the functions of LAMP/CD68 like protein in *C. elegans* including its deficiency [42] were thoroughly evaluated. Novel information acquired by studies of *C. elegans* orthologs of these non-catalytic human lysosomal proteins provided important insights into biological function of these proteins.

The amount of data about the functions of luminal lysosomal hydrolases in *C. elegans* is restricted to sparse reports, some of them are appended to this thesis (publication 1 and submitted manuscript).

2.2. Lysosomal biology

2.2.1. Introduction

Christian de Duve used the term *"lysosome"* for the first time in the year 1955 [43]. Lysosomes were initially discovered by cell fractionation assays. Lysosomal morphology was characterized by electron microscopy shortly after [44].

Lysosomes are membrane-bound organelles which are found in all mammalian cells apart from red blood cells. Lysosomes are responsible for the controlled intracellular digestion of macromolecules. This dynamic cellular compartment contains more than 60 luminal hydrolytic enzymes. All hydrolases are functioning at acidic pH, which is maintained by vacuolar-type H^+-ATPase and Cl^- channel protein in the lysosomal membrane [45, 46]. The proteome of lysosomal membranes (130-150 different proteins) has been characterized in the year 2007 by Schroder et. al [47]. The most extensively studied lysosomal membrane proteins are highly glycosylated lysosomal associated membrane proteins (LAMPs) [48-50] and lysosomal integral membrane proteins (LIMPs) [51, 52].

Lysosomes are only an integral part of extremely dynamic endosomal–lysosomal system, which is composed of continually communicating membrane bound vesicles involved in vectorial and regulated cargo transport and recycling.

There are at least three pathways for entry of substrates to lysosomes – endocytosis [53, 54], autophagocytosis [55-57] and phagocytosis [58]. Their individual utilization may be cell type specific.

2.2.2. Transport of lysosomal proteins to lysosomes

Lysosomal hydrolases and membrane proteins are synthesized in the rough endoplasmic reticulum (ER) and are transported through the Golgi apparatus to the *trans* Golgi network (TGN). The transport vesicles arise by budding off from the TGN and deliver

these proteins to late endosomes, which serve as molecular sorters in the endosomal-lysosomal system.

Most of the soluble lysosomal enzymes are synthesized with N-linked high mannose-type oligosaccharides [59]. The proteins move by vesicular transport from the rough ER to the Golgi apparatus where they go through various post-translational modifications. The most important modification for lysosomal targeting and sorting is the formation of the mannose-6-phosphate (M6P) recognition marker which is recognised by mannose-6-phosphate receptors (MPRs). The receptor proteins bind to lysosomal hydrolases on the luminal side of the TGN membrane and deliver them to LE, where the low pH induces dissociation of lysosomal enzymes from MPRs. MPRs are recycled back to the Golgi or to the plasma membrane.

In addition to MPR–mediated trafficking there are alternative mechanisms of delivery of soluble lysosomal enzymes to the endosome-lysosome system. Prosaposin, a precursor of four lysosomal saposins (A – D), acid sphingomyelinase (ASM) and GM2 activator protein, all belonging to the saposin – like protein (SAPLIP) family, share a common intracellular receptor - sortilin for sorting from TGN to the lysosomes [60-63]. Beta-glucocerebrosidase employs LIMP2 as a receptor molecule in the lysosomal membrane [64]. In addition, acid phosphatase most probably utilizes mannose-phosphate mediated recycling pathway to lysosomes from cytoplasmic membrane [65].

The lysosomal membrane proteins (LMP), such as LAMPs, LIMPs and other, are sorted from the TGN to the lysosomes by an M6P - independent pathway. They are delivered to lysosomes on the basis of either tyrosine or di-leucine –targeting signals in their cytoplasmic C-terminal tails [66, 67].

2.3. Lysosomal storage diseases

2.3.1. Main features

Lysosomal storage diseases (LSDs) are a group of inherited metabolic diseases (IMD, inborn errors of metabolism), which are characterised by an accumulation of undegraded material in the lysosomes. The global incidence of LSDs as a group is about 1:6000 newborns, but individually they are mostly very rare [68, 69].

There are 48 different storage disorders described to date [70]. Majority of LSDs are inherited in an autosomal recessive manner, with the exception of several X-linked disorders (see below).

LSDs are characterised by incomplete degradation of macromolecules and accumulation of partially degraded material in the lysosomes which leads to cellular and tissue damage and subsequent organ dysfunction. Current concept of pathogenesis of lysosomal storage accepts a paradigm of complex systemic pathologic consequences due to single protein deficiencies. The predominant fraction of LSDs is caused by the deficiency of lysosomal hydrolases or their activators, however, several storage disorders result from the deficits of non-catalytic proteins (e.g. Niemann-Pick type C1 a C2 [71-73] or Mucolipidosis type II (I-cell disease) [74, 75].

LSDs have a broad spectrum of clinical phenotypes. Furthermore a single disorder can vary in the age of onset and severity of symptoms including central nervous system (CNS) manifestation. Common symptoms of many LSDs include organomegaly, CNS dysfunction, bone abnormalities and coarse facial features.

The predominating approach to the treatment of LSDs is enzyme replacement therapy (ERT), which is based on substitution of the defective protein with its active counterpart. ERT is utilized for treatment of Gaucher disease [76], Fabry disease [77], Hurler disease (MPS I)

[78], Pompe disease [79] and enzymes for treatment of other LSDs are currently tested in clinical trials. ERT has been demonstrated to improve non-neuropathic symptoms but is currently virtually ineffective for neurological affection due to the blood-brain barrier (BBB).

Bone marrow transplantation also provides replacement of defective protein and was successfully applied in some forms of mucopolysaccharidosis, in non-neuropathic Gaucher disease [80] and other disorders.

Gene therapy is an attractive therapeutic alternative [81-83] however in spite of the numerous studies over the decade there have still remained many limitations (transient expression, BBB, humoral responses) for effective gene therapy of LSDs in humans.

Additional currently used and explored approach is substrate reduction therapy (SRT) which modulates the availability of substrate by its modification or inhibition of its synthesis [84-86].

The most current therapeutic strategy - enzyme enhancement therapy (EET) [76], intends to stabilize and increase the residual activity/function of the mutated protein by using low-molecular-weight chaperones [87] that can specifically and reversibly bind and stabilize the mutant proteins. Therapeutic use of specifically acting chaperones has been described in Fabry disease [87, 88] and Gaucher disease [87].

2.3.2. Lysosomal storage diseases of our interest

We have focused on LSDs with impaired metabolism of glycans, namely on Fabry, Schindler, Pompe diseases and muccopolysaccharidosis (MPS) type IIIC. We hypothesized that the worm, which has active glycan metabolism, will have orthologs corresponding to human enzymes deficient in the above disorders.

2.3.2.1. Fabry disease

Fabry disease (MIM no. 301500) is an X-linked recessive disorder of glycosphingolipid catabolism due to deficiency of lysosomal α-galactosidase A (α-D-galactoside galactohydrolase; EC 3.2.1.22; α-GAL A). This enzymatic deficiency results in systemic lysosomal deposition of neutral glycosphingolipids with terminal α-galactosyl moieties [89].

Alpha-galactosidase A gene has been localized to a region on the long arm of the X chromosome, Xq22. The gene consists of seven exons and its length is approximately 12 kbp. The full-length cDNA has 1393 bp and contains 60 bp of 5´ untranslated region, and encodes a precursor protein of 429 amino acids including 31 amino acids long signal peptide.

Alpha-GAL A belongs to the glycoside hydrolase family 27, clan D [90]. Members of the family have been identified in animals, plants and microorganisms and they all share similar active site and reaction mechanism. The X–ray crystallographic structures of human α-GAL [91] and rice α-GAL [92] have been determined. Native human α-GAL A is a protein of about 101 kDa that forms a homodimer. The enzyme is relatively heat–labile glycoprotein. Alpha-GAL A monomer contains four potential N-glycosylation sites. Human α-GAL A has a homodimeric quarternary structure and each monomer unit is composed of two distinct domains. Domain I contains the active site and adopts $(\beta/\alpha)_8$ barrel structure - a common motif in many glycoside hydrolases. Domain II has eight antiparallel β strands, packed into two β sheets in a β sandwich fold that contains a Greek key motif [91].

The clinical manifestations of affected hemizygotes usually include angiokeratomas of the skin, paresthesias and renal and cardiac involvement resulting in organ failure. Heterozygous females may express moderate or even severe forms of the disorder, because of skewed X chromosome inactivation [93].

Diagnosis is based on measuring α-GAL A activity with addition of α-*N*-acetyl-galactosaminidase inhibitor - *N*-acetylgalactosamine [94]. Reliable detection of heterozygotes is based on DNA analysis.

Treatment of Fabry patients is based on ERT by using one of the recombinant α-galactosidases A, agalsidase α (Replagal^R) or agalsidase β (Fabrazyme^R).

2.3.2.2. Schindler disease

Schindler disease (MIM no. 609241-2) is very rare autosomal recessive deficiency of lysosomal α-*N*-acetylgalactosaminidase (α-N-acetyl-D-galactosamide:N-acetylgalactos-aminohydrolase; EC 3.2.1.49; α-NAGA). The enzymatic defect leads to the accumulation of glycopeptides, glycosphingolipids and oligosaccharides with α-*N*-acetylgalactosaminyl residues in various tissues and fluids [95].

Early studies suggested that human α-*N*-acetylgalactosaminidase, which was previously referenced as lysosomal α-galactosidase B, is a glycoform of α-galactosidase A because of their similar physical properties. Futher studies indicated that these enzymes (α-GAL A and α-NAGA) are products of two different genes [96]. The two genes differ in the number of exons and in the number, position and orientation of *Alu* repeats. Exons 2 – 7 of the *α-NAGA* gene show considerable similarity to the first six exons of *α-GAL A* gene. Because of the remarkable amino acids identity (49%) and similarity between the two genes (63%), Wang and co-workers suggested that these two genes evolved by duplication and divergence from a common ancestral gene [97].

Alpha-NAGA gene has been localized to the chromosomal region 22q13.1 – 13.2. Its gene consists of nine exons. The 3.6 kb full – length cDNA codes a precursor peptide of α-NAGA consists of 411 aminoacids including a 17 aminoacids signal peptide.

Human α-NAGA is a relatively thermostable enzyme with a native molecular weight of about 110 kDa and homodimeric structure. α-NAGA is a member of the glycoside hydrolase family 27 clan D like α-GAL A. Alpha-NAGA monomer contains six potential N-glycosylation sites. The structure of chicken α-NAGA has been determined by X – ray crystallography [98] and was found to be highly similar to the structure of human α-GAL A (see above).

Schindler disease is clinically heterogenous. Phenotype spans from severe infantile neurodegenerative form to adult-onset disease dominated by angiokeratomas and mild intellectual impairment [95].

The diagnosis is based on the determination of α-NAGA activity in plasma or cultured skin fibroblasts.

Currently there is no specific treatment for the Schindler disease available.

2.3.2.3. Glycogen storage disease type II (Pompe disease)

Glycogen storage disease type II (GSD II), also named Pompe disease (MIM no. 232300), is an autosomal recessive disorder of glycogen metabolism resulting from the deficiency of the lysosomal acid α-glucosidase (alpha-D-glucoside glucohydrolase; EC 3.2.1.20; GAA). The enzyme deficiency results in intralysosomal accumulation of glycogen in various tissues and organs [99].

GAA gene is localized at the long arm of chromosome 17 (17q25.2-q25.3), is 20 kbp long and consists of 20 exons. The cDNA is over 3.6 kb long, with 2859 nucleotides of coding sequence.

GAA is a glycoprotein that catalyzes the hydrolysis of α-1,4- and α-1,6-glucosidic bonds. Human GAA consists of 952 amino acids and the molecular weight of the non-glycosylated protein was estimated to be 105 kDa. GAA contains seven potential N-glycosylation sites. The enzyme is additionally modified by both N- and C- terminal proteolytic cleavage, primarily within lysosomes [99].

GAA belongs to the glycosyl hydrolases family 31 (GH31) [90, 100] which includes many others hydrolases from different eukaryotic and prokaryotic organism, all having significant sequence similarity [101, 102]. Human genome contains at least four other genes belonging to the family GH31 - sucrase – isomaltase (SUIS) and maltase – glucoamylase (MGA) with acid pH optimum and glucosidase II alpha subunit (GANAB) and neutral α-glucosidase C (GANC) functioning at neutral pH. All five proteins share common GH31 active side sequence. YicI from *Escherichia coli* [101], MalA from *Sulfolobus solfataricus* [103] and N-terminal subunit of human maltase-glucoamylase (NtMGA) [104] are the only enzymes of family GH31, for which the three-dimensional (3D) structure has been determined. The catalytic domain is composed of $(\beta/\alpha)_8$ barrel structure.

Clinical presentation of GSD II spans a range of phenotypes, all of which include varying degrees of myopathy.

Clinical diagnosis is based on the determination of GAA activity. Acarbose, as a potent inhibitor of SUIS and MGA, is used for the direct and specific measuerement of GAA activity [105].

Therapy is based on ERT by using recombinant GAA (aglucosidase α, Myozyme) [79].

Based on the data that demonstrate the importance of glycogen metabolism in nematodes [106, 107], we reasoned that *C. elegans* may be a suitable model for GSD II.

2.3.2.4. Mucopolysaccharidosis III C (Sanfilippo syndrome C)

The mucopolysaccharidosis III C (MPS IIIC), also called Sanfilippo syndrome C (MIM no. 252930), is a rare autosomal recessive disorder of mucopolysaccharide catabolism caused by deficiency of the lysosomal membrane enzyme heparin acetyl-coenzyme A (CoA):α-glucosaminide *N*-acetyltransferase (N-acetyltransferase, HGSNAT; EC 2.3.1.78), which leads to the intralysosomal storage of heparan sulphate in all organs and its excretion in urine [108].

Until recently, N-acetyltransferase gene had not been identified and its protein structure characterized. Ausseil and co-workers mapped N-acetyltransferase gene to an 8.3 cM (16Mbp) interval in the pericentromeric region of chromosome 8 [109]. The N-acetyltransferase gene has been identified recently [110] (publication 2). The gene maps to 8p11.1 and encodes a 73 kDa protein with predicted multiple transmembrane domains and glycosylation sites (the transmembrane protein 76 (TMEM 76), identical to HGSNAT).

MPS IIIC is characterised by severe CNS degeneration, while somatic symptoms (hepatomegaly, short stature, joint stiffness, coarse facial features and hypertrichosis) are mostly mild [108].

The diagnosis of MPSs is based on the analysis of urinary GAGs and is confirmed by the enzymatic activity assay [111, 112].

2.3.3. Lipid rafts and composition of lysosomal membrane

It has been shown that N-acetyltransferase is associated with lipid rafts in lysosomal membrane [113]. Lipid rafts are dynamic and heterogenous membrane microdomains that are enriched in cholesterol and glycosphingolipids and have specific lipid and protein composition. Lipid rafts were first described in the cytoplasmic membrane and lately also in other intracellular compartment, such as ER, GN, mitochondria and lysosomal-endosomal

system [113-115]. Lipid rafts play active roles in various physiological processes including signal transduction, vesicle trafficking (sorting in TGN and endosomes) [116], cell adhesion and motility, entry of pathogenic viruses or bacterial toxins [117]([118]. New information about the functions and composition of lysosomal membrane microdomains and their roles in molecular pathology of LSDs is continually becoming available.

3. Aims of the study

Study of cellular pathology of human lysosomal diseases is very important and interesting because it enables us to understand the related biological processes in cells. Not everything is practicable to study on patients, so it is important and needful to use animal models. However, work with mammalian animal models is on overall very complicated and demanding. *C. elegans* is a simple multicellular organism which was previously used to successfully study several human diseases including some lysosomal storage disorders caused by deficiency of lysosomal non-catalytic proteins. It represents ideal intermediate stage between study of tissue culture and mammalian models.

Subject of this Ph.D. thesis was to study selected deficits of lysosomal glycoconjugates degradation (Fabry, Schindler, Pompe and Mucopolysaccharidosis IIIC diseases) in term of cell biology, biochemistry and genetics and find out if *C. elegans* is suitable model for lysosomal enzymopathies. First task was to find and characterize the true *C. elegans* orthologs and then perform systematic RNA-mediated interference assays of these orthologous genes with subsequent characterization of phenotype of interfered organisms. Not all lysosomal proteins have their orthologs in *C. elegans* and one example is human Acetyl-CoA: glucosaminide N-acetyltransferase (HGSNAT), deficit of which leads to MPS IIIC. Based on this finding we could not study this lysosomal membrane protein in *C. elegans*. I participated in the discovery of the gene coding HGSNAT and another aims of this study were to perform basic bionformatic analysis and characterize tissue and cellular distributions of HGSNAT mainly at microscopical level.

This work is composed of three sections and partial goals of each part are listed below.

3.1. Aims of the first part of the study

- To identify *C. elegans* orthologs of human α-galactosidase and α-N-acetyl-galactosaminidase which are presumed to share a common ancestor.
- To verify the predicted gene and characterize it on the cDNA level.
- To study its biological functions during *C. elegans* lifetime by RNA-mediated interference.
- To study its expression pattern by using extrachromosomal GFP array.
- To study the phylogeny of the orthologous genes.
- To study expression of *C. elegans* ortholog in wild-type and knockout mouse fibroblasts.
- To evaluate a suitability of *C. elegans* as a model organism for studying two lysosomal enzymopathies - Fabry and Schindler diseases.

3.2. Aims of the second part of the study

- To identify *C. elegans* orthologs of human acid α-glucosidase.
- To verify the predicted or partially confirmed genes and characterize them on the cDNA level.
- To study their biological functions during *C. elegans* lifetime by RNA-mediated interference.
- To characterize acquired deletion strains generated by *C. elegans* Gene Knockout Consortium.
- To study expression pattern of orthologous genes with acidic pH optimum by using extrachromosomal GFP arrays.
- To study the phylogeny of the orthologous genes.
- To evaluate a suitability of *C. elegans* as a model organism for studying glycogen lysosomal storage (Pompe disease).

3.3. Aims of the third part of the study

- To verify the sequence of the predicted gene of human heparin acetyl-coenzyme A: α-glucosaminide N-acetyltransferase (HGSNAT) on the cDNA level.
- To identify splice variants of HGSNAT and human tissues in which is HGSNAT expressed.
- To perform basic bioinformatic analyses.
- To identify and characterize *C. elegans* ortholog of human HGSNAT.
- To study the cellular distribution of human HGSNAT and its relation to lysosomal microdomains by immunofluorescence microscopy.

4. Materials and methods

4.1. General techniques

4.1.1 Preparation of genomic DNA and cDNA

Mixed stage *C. elegans* culture and cultured skin mouse or human fibroblasts were used for preparation of total RNA [119] which was reverse-transcribed using SuperScriptII reverse transcriptase (Invitrogen) and oligo dT18 according to protocol supplied by the manufacturer (Invitrogen). Genomic DNA was isolated from cultured mouse or human skin fibroblasts by standard phenol extraction method [120].

4.1.2 Polymerase chain reaction (PCR)

PCR reactions were performed according to generally accepted protocols [121] using specific primers and templates (gDNA, cDNA, plasmid DNA, and Human Multiple Tissue cDNA (MTC) Panel 1 - CLONTECH Laboratories, Inc).

4.1.3 Cloning of DNA, isolation of plasmids and DNA sequencing

Cloning into TOPO vectors (pCR 2.1, pCRII and pCR-XL) was performed according to manufacturer´s protocol with TOPO TA cloning kits (Invitrogen). T4 DNA Ligase was used (Invitrogene, Fermentas) for cloning into other vectors (L4440, pPD95.75, pPD95.67 and pCMVTag1). Gel purified DNA fragments (High Pure PCR Product Purification Kit, Roche) were ligated overnight into previously linearized vectors (see particular sections) at 16 ^{o}C. Transformation of competent cells TOP10 (Invitrogen) was performed by heat shock at 42 ^{o}C for 30s. Plasmid DNAs were isolated by JETquick Plasmid Miniprep Spin Kit (Genomed) or in case of transfection by EndoFree Plasmid Maxi Kit (Qiagen) according to manufacturer´s protocols. We analyzed the plasmids for inserts by restriction analysis and/or by PCR screening. DNA sequences of inserts and whole costructs were verified by direct sequencing on the automated fluorescent sequencer Li-Cor (LI-COR, Inc.), AlfExpress (Pharmacia Biotech) or ABI 3100 – Avant sequencer (Applied Biosystems, USA).

4.1.4 Signal sequence, transmembrane domains and potentional N-glycosylation sites prediction

All these predictions were performed by CBS prediction servers [122].

4.1.5 Total protein determination

Concentration of total protein in the samples was determinated by Hartree method (modification of Lowry method) [123].

4.1.6 Microscopic techniques

Worms and cultured mouse and human skin fibroblasts were evaluated under epifluorescence microscope (Nikon, TE 2000E) equipped with Nomarski DIC optics and confocal laser scanning head C1*si* (for detailed microscope setup see http://udmp.lf1.cuni.cz/).

4.2. *C. elegans* related techniques

4.2.1 *C. elegans* strains and cultivation

The wild-type Bristol N2 and other strains of *C. elegans* were cultured under standard laboratory conditions as described previously [2]. Deletion mutants of *aagr-1* (*ok2317*) and *aagr-4* (*ok1423*) were generated by the *C. elegans* Gene Knockout Consortium [124].

4.2.2 BLAST searches

Wormbase (2002-2008 consecutive and freeze versions, http://www.wormbase.org/) databases were repeatedly searched for human α-GAL, α-NAGA and GAA orthologs in C. elegans using BLASTP program set at default values [125]. Amino acid sequence of human lysosomal α-GAL (GenBank acc. no. NP_000160), α-NAGA (GenBank acc. no. NP_000253) and GAA (GenBank acc. no. NP_000143) were used as a query sequences [126].

4.2.3 RNA mediated interference

The entire coding sequence of *gana-1* was used for RNAi experiments. For RNAi assays of particular *aagr*s we selected regions of *aagr-1-4* cDNAs with the lowest similarity/identity between otherwise homologous sequences.

PCR products were cloned into the double promoter pCRII-TOPO vector (Invitrogen) for the purposes of successive microinjection mediated RNAi experiments. Resultant constructs were separately linearized and used as templates for in-vitro transcription employing In Vitro Transcription KIT (Promega) using T3 and SP6 RNA polymerases. Single stranded antisense RNA molecules were annealed for 30 minutes at room temperature to generate double stranded RNAs (dsRNAs) which were then treated with DNase I (New England Biolabs). Standard protocols were used for dsRNA microinjection [9]. F_1 progeny of injected animals was screened for morphological RNAi effects.

PCR products were cloned into L4440 double T7 promoter vector (supplied by Dr. Andrew Fire, Standford University) for RNAi feeding experiments. The plasmids were transformed into *E. coli* strain HT115 [8] carrying IPTG inducible T7 RNA polymerase. Worms were fed on induced HT115 *E. coli* carrying the relevant L4440 construct. Worms fed on HT115 carrying L4440 plasmid without insert were used as a control. F_1 and F_2 progeny was screened for morphological phenotypes.

Interfered nematodes were treated as described in section 4.2.4 for biochemical measurements.

4.2.4 Determination of α-galactosidase, α-N-acetylgalactosaminidase, α-glucosidase and β-hexosaminidase (control) activities

Enzyme activities were determined in the crude homogenate of worms by using methylumbelliferyl substrates. For some measurements of α-galactosidase and α-N-acetyl-galactosaminidase activities inhibitors N-acetyl-D-galactosamine (0,1 M) (Sigma), D-galactose (0,1 M) (Sigma) or D-glucose (0,1 M) (Sigma) were added and for selected measurements of α-glucosidase activity selective inhibitor acarbose (8 μM) (Sigma) [105] was added to the reaction mixture.

4.2.5 Transgenic GFP expression

Mixed stage N2 nematode culture genomic DNA was used as a template to amplify inserts including regulatory and part or whole coding sequence of the analysed genes. The PCR products were cloned in-frame with GFP coding sequence of the vectors pPD95.67

containing a nuclear localization sequence (NLS) and/or pPD95.75 without NLS (both vectors supplied by Dr. Andrew Fire, Standford University). Extrachromosomal transgene expressing lineages were generated by co-injecting plasmid DNA along with the marker plasmid pRF4 (containing the *rol–6(su1006)* mutant collagen gene) at the total concentration of 100 ng/µl into L4/young adult germline syncytium of N2 worms [127]. Presence of the transgene was verified both in plasmid DNA and RNA by PCR and sequencing. The transgenic progeny was screened for GFP signal by epifluorescence microscopy (see section 4.1.6).

4.2.6 Alkalization of lysosomes

Worms were treated with either one of two agents (NH_4Cl and concanamycin A (CON A)) [46, 128], which are known to specifically increase pH in the acidic cellular compartments. For the NH_4Cl method, animals were resuspended in 0, 10, 25, 50, 75 and 100 mM aqueous solutions of NH_4Cl. Small aliquots of worms were examined microscopically after 30 min, 2, 4, 6, 8 and 24 hours. Alternatively, animals were soaked in 0, 10, 20, 50, 100, 150, 200 nM solutions of CON A in aqueous media. Small aliquots of worms were evaluated after 30 min, 1, 2, 3, 6 and 24 hours.

4.2.7 Immunofluorescence staining of worms

Mixed stage pJH3 and N2 cultures were harvested from NGM OP50 plates and washed thoroughly in M9 buffer to deplete intestinal bacteria. The fixation and imunnofluorescence staining procedures were based on the approaches of Nonet et al. [129]. All antibody incubations were performed overnight at room temperature. Primary antibody (polyclonal rabbit anti-GFP IgG (Abcam)) was diluted 1:500. Secondary antibody (goat anti-rabbit IgG Alexa Fluor 488 labeled (Molecular Probes)) was diluted 1:1000. Specimens were mounted onto the agarose pads with additional DAPI or SYTOX orange (Molecular Probes) staining of the nuclei. Microscopic examination was performed as described in section 4.1.6.

4.2.8 Western blotting

Proteins (25 – 50 µg of total protein per lane) from the homogenate of worms were separated by SDS-PAGE gradient gel (4 to 20 % polyacrylamide) and transferred onto nitrocellulose membrane by semi-dry blotting according to a common protocol with chemiluminiscence detection (SuperSignal, West Pico) [130]. Primary antibody was rabbit polyclonal anti-GFP IgG (Abcam, dilution 1:5000), the secondary antibody was goat anti-rabbit IgG/Px (Pierce, diluted 1:20000).

4.2.9 Isolation and characterization of deletion mutants

Deletion mutants of *aagr-1* (*ok2317*) and *aagr-4* (*ok1423*) were kindly generated by *C. elegans* Gene Knock-out Consortium [124]. Both deletion strains were backcrossed repeatedly (at least 3 times) according to established protocols [131]. The backcrossed worms were checked for morphological or otherwise observable phenotypes and the enzyme activities were measured as described in section 4.2.4.

4.2.10 Multiple protein alignments and phylogenetic tree construction

Consensual translations of the sequenced cDNA´s and evolutionary relevant protein sequences were aligned using ClustalW algorithm [132] and Blosum62 matrix and the alignments were manually edited. Phylip 3.57c and 3.6 software packages [133] were used for phylogenetic tree construction employing maximum likelihood, maximum parsimony and distance matrix methods.

4.2.11 Homology modeling

4.2.11.1 3D model of GANA-1

X-ray structure of chicken α-NAGA [98], rice α-GAL [92] and human α-GAL [91] were used as structural templates for model construction of GANA-1 using automatic algorithms of SwissModel server [134]. The model was manually inspected and energy was minimized in DeepView computer program [135].

4.2.11.2 3D models of AAGR-1-4 and molecular docking

Protein sequence alignments of *E.coli* YicI [101], *S. solfataricus* MalA [103], *H.sapiens* NtMGA [104] and *C. elegans* AAGR-1-4 were calculated using ClustalW algorithm [132] and Blosum62 matrix. Structural models were prepared using MODELLER 9 software [136] with the *E.coli* YicI, *S. solfataricus* MalA and *H.sapiens* NtMGA as templates. Unstructured loops (insertions in AAGR-1 and -2, in special) were energy refined and the final models were selected based on DOPE score (Discrete Optimized Protein Energy) and visual check. Quality of the models was assessed by PROCHECK [137] and MolProbity [138] programs and individual structural domains were pairwise compared using DaliLite server [139]. Docking of acarviosine was done by Autogrid 4 and Autodock 4 [140].

4.3. Cell culture related techniques

4.3.1 Cell culture

Mouse skin fibroblasts (wild type (wt) and two knockout lines for α-galactosidase and α-N-acetylgalactosaminidase were supplied by Prof. Detlev Schindler, Wurzburg University). Human skin fibroblasts from patients and control individuals were cultured according to standard procedure in DMEM (Sevapharma a.s) supplemented with 10% fetal calf serum (FCS) (PAA laboratories), 2% $NaHCO_3$ (Sevapharma a.s) and 1% gentamycin (Lek Pharmaceutical and Chemical Company) or penicillin/streptavidin (Diagenes s.r.o.). Cell cultures were grown in 25 cm^2 culture flasks (TPP) in 5% CO_2 incubator (Jouan IGO 150). All cell culture procedures were carried out in the laminar flow box (Biohazard, Clean Air CA/RE3).

4.3.2 Expression of gana-1 in eukaryotic expression system

4.3.2.1 Preparation of expression constructs

We used epitope tagging vector pCMV-Tag 1 (Stratagene) designed for gene expression in mammalian cells. Entire coding sequence of *gana-1* was inserted into the pCMV-Tag 1 vector with either FLAG or c-myc epitopes. We acquired six *gana-1* constructs. The first was tagged with both epitopes, the Flag (N-terminal) and c-myc (C-terminal). The remaining five constructs were tagged with Flag epitope either on N-terminal or C-terminal and three are tagged internal on various place of *gana-1* sequence.

4.3.2.2 Transfection of mouse skin fibroblasts

Mouse skin fibroblasts were transfected with the above described constructs (pCMV-Tag1+*gana-1*) using Lipofectamine 2000 reagent (GibcoBRL) or Amaxa nucleofector technology [141](Amaxa Biosystems).

Transient cell lines were fixed 20 hours after transfection and examined by immunofluorescence staining (for details see below). The stable cell lines were allowed to grow for 72 hours and after trypsinization they were cultured in the complex medium with 0,8 μg/ μl of selective antibiotic geneticin (G418) (KRD) to maintain stable cell lines.

4.3.3 Immunofluorescence staining of transfected cells

Transfected cells were fixed in 100% methanol at -20 ^{o}C for 10 min. As primary antibodies monoclonal mouse anti c-myc IgG1 (Sigma, dilution 1:150), polyclonal rabbit anti-Flag IgG (Sigma, dilution 1:100) and monoclonal rat anti mouse CD107a (Lamp-1) (Fitzgerald, dilution 1:200) were used. Secondary antibodies were goat anti-mouse IgG1 Alexa Fluor 488 labeled, goat anti rabbit IgG Alexa Fluor 568 labeled and donkey anti rat IgG Alexa Fluor 488 labeled (all diluted 1:1000, Molecular probes).

4.3.4 Western blotting

Western blotting procedure was performed as described above (see section 4.2.8). The proteins (25 μg of total protein per lane) were separated in 12% SDS-PAGE gel. Polyclonal rabbit anti-Flag IgG (Sigma, dilution 1:4000) was used as the primary antibody and goat anti-rabbit IgG/Px (Pierce, dilution 1:10000) was used as the secondary antibody. Fusion protein C-Terminal FLAG-BAP (Sigma) was used as the positive control (10 ng per lane).

The specifity of antibody against HGSNAT was verified in homogenates of leukocytes by Western blotting. We used polyclonal rabbit anti HGSNAT (2785-6) (diluted 1:3000) as the primary antibody and goat anti rabbit IgG/Px (Pierce) (diluted 1:12000) as the secondary antibody.

4.3.5 Measurement of influence of HGSNAT antibody on HGSNAT and β-hexosaminidase activities

For assays were used artificial substrates tagged with 4-methylumbelliferone. In the case of HGSNAT assay we added 10 μl of 6 mM acetyl CoA (Sigma). The amount of polyclonal rabbit anti HGSNAT antibody (2785-6) was ranged from 0 to 4.5 μg per reaction.

4.3.6 Immunofluorescence staining and colocalization studies of HGSNAT

The immunofluorescence staining was performed according to a common protocol and the specific conditions were adjusted according to primary antibodies and their combinations used. The cells were fixed in 100% methanol at -20 ^{o}C for 10 min or in 4% paraformaldehyde (PFA) at 4 ^{o}C for 10 min with or without following permeabilization step with 0.1% TRITON X-100 performed at room temperature for 10 min. After the protein blocking step (5% FBS in 1x PBS, 30 min, RT) samples were incubated with primary antibodies diluted in 5% FBS in 1x PBS for 1 hour at 37 ^{o}C or overnight at 4 ^{o}C. Appropriate Alexa Fluor labeled antibodies (Molecular probes) were used as secondary antibodies and were diluted 1:1000 and incubated 1 hour at 37^{o}C. Nuclei of cells were stained with DAPI (1:1000, 15 min, 37 ^{o}C). Specimens were mounted with aqueous mounting medium IMMU-MOUNT (Thermo Scientific).

5. Results and discussions

5.1. *Caenorhabditis elegans* as a model organism for selected lysosomal storage diseases

The initial results based on comparison of the conservation of sequence of selected lysosomal proteins *in silico* and measuring their activities in mixed N2 *C. elegans* cultures showed that there is a good conservation (orthologous genes) and function (enzymatic activity) of the selected lysosomal proteins between man and *C. elegans*, and that *C. elegans* may be a suitable model system for the study of LSDs. Based on the highest similarity and type of LSD we resorted to further study *C. elegans* orthologs of α-galactosidase, α-N-acetylgalactosaminidase and α-glucosidase whose deficiencies lead to Fabry, Schindler and Pompe disease, respectively.

5.1.1. *Caenorhabditis elegans* as a model organism for Fabry and Schindler disease (publication 1)

5.1.1.1 Blast search and verification of the predicted gene structure

We were able to find only one predicted open reading frame (ORF) orthologous to human α–GAL and α–NAGA in the complete *C. elegans* genome [142]. It was designated R07B7.11 and it had significant sequence similarity to both human genes, which were previously proposed to evolve from the common ancestral gene. Similar results were obtained while searching *C. briggsae* genome [142]. R07B7.11 was later designated *gana-1* (α-GAL and α-NAGA ortholog (publication 1)). *Gana-1* consists of 5 exons and 4 introns and was initially annotated as a potential ortholog of human α–NAGA.

The sequence of the predicted gene was verified by sequencing of the PCR products from genomic DNA and cDNA. We analysed the whole coding region including 3′ and 5′ untranslated regions (UTR). The sequence analysis revelaed SL1 mode of *trans* splicing. The length of genomic DNA from start to stop codons is 1681 bp. The entire ORF of *gana-1* has 1356 bp and encodes a protein of 451 aminoacids. We have found no alternative splicing by RT-PCR, a feature similar to both human and mouse orthologs and we noted no signs of RNA editing in clones derived from the *gana-1* cDNA.

GANA-1 signal sequence prediction was performed by SignalP server [122]. Prediction results revealed high N-terminal signal peptide prediction probability (1.000) and two possible signal sequence cleavage sites (between residues 15-16 or 21-22).

5.1.1.2 Biochemical studies

We found both enzyme (α–GAL and α–NAGA) activities in the homogenates from *C. elegans* N2 strain using methylumbelliferyl (MU) substrates [143]. The activity of α–NAGA measured after incubation at 37 °C was 430 nmol.mg-1.h-1 with MU-α–N-acetyl-galactosaminide as a substrate and the activity of α–galactosidase A using MU-α–galactopyranoside was 43 nmol.mg-1.h-1. Alpha–NAGA and α–GAL activities measured after incubation at 25 °C were 317 and 28.7 nmol.mg-1.h-1 respectively.

We performed inhibitory assays in the homogenates from *C. elegans* N2 strain. In case of α–galactosidase A, the degradation of the MU-α–galactopyranoside was significantly inhibited (up to 95%) in the presence of D-GalNAc, whereas, in the presence of D-Gal the degradation of the same substrate was inhibited up to 75%. In the assay of α–NAGA, the

degradation of the MU-α–N-acetylgalactosaminide was inhibited up to 97% by D-GalNAc and up to 90% by D-Gal. No inhibition of α–NAGA and α–GAL A activity by D-glucose was observed.

We observed significant effect of both inhibitors on both activities and mainly the strong inhibitory effect of D-GalNAc on the α–GAL activity, which is not present in human α–GAL. These results support the hypothesis that *C. elegans* has only one enzyme with both α–GAL A and α–NAGA activities.

5.1.1.3 RNA-mediated interference

To evaluate the function of *gana*-1 we performed RNA interference (RNAi) experiments. RNAi assays were directed against the complete coding region of *gana-1*. However, we were not able to observe any abnormal morphological phenotype. In order to foster RNAi we resorted to combination of microinjection and feeding approaches and biochemically evaluated both activities of *gana-1*. Measurement of *gana-1* activity in four individual experiments showed simultaneous decrease of both α–GAL and α–NAGA activities in interfered worms. In all RNAi experiments both α–GAL and α–NAGA activities decreased proportionally, usually by tens of percent of activity of appropriate controls. The activity of β-hexosaminidase, which was used as the control enzyme, did not differ between the control and interfered worms. These results support the specificity of *gana-1* RNAi and our hypothesis that the *gana-1* has both enzyme activities.

5.1.1.4 Expression of *gana-1*

We created transgenic worms carrying expression construct containing the complete coding region of *gana-1* C-terminally tagged with green fluorescence protein (GFP). Although we verified the presence of the *gana-1*::GFP transgene in the transgenic worms on the level of gDNA, cDNA and protein (Western blotting), we were not able to observe any GFP signal. Because Western blotting showed the presence of fusion protein of expected size, we assumed that the absence of GFP signal resulted from pH-dependent quenching of GFP fluorescence emission [144].

We used two agents [46, 128] that specifically alkalize acidic cellular compartments. Worms carrying *gana-1*::GFP transgene were soaked in NH_4Cl or concanamycin A (CON A) solutions. After soaking, we observed a distinct GFP signal in the membrane bound compartment of endocytically active coelomocytes. Intensity of the GFP signal was dependent on the concentration and the time of incubation of the alkalizing reagent used. We consider the reappearance of the GFP signal after treatment of the transgenic worms with compounds increasing pH in acidic cellular compartments as indirect proof of lysosomal localisation of the fusion protein. The GFP signal in coelomocytes had the same coarsely granular pattern as that observed after immunostaining (discussed below).

Immunofluorescence detection of GFP fusion protein showed a specific and coarsely granular cytoplasmic pattern of mosaic transgene expression in body wall muscle, intestinal cells and coelomocytes in approximately 30% of the population.

5.1.1.5 Bioinformatic studies on *gana-1*

Bioinformatic studies such as multiple alignment, phylogenetic analysis and homology modeling suggest a close evolutionary relationship of GANA-1 to both human α-GAL A and α-NAGA and confirm the hypothesis that *gana-1* probably developed from the hypothetical ancestral gene before the duplication event.

5.1.1.6 Expression of *gana-1* in eukaryotic expression system (unpublished data)

To characterize the function and expression of GANA-1 *in vivo* and to support our hypothesis that GANA-1 has both α–GAL and α–NAGA activities, we decided to express *gana-1* in normal mouse fibroblasts and mouse fibroblasts deficient in either α–Gal or α–Naga. Our second aim in this part of the study was to find if GANA-1 compensated α–GAL and α–NAGA activities in mammalian cells.

We prepared six expression constructs containing the complete coding region of *gana-1* in the pCMV-Tag 1 vector (Stratagene) tagged on different site with FLAG and/or c-myc. Initial results of transfection verified functionality of all constructs and we observed granular cytoplasmic pattern of *gana-1* overexpression in all three types of mouse fibroblasts. Unfortunately, colocalization studies with Lamp 1 did not provide confirmation of the lysosomal localization of the overexpressed *gana-1*. Because of low efficiency of transient transfection (only about 2%), we tried to obtain stable cell lines expressing *gana-1* under the selection of antibiotic geneticin (G418). The presence of the *gana-1* transgene in the mouse fibroblasts was confirmed on the level of genomic DNA and in case of *gana-1* C-terminally tagged with FLAG also on the level of RNA (cDNA). However, we were not able to confirm the presence of GANA-1 fusion protein by Western blotting or immunosfluorescence assays. We can only hypothesize about the reasons of low GANA-1 expression and/or its enhanced degradation. Our findings can also be attributed to different glycosylation patterns [31] and/or dissimilar signal peptides in mammalian and *C. elegans* cells.

5.1.2. *Caenorhabditis elegans* as a model organism for Pompe disease (submitted manuscript)

5.1.2.1 Blast search and sequence verification of predicted genes

BlastP searches for *C. elegans* orthologs of human GAA in the Wormpep database revealed four acid alpha glucosidase related genes (*aagr-1-4*). All four found coding sequences were annotated as GH31 family members.

We verified the available *in silico* predictions by sequencing of all four genes (*aagr-1-4*) and found that they all contain SL1 *trans* splicing element despite short distance (approx. 650 bp) between *aagr-1* and upstream (D2096.12) gene which belongs to CEOP4284 transcriptional operon [145]. The first gene in the CEOP4284 operon is UDP-glucuronic acid decarboxylase (*sqv-1*) [146] which is *trans* spliced in SL1 mode. The sequence analysis of the second gene D2096.12 in the operon verified it's gene organization and complete sequence and revealed SL2 mode of *trans* splicing.

5.1.2.2 Signal peptide and intracellular targeting predictions

SignalP predictions revealed presence of N-terminally situated signal peptide with strong probability for all AAGRs. The lengths of signal peptide sequences correspond with the signal sequence cleavage sites: 22-23 (AAGR-1), 18-19 (AAGR-2), 19-20 or 22-23 (AAGR-3) and 16-17 (AAGR-4). TargetP server predicted all AAGRs as non-secretory, non-mitochondrial, intracellular targeting proteins.

5.1.2.3 Biochemical studies

GAA activities were assessed in the homogenates from *C. elegans* N2 strain when measured with methylumbelliferyl substrates at two different pH values (4.0 and 6.5) and two different incubation temperatures (25 °C and 37 °C). We assumed that values acquired by measuring according to this protocol at acidic pH represent sum of enzymatic activities of

several enzymes that are predicted in *C. elegans*. The overall values measured at 25 °C were 53 and 67.5 nmol $mg^{-1}h^{-1}$ for pH values 4.0 and 6.5 respectively. Activity values measured at 37 °C were 165 and 277 nmol $mg^{-1}h^{-1}$ respectively.

It has been reported that addition of acarbose [105], which is a potent inhibitor of maltase-glucoamylase and sucrase-isomaltase, to the reaction mixture at acidic pH selectively eliminates these interfering activities. Thus values measured with acarbose at acidic pH should directly reflect acid alpha glucosidase activity. On the other hand the effect of acarbose on the neutral glucosidases has been insignificant. Table 1 show influence of acarbose on GAA activities.

In order to differentiate AAGRs functioning at the acid pH from the ones acting at neutral pH we further performed selective RNAi experiments and deletion mutant analyses as described below.

5.1.2.4 RNA-mediated interference

We performed selective separate RNA interference experiments of all four *aagr* genes. We selected the sequence regions for RNAi assays on the basis of the cDNA multiple alignment. To avoid or at least minimize cross-interference between otherwise homologous sequences we tried to find and employ regions with the lowest similarity/identity as possible.

Equally as in case of other lysosomal hydrolase ortholog *gana-1* (publication 1) we were not able to observe any morphological or other observable phenotype. The minimal RNAi phenotype was very probably caused by high residual enzymatic activity. Measurement of GAA activities at two distinct pH (4.0 and 6.5) and parallel acarbose inhibition showed separation of the neutral and acid glucosidase activities to different AAGRs (Table 1). We found the most relevant drop of activity values after RNAi of *aagr-2* at the acidic pH and in case of *aagr-3* RNAi at neutral pH, up to the levels of 20 % of control values. Glucosidase activities after RNAi of *aagr-1* was decreased more dramatically in the acidic pH (average value 68% of controls) compared to neutral pH (86% of control values). These changes were less dramatic compared to decrease of *aagr-2* and *aagr-3* activity after relevant RNAi. The decrease of GAA activities after RNAi of *aagr-4* was minimal.

Experiment No.		RNAi	α-glucosidase activity [nmol/$mg_{protein}$*h]							
			pH 4 acarbose -		**pH 4 acarbose +**		**pH 6,5 acarbose -**		**pH 6,5 acarbose +**	
			activity	% of control	activity	% of control	activity	% of control	activity	% of control
I.	**acidic**	***aagr-1***	141	**54**	10	**15**	252	**94**	215	**103**
		aagr-2	26	**10**	20	**31**	188	**70**	183	**88**
		control	261	**100**	64	**100**	268	**100**	208	**100**
V.	**neutral**	***aagr-3***	178	**106**	40	**103**	62	**25**	19	**9**
		aagr-4	166	**99**	37	**94**	203	**83**	157	**76**
		control	167	**100**	39	**100**	246	**100**	206	**100**

Table 1: Influence of acarbose on activities of all AAGRs in selected RNAi experiments. Table shows variable impact of acarbose on GAA activities measured at acidic (4.0) or neutral (6.5) pH after selected RNAi experiments of separate *aagr-1-4* compared to controls. Influence of acarbose on neutral activities of AAGR-3 and -4 was insignificant. All measurements were done at 37 ^{o}C with 8 μM acarbose.

We observed variable influence of acarbose on the GAA activity (Table 1) in the individual RNAi experiments. These experiments suggested AAGR-2 as the acarbose

inihibited acidic AAGR and AAGR-1 as the least acarbose sensitive acidic AAGR. This observation was futher supported by the analysis of *aagr-1* deletion mutant (see later). Based on this finding we consider AAGR-1 the most probable true ortholog of human acid α-glucosidase.

5.1.2.5 Isolation and characterization of the *aagr-1* and *aagr-4* deletion mutants

Deletion mutants of *aagr-1* (*ok2317*) and *aagr-4* (*ok1423*) were kindly generated by *C. elegans* Gene Knock-out Consortium [124]. We used the PCR primer data provided by the *C. elegans* Gene Knock-out Consortium to analyse the extent of the deletions.

The sequence analysis of the gDNA of the *ok2317* strain disclosed 1162 bp long deletion that leads to the loss of 218 amino acids of the primary structure of the AAGR-1 protein. Detailed microscopic analysis of the *ok2317*animals did not reveal any abnormal morphological phenotype. We performed GAA activity measurements at pH values 4.0 and 6.5 and parallel acarbose inhibition in the back-crossed nematode cultures homogenates of *ok2317* strain. We observed that acidic GAA activity significantly decreases as compared to standard N2 Bristol strain and that the residual acidic activity is strongly inhibited by acarbose. These results are in full accordance with the activity values acquired after RNAi assays of *aagr-1* in N2 Bristol strain and thus support our previous finding that AAGR-1 is the least acarbose sensitive acidic AAGR and thus the most probable ortholog of human acid α-glucosidase.

Unfortunately, the *aagr-1* deletion mutant does not clearly replicate GSD type II (Pompe) phenotype. We explain this finding primarily by the existence of the second enzyme (*aagr-2*) with acidic glucosidase activity in *C. elegans* which probably compensates the deficient acidic glucosidase activity of *aagr-1*. Based on this assumption, it is possible that double knockout of both enzyme with acidic GAA activity in *C. elegans* AAGR-1 and AAGR-2 could be suitable model for study Pompe disease.

The sequence analysis of the *ok1423* strain revealed 1832 bp long deletion in the *aagr-4* gene that leads to the loss of 281 amino acids from the conceptual translation. We did not see any abnormal morphological phenotype by microscopic analysis. Consequential measurement of GAA activities in the homogenates of deleted *vs.* standard N2 Bristol strain at pH values 4.0 and 6.5 showed the insignificant impact of the deletion on enzymatic activities at both pH levels. To investigate the contribution of the two predicted neutral activity enzymes, AAGR-3 and AAGR-4, to the global neutral GAA activity, we performed RNAi assays against *aagr-3* mRNA in *ok1423* back-crossed strain. The acquired activity values measured at neutral pH corresponded with the values obtained by the RNAi experiments against *aagr-3* in N2 Bristol strain. Activity values measured at acidic pH did not differ from the controls. Based on these results we conclude that AAGR-4 provides only a minor portion of the global GAA activity.

5.1.2.6 Transcriptional GFP fusions of *aagr-1* and *aagr-2* genes

In order to evaluate the expression patterns of the acid GAA orthologs AAGR-1 and AAGR-2 we prepared transcriptional GFP fusion constructs containing regulatory sequences of the *aagr-1* and *aagr-2* genes.

5.1.2.6.1 *Aagr-1*

Transcriptional extrachromosomal GFP construct include 2106 bp of *aagr-1* regulatory sequence, which cover 1340 bp upstream of *aagr-1* ATG and complete intron 1. GFP

expression was limited to six coelomocytes and in some cases to intestinal cells and was observed in L4 and adult nematode stages.

5.1.2.6.2 *Aagr-2*

Transcriptional extrachromosomal GFP construct of *aagr-2* covered 3431bp upstream of *aagr-2* ATG. Positive signal was spatially limited to membrane-bounded vacuoles of 6 coelomocytes and the diffuse GFP signal was observed also in the pseudocoelom. Signal in the pseudocoelom was highly suggestive of coelomocytic secretory origin. Temporally the expression was observed in L4 and adult nematode stages, similarly to GFP expression of *aagr-1*.

5.1.2.7 Bioinformatic studies on *aagr-1-4*

Multiple alignment of amino acid sequences of selected eukaryotic proteins from GH31 family and AAGRs demonstrated conservation of primary protein sequence within the extent of GH31 module [101, 147]. The multiple alignment and unrooted phylogenetic tree divided protein sequences into two discrete clades. Proteins forming the first clade were confirmed or predicted to have acid pH optima of enzymatic activity (GAA, SUIS, MGA) and included AAGR-1 and -2 and proteins from the second clade have neutral pH optima of enzymatic activity (GANC, GANAB) and included AAGR-3 and -4. The immediate surroundings of the active site nucleophile (WiDMnE) was the most conserved region in the aligment.

Homology modeling was based on multiple alignment of *E. coli* YicI [101], *S. solfataricus* [103], *H. Sapiens* NtMGA [104] and all four AAGRs. The best template for all AAGRs was 3D structure of NtMGA [104] with inserted inhibitor acarbose. The sequence identity of the catalytic domain between the template NtMGA and AAGR-1 and -2 was higher when compared to AAGR-3 and -4. Moreover, the entire N-terminal domain of AAGR-3 and -4 shares low level of identity with all templates because of their higher similarity with GANAB and GANC proteins.

We observed only few substitutions in the active site of AAGR-1-4. The most important difference between templates and model molecules was the substitution tyrosine/tryptophan (Y/W) in the active site which may discriminate between proteins which are inhibited/uninhibited by acarbose. The residues Y299 of NtMGA aligns to Y184 in MalA and structurally corresponds to Y304 in AAGR-2 (proteins inhibited by acarbose) but is substituted by W316, W361 and W353 in AAGR-1, -3 and -4 (proteins insensitive to acarbose). These results further confirm AAGR-1 as the true ortholog of human acid α-glucosidase.

5.1.3. Characterisation of human Acetyl-CoA: α-glucosaminide N-acetyl-transferase on the cellular level (publication 2 and supplementary materials to this publication)

Based on the results of linkage analyses that narrowed the candidate region for human heparin acetyl-CoA:α-glucosaminide N-acetyltransferase (HGSNAT, N-acetyltransferase) to a 2.6-cM interval between *D8S1051* and *D8S1831*, we identified the TMEM76 gene. TMEM76 was located within the candidate region as the gene that encodes lysosomal HGSNAT and whose deficit causes mucopolysaccharidosis IIIC (MPS IIIC, or Sanfilippo C syndrome) (for details see publication 2). Concurrently with publication of our results another group reported the same gene found by proteomic analyses [110].

5.1.3.1 Sequence verification of predicted gene

We verified the sequence of the predicted gene by sequencing of the full-length cDNA (4.5 kb) which is composed of two polyadenylation signals and 1992 bp long coding sequence containing 18 exons. We found that HGSNAT is ubiquitously expressed in various human tissues. In addition to the full-lenght transcript we amplified two shorter ones by RT-PCR. We identified one alternative transcript with spliced out exons 9 and 10 resulting in an in-frame deletion of 64 amino acids. We suppose that this transcript does not encode an active enzyme because it was detected in two patients with MPS IIIC who had almost complete loss of HGSNAT activity. The second shorter transcript lacked exons 3, 9 and 10 and led to an in-frame deletion of 107 amino acids.

5.1.3.2 Basic bioinformatic analysis

An integrated bioinformatic search and predictions performed by SignalP, TMHMM and NetNGlyc servers [122] reveal that HGSNAT encodes a protein composed of 663 amino acids (73 kDa) with N-terminal signal peptide, 11 predicted transmembrane domains and 4 potentional N-glycosylation sites.

Surprisingly, although it is present in a variety of mammalian genomes, the sequence homology searches demonstrated that HGSNAT does not have a structural similarity neither to any known prokaryotic or eukaryotic N-acetyltransferase (including *C. elegans*) nor to other lysosomal proteins. HGSNAT shares homology with conserved uncharacterized family of bacterial membrane proteins COG 4299. Based on this finding we did not study HGSNAT in *C. elegans*.

5.1.3.3 Cellular distribution of HGSNAT (supplementary material to the publication 2)

We have obtained an affinity purified rabbit polyclonal antibody against a peptide epitope from one of the HGSNAT extramembrane domains. The specifity of the antibody was verified by Western blotting and by measuring of dependence of HGSNAT activity on concentration of the antibody. The activity of HGSNAT was inhibited by increasing concentration of antibody in contrast to activity of control enzyme β-hexosaminidase that did not change during experiments.

Immunofluorescence microscopy showed that in control cultured human fibroblasts the anti-HGSNAT antibody stained a subpopulation (approximately 25- 50% of LAMP2-positive vesicles) of lysosomes. The signal did not systematically co-localize with any of the other organellar markers: PDI (endoplasmic reticulum), Golgi 58K (Golgi apparatus), COXI (mitochondria).

The systematic co-localization studies with EEA1 (early endosomal compartment), mannoso-6-phosphate receptor (M6PR, late endosomal compartment), cathepsin D (lysosomal luminal protein) and LAMP 2 (lysosomal associated membrane protein) showed that the anti-HGSNAT antibody is not detected neither in early nor late endosomes but stained only discrete regions of the lysosomal membrane probably on its luminal site. The immunoelectron detection of HGSNAT in enriched lysosomal franctions further confirmed the localization in discrete regions of lysosomal membranes on luminal site. These results corresponded well with the microdomain localization of HGSNAT previously proposed by biochemical methods [113].

In spite of apparent microdomain localization, HGSNAT did not co-localize with tested protein and lipid microdomain markers such as flotillin 1, GM1 ganglioside, globotriaosylceramide (Gb3) or cholesterol (filipin staining). HGSNAT, however, partially co-localized with sortilin, the alternative receptor for transport of soluble or membrane-associated

lysosomal proteins into lysosomes [60, 62]. Based on the result we hypothesize that lysosomal sorting of the HGSNAT can be sortilin dependent.

We studied the cellular distribution of HGSNAT in selected lysosomal storage disorders. The transport of HGSNAT apparently does not depend on MPR as it reached lysosomes in I-cell disease cells (N-Acetylglucosamine-1-phosphotransferase deficiency) [148]. The subcellular distribution of HGSNAT also does not seem to be affected by deficiencies in other proteoglycan hydrolytic lysosomal pathways as demonstrated in selected mucopolysaccharidoses (I, II, IIIA and IIIC) cell lines.

We were further interested in distribution of HGSNAT compared with its substrate – heparan sulphate. Surprisingly we were not able to demonstrate systematic co-localization of HGSNAT and heparan sulphate (HS) in any kind of evaluated cell lines. We explain this finding by the detection limits of the antibody and indirect immunofluorescence techniques.

The supplementary material provides evidence of the presence of microdomains in lysosomal membrane on microscopy level and confirms the microdomain localisation of HGSNAT.

6. Conclusions

6.1 General conclusions

The principal theme of this thesis was the study of selected deficits of lysosomal glycans metabolism - Fabry, Schindler, Pompe and Mucopolysaccharidosis IIIC diseases. The majority of this work was focused on simple metazoan model organism *Caenorhabditis elegans* and its use in modeling of lysosomal enzymopathies. The most important step was the identification of true nematode´s orthologs of selected human hydrolases and their characterisation. The main approach in modeling of diseases was RNA-mediated interference of relevant orthologous genes. We found that RNAi did not provide sufficient expression knock-down of hydrolases, which is necessary for development of lysosomal storage. Based on these results we propose that deletion mutants of relevant lysosomal hydrolases with zero activity may serve as valuable models for studying human LSDs. Even though we did not manage to generate strains with lysosomal storage, study of *C. elegans* orthologs of lysosomal glycosidases provided important information about these proteins and their phylogeny which could be applied in further studies of these diseases. In addition, as was shown in many other studies [32], *C. elegans* is a valuable model organism for studying human pathology states even in cases when the nematode´s mutant phenotype does not fully replicate human disease pathology.

The last part of this thesis dealt with the characterisation of lysosomal membrane protein HGSNAT mutated in mucopolysaccharidosis IIIC, which catalyzes a key step in degradation of glycosaminoglycan heparan sulphate. We found that HGSNAT does not have any *C. elegans* ortholog. The study of the cellular distribution of HGSNAT showed that HGSNAT is situated in discrete regions of lysosomal membranes and is present only in a subpopulation of lysosomal cellular pool. Our study gave evidence of presence of lysosomal membrane microdomains.

6.2 Individual conclusions

We found only one *C. elegans* ortholog (GANA-1) of both human α-GAL A and α-NAGA proteins. Biochemical, RNAi, GFP expression and bioinformatic analyses confirmed our assumption that GANA-1 has both α-GAL and α-NAGA activities and is localized in acidic cellular compartment. In addition, phylogenetic and homology modeling analyses confirm the hypothesis that α-Gal A and α-Naga genes arose by duplication from a hypothetical ancestral gene and that *gana-1* probably developed from this hypothetical ancestral gene before the duplication event. We propose that a deletion mutant of *gana-1* may provide a suitable model for studying Fabry and Schindler diseases in *C. elegans*.

We found four potential *C. elegans* orthologs (*aagr-1-4*) of human acid α-glucosidase. We determined that AAGR-1 and -2 have predominant acidic and AAGR-3 and -4 neutral GAA activity based on results of biochemical, RNAi, GFP expression, bioinformatic and mutant analyses. We demonstrated that AAGR-2 is the prevalent contributor to the total acidic and AAGR-3 to the total neutral GAA activity and that expression of the acidic proteins AAGR-1 and -2 is localized to a subset of endocytically active cells. Our results shown that AAGR-1 is the least acarbose sensitive acidic AAGR which was further supported by the analysis of *aagr-1* deletion mutant. Based on these outcomes we consider AAGR-1 likely true ortholog of human acid α-glucosidase. However, deletion mutant of *aagr-1*, which we had at our disposal, does not clearly replicate GSD type II (Pompe) lysosomal storage phenotype.

That is apparently due to existence of the second enzyme AAGR-2 with acidic GAA activity which sufficiently compensates the deficient AAGR-1 activity. We speculate that double knockout of both *C. elegans* gene *aagr-1* and *-2* could be a suitable model for GSD type II.

We identified the gene coding HGSNAT based on the genetic linkage analysis. Sequence verification confirmed that cDNA of HGSNAT is 4.5 kb long and contains 1992 bp long coding sequence composed of 18 exons. HGSNAT is ubiquitously expressed in various human tissues. Bioinformatic analyses reveal that HGSNAT contains N-terminal signal peptide, 11 predicted transmembrane domains and 4 potential N-glycosylation sites. Interestingly, the sequence homology searches indicate that human HGSNAT does not share homology with any known prokaryotic or eukaryotic N-acetyltransferase (including *C. elegans*) except its mammalian orthologs. Immunofluorescence microscopy analyses demonstrated that HGSNAT is present only in a fraction of lysosomes and is localised in microdomains of lysosomal membranes. The partial co-localisation of HGSNAT and sortilin suggests sortilin dependent lysosomal sorting of HGSNAT. The cellular distribution of HGSNAT does not seem to be influenced by lysosomal storage.

7. Abbreviation

Aagr*:** acid α-glucosidases related, **ASM**: acid sphingomyelinase, **BBB**: blood – brain barrier, **bp**: base pairs, **BSA**: bovine serum albumin, ***C. briggsae: *Caenorhabditis briggsae*, ***C. elegans***: *Caenorhabditis elegans*, **cDNA**: complementary DNA, **CGC**: *Caenorhabditis* Genetics Center, **CNS**: central nervous system, **Co A**: coenzyme A, **CON A**: concanamycin A, **D-Gal**: D-galactose, **D-GalNAc**: N-acetyl-D-galactosamine, **DMEM**: Dulbecco's modified eagle's medium, **DNA**: deoxyribonucleic acid, **DOPE**: Discrete Optimized Protein Energy,
dsRNA: double-stranded RNA, ***E. Coli***: *Escherichia coli*, **EEA**: early endosome antigen, **EET**: enzyme enhancement therapy, **ER**: endoplasmic reticulum, **ERT**: enzyme replacement therapy, **EST**: expressed sequence tag, **FCS**: fetal calf serum, **GAA**: acid α-glucosidase, **GANA-1:** α-GAL and α-NAGA *C. elegans* ortholog, **GANAB**: glucosidase II alpha subunit, **GANC**: neutral α-glucosidase C, **Gb 3**: globotriaosylceramide, **gDNA**: genomic DNA, **GFP**: green fluorescent protein, **GH31**: glycosyl hydrolases family 31, **GSD II**: glycogen storage disease type II, **HGSNAT**: heparin acetyl-coenzyme A:α-glucosaminide *N*-acetyltransferase, **HS**: heparan sulphate, **IMD**: inherited metabolic diseases, **IPTG**: isopropyl-β-D-thiogalactopyranoside, **kb**: kilobase, **L1-4:** larval stages 1-4, **LAMP**: lysosomal associated membrane protein, **LIMP**: lysosomal integral membrane protein, **LMP**: lysosomal membrane protein, **LSD**: lysosomal storage diseases, **M6P**: mannose-6-phosphate, **MGA**: maltase – glucoamylase, **MLII**: mucolipidosis II, **MPR**: mannose-6-phosphate receptor, **MPS**: mucopolysaccharidoses, **mRNA**: messenger RNA, **MU**: methylumbelliferyl, **NGM**: nematode grow medium, **NLS:** nuclear localization sequence, **NtMGA**: N-terminal subunit of human maltase-glucoamylase, **ORF**: open reading frame, **OST**: open reading frame sequence tag, **PAGE**: polyacrylamide gel electrophoresis, **PBS**: phosphate buffered saline, **PCR**: polymerase chain reaction, **PDI**: protein disulfide isomerase, **PSF**: point spread function, **RNA**: ribonucleic acid, **RNAi** : RNA - mediated interference, **RT**: room temperature, **RT-PCR**: reverse transcription polymerase chain reaction, **SAPLIP**: saposin – like protein, **SDS**: sodium dodecyl sulphate, **SL**: splice leader, **SNP**: single nucleotide polymorphism, **SRT**: substrate reduction therapy, **SUIS**: sucrase – isomaltase, **swPCR**: single worm PCR, **TGN:** *trans* Golgi network, **TMEM**: transmembrane protein, **UTR**: untranslated region, **α-GAL**: α-galactosidase, **α-Glu**: α-glucosidase, **α-NAGA**: α-*N*-acetylgalactosaminidase, **β-Hex**: β-hexosaminidase.

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9. List of publications

9.1 Publications related to the thesis

1) Characterization of gana-1, a Caenorhabditis elegans gene encoding a single ortholog of vertebrate alpha-galactosidase and alpha-N-acetylgalactosaminidase.
Hujová J.[*],Sikora J.[*],Dobrovolný R.[*],Poupětová H.,Ledvinová J.,Kostrouchová M.,Hřebíček M.
BMC Cell Biol 2005 Jan 27; 6(1):5. **(IF2007 = 3.092)**
[*]equal contributors

2) Mutations in TMEM76 Cause Mucopolysaccharidosis IIIC (Sanfilippo C Syndrome).
Hrebicek M., Mrazova L., Seyrantepe V., Durand S., Roslin NM., Noskova L., Hartmannova H., Ivanek R., Cizkova A., Poupetova H., Sikora J., **Urinovska J.,** Stranecky V., Zeman J., Lepage P., Roquis D.,Verner A., Ausseil J., Beesley CE., Maire I., Poorthuis BJ., van de Kamp J., van Diggelen OP., Wevers RA., Hudson TJ., Fujiwara TM., Majewski J., Morgan K., Kmoch S., Pshezhetsky AV.
Am J Hum Genet. 2006 Nov;79(5):807-819. Epub 2006 Sep 8. **(IF2007 = 11.092)**

Members of glycoside hydrolases family 31 in *Caenorhabditis elegans*: Search for the worm's lysosomal acid α-glucosidase.
Uřinovská J.*, Sikora J.*, Poupětová H, Majer F, Hlavatá J, Jelínek J, Kostrouchová M, Hřebíček M.
Submitted manuscript to BMC Cell Biology
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9.2 Other publications

Eight novel ABCD1 gene mutations and three polymorphisms in patients with X-linked adrenoleukodystrophy: The first polymorphism causing an amino acid exchange.
Dvorakova L.,Storkanova G.,Unterrainer G.,**Hujova J.,**Kmoch S.,Zeman J.,Hrebicek M.,Berger J.
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9.3 Published abstracts

XVII. biochemický sjezd, Prague, Czech republic, September 2000
Dvořáková L., Štorkánová G., Hřebíček M., Zeman J., **Hujová J.**, Unterrainer G., Berger J.
Mutation analysis and identification of carriers in X-linked adrenoleukodystrophy (X-ALD) families.The first polymorphism causing an amino acid exchange within the ALDP-gene.
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Sikora J., **Hujova J.**, Dobrovolny R., Holanova D., Asfaw B., Poupetova H., Ledvinova J., Kostrouchova M., Hrebicek M.
Caenorhabditis elegans as a model for lysosomal glycosidase deficiencies.
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53rd Annual Meeting of The American Society of Human Genetics, Los Angeles, USA, November 2003
Hrebicek M., Sikora J., **Hujova J.**, Holanova D., Poupetova H., Asfaw B., Ledvinova J., Kostrouchova M.
Evaluation of *Caenorhabditis elegans* model for lysosomal diseases caused by deficits of glycosidases.
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9.4 Other selected presentations

18. pracovní dny Dědičné Metabolické Poruchy, Slušovice, Czech republic, May 2003
Sikora J., **Hujova J.**, Dobrovolny R., Holanova D., Asfaw B., Poupetova H., Ledvinova J., Kostrouchova M., Hrebicek M.
Caenorhabditis elegans as a model for lysosomal glycosidase deficiencies.
First price for the scientists under 30 years of age.

14th ESGLD Workshop, Poděbrady, Czech Republic, September 2003
Hujova J., Dobrovolny R., Sikora J., Holanova D., Asfaw B., Poupetova H., Ledvinova J., Kostrouchova M., Hrebicek M.
Caenorhabditis elegans as a model for Fabry and Schindler diseases.

14th ESGLD Workshop, Poděbrady, Czech Republic, September 2003
Sikora J., **Hujova J.**, Dobrovolny R., Holanova D., Asfaw B., Poupetova H., Ledvinova J., Kostrouchova M., Hrebicek M.
Caenorhabditis elegans as a model for acid α-glucosidase deficiency.

European Worm Meeting , Casino Kursaal, Interlaken, Switzerland, May 2004
Hujova J., Dobrovolny R., Sikora J., Holanova D., Asfaw B., Poupetova H., Ledvinova J., Kostrouchova M., Hrebicek M.
Caenorhabditis elegans has only one α-galactosidase/ α -N-acetylgalaktosaminidase ortholog

5th students´ scientific conference 1. LF UK, Prague, Czech Republic, May 2004
Sikora J., **Hujova J.**, Dobrovolny R.
Caenorhabditis elegans has only one α-galactosidase and α -N-acetylgalaktosaminidase ortholog
First price.

15th ESGLD Workshop, Oslo, Norway, September 2005
Uřinovská J., Sikora J., Dobrovolný R., Poupetova H., Ledvinova J., Kostrouchova M., Hřebiček M.
Characterization of *gana-1*, a *Ceanoharbditis elegans* gene encoding a single ortholog of vertebrate α-galactosidase and α-N-acetylgalactosaminidase

16th ESGLD Workshop, Perugia, Italy, September 2007
Uřinovská J., Sikora J., Poupetova H., Hlavata J., Kostrouchova M., Hřebiček M.
Glycosyl hydrolases family 31 members in *Caenorhabditis elegans*: Search for worm´s lysosomal acid α-glucosidase.

47th ASCB Annual Meeting, Washington, DC, December 2007
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Microscopic evidence for HGSNAT-containing microdomains in lysosomal membrane.