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Review

Molecular basis of multiple sulfatase deficiency, mucolipidosis II/III and Niemann–Pick C1 disease — Lysosomal storage disorders caused by defects of non-lysosomal proteins ☆

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ABSTRACT

Multiple sulfatase deficiency (MSD), mucolipidosis (ML) II/III and Niemann–Pick type C1 (NPC1) disease are rare but fatal lysosomal storage disorders caused by the genetic defect of non-lysosomal proteins. The NPC1 protein mainly localizes to late endosomes and is essential for cholesterol redistribution from endocytosed LDL to cellular membranes. NPC1 deficiency leads to lysosomal accumulation of a broad range of lipids. The precise functional mechanism of this membrane protein, however, remains puzzling. ML II, also termed I cell disease, and the less severe ML III result from deficiencies of the Golgi enzyme *N*-acetylglucosamine 1-phosphotransferase leading to a global defect of lysosome biogenesis. In patient cells, newly synthesized lysosomal proteins are not equipped with the critical lysosomal trafficking marker mannose 6-phosphate, thus escaping from lysosomal sorting at the trans Golgi network. MSD affects the entire sulfatase family, at least seven members of which are lysosomal enzymes that are specifically involved in the degradation of sulfated glycosaminoglycans, sulfolipids or other sulfated molecules. The combined deficiencies of all sulfatases result from a defective post-translational modification by the ER-localized formylglycine-generating enzyme (FGE), which oxidizes a specific cysteine residue to formylglycine, the catalytic residue enabling a unique mechanism of sulfate ester hydrolysis. This review gives an update on the molecular bases of these enigmatic diseases, which have been challenging researchers since many decades and so far led to a number of surprising findings that give deeper insight into both the cell biology and the pathobiochemistry underlying these complex disorders. In case of MSD, considerable progress has been made in recent years towards an understanding of disease-causing FGE mutations. First approaches to link molecular parameters with clinical manifestation have been described and even therapeutical options have been addressed. Further, the discovery of FGE as an essential sulfatase activating enzyme has considerable impact on enzyme replacement or gene therapy of lysosomal storage disorders caused by single sulfatase deficiencies.

1. Introduction

Lysosomal storage disorders (LSDs) arise when one or several lysosomal proteins, in particular catabolic hydrolases, are deficient, so that the substrates of the affected enzyme(s) are no longer degraded and thus accumulate. Apart from the many specific lysosomal enzyme deficiencies there are two fatal diseases in which an entire family or even the majority of all lysosomal enzymes do not fulfill their functions. Both syndromes, mucolipidoses (ML) II/III and multiple sulfatase deficiency (MSD), result from deficiencies of specific post-translational modifications, namely the generation of mannose 6-phosphate or Cα-formylglycine (FGly) residues, respectively. Mannose 6-phosphate is introduced into the majority of newly synthesized lysosomal enzymes in the Golgi apparatus, while FGly modification of nascent sulfatases occurs in the ER. Niemann–Pick type C1 (NPC1) disease is a third LSD originating from the defect of a largely non-lysosomal protein. The NPC1 protein localizes mainly to late endosomes and fulfills an unknown function, deficiency of which leads to lysosomal accumulation of a broad range of lipids.

☆ Databases: *SUMF1* – OMIM: 607939, 272200 (MSD); *NPC1* – OMIM: 607623, 257220 (NPC1 disease); *GNPTA* – OMIM: 607840, 252500 (MLII alpha/beta), 252600 (MLIII alpha/beta); *GNPTG* – OMIM: 607838, 252605 (MLIII gamma).

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MSD serves as a paradigmatic example of how an inherited disease can guide scientists to discover fundamental new aspects in basic research, which in turn have direct impact for therapy. The key finding that MSD patient cells express wild-type cDNAs yielding full-length sulfatase polypeptides that, however, lack enzymatic activity [1] led to the prediction and, finally, discovery of a novel post-translational modification of sulfatases, namely the generation of FGly by oxidation of a specific cysteine residue [2]. The critical role of this novel amino acid and its highly reactive and, in effect, toxic formyl group was later understood to originate from the formation of a (non-toxic) aldehyde hydrate within the active site of sulfatases carrying two geminal hydroxyl groups which both are required for sulfatase ester hydrolysis according to a novel mechanism [3,4]. In fact, the hydrate water molecule allows for an 'intramolecular hydrolysis' of a covalent sulfo-enzyme intermediate without the requirement of activating an 'external' water molecule, e.g., by metal ions as employed by other esterases [5].

Also FGE, the enzyme generating this unusual amino acid, was and still is full of surprises both with regard to its structure and function as a metal-independent oxygenase and its unusual cell biology, as is described in further detail below. Of the findings so far, two aspects had immediate relevance for therapeutic options. The finding that FGE is secreted, taken up by other cells and transported all the way back to the ER of recipient cells [6] is the basis for a scenario to apply FGE in enzyme replacement therapy to treat MSD, a rather unusual approach for an enzyme defect of the ER. Secondly, FGE already now is an important tool for the production of highly active sulfatases to be used for enzyme replacement therapy of lysosomal storage disorders caused by single sulfatase deficiencies [7] (M. Heartlein, personal communication).

Thus, multiple lessons have been learned from MSD so far, which underlines the general importance of studying inherited diseases even if only very few patients are affected. On the other hand, many questions need to be answered to unravel the steps from the basic molecular defect to the manifestation of the disease, each step with the potential to be targeted for therapeutic purposes and, altogether, to gain deeper insight into complex pathophysiological mechanisms. Some of these issues will be illustrated below for MSD, and in less depth, also for ML II/III and NPC1-disease, three lysosomal storage disorders caused by deficiencies of non-lysosomal key players in cell biology (though in the case of NPC1 the precise role awaits to be uncovered).

2. Mucolipidoses II/III — diseases of lysosomal protein trafficking

The mucolipidoses ML II alpha/beta, ML III alpha/beta and ML III gamma are three related autosomal-recessive lysosomal storage disorders in which the transport of newly synthesized lysosomal proteins to the lysosomes is impaired due to a genetic defect of the Golgi enzyme uridine diphosphate (UDP)-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine 1-phosphotransferase (EC 2.7.8.15), commonly termed GlcNAc phosphotransferase [8]. Although these diseases are rare in occurrence (see Table 1), they have provided important insight into the transport of lysosomal hydrolases [9,10]. The recent reclassification of the ML II and ML III disorders has replaced the previous nomenclature as shown in Table 1 [11].

Table 1
Classification of mucolipidoses II and III

Disorder	Affected gene (chromosomal location)	Previous nomenclature	OMIM#	Birth prevalence per 100,000
ML II alpha/beta	*GNPTAB* (12q23.3)	ML II, I-cell disease	252500	0.16
ML III alpha/beta	*GNPTAB* (12q23.3)	ML IIIA, classic pseudo Hurler polydystropy	252600	0.08
ML III gamma	*GNPTG* (16p13.3)	ML IIIC, ML III variant	252605	

OMIM catalog numbers (Online Mendelian Inheritance in Man, John Hopkins University) and prevalence of the mucolipidoses per 100,000 births according to [9].

2.1. Molecular and cell biological basis of ML II/III

In higher eukaryotes, most lysosomal hydrolases are targeted to the lysosomes via the mannose 6-phosphate pathway which requires the posttranslational modification of newly synthesized lysosomal proteins with mannose 6-phosphate as a recognition marker (reviewed in [12]). As depicted in Fig. 1, along the biosynthetic pathway the mannose 6-phosphate residue is generated in two subsequent enzymatic reactions [13]. Following *N*-glycosylation of asparagine residues in the ER, GlcNAc phosphotransferase in the Golgi catalyzes the transfer of GlcNAc 1-phosphate to the C6 position of selected α1,2-linked mannose residues on high mannose-type oligosaccharides [14–17]. The topogenic structure required for the recognition of lysosomal hydrolases by GlcNAc phosphotransferase is still not well defined. The presence of adequately spaced lysine residues appears to be necessary, although no common recognition motif could be identified even among closely related lysosomal enzymes [18]. In the second step, the mannose 6-phosphate marker is exposed via removal of the *N*-acetylglucosamine residue by the uncovering enzyme *N*-acetylglucosamine 1-phosphodiester *N*-acetylglucosaminidase (NAGPA, EC 3.1.4.45) localized in the trans Golgi network [19,20]. Subsequently, lysosomal proteins can be recognized by the mannose 6-phosphate receptors (MPR46/MPR300) and shuttled to early or late endosomes where they dissociate from their receptors due to the low pH and are further transported to the lysosomes (reviewed in [21]). Interestingly, inherited genetic disorders are only known for GlcNAc phosphotransferase, but not for the uncovering enzyme NAGPA [10,19].

GlcNAc phosphotransferase has been characterized as an enzyme complex with a hexameric $\alpha_2\beta_2\gamma_2$ subunit structure [22]. It has been suggested that the α and β subunits contain the catalytic activity, whereas the γ subunit is responsible for the recognition of lysosomal proteins [8,23]. The subunits of the enzyme are encoded by two genes, *GNPTA* (also termed *GNPTAB*) and *GNPTG*. The *GNPTA* gene is located on chromosome 12q23.3 and encodes an αβ precursor protein with a complex modular domain structure of 1256 amino acid residues that is proteolytically processed to release the α and β subunits, both equipped with a transmembrane domain [24,25]. The gene *GNPTG* localizes to chromosome 16p13.3 and codes for the γ subunit, a soluble protein of 305 amino acid residues that associates with the α/β subunits, yielding a membrane-bound enzyme complex of 540 kDa in the *cis* Golgi [22,23,25].

Mutations in the *GNPTA* gene as observed in ML II alpha/beta and ML III alpha/beta result in a reduction of GlcNAc phosphotransferase activity [24–31]. As a consequence, the lysosomal proteins lacking mannose 6-phosphate residues are unable to bind to mannose 6-phosphate receptors, resulting in hypersecretion into the extracellular space and the body fluids instead of lysosomal targeting. Similar to other LSDs, undigested macrocompounds accumulate in the lysosomes. Total or near-total deficiency of GlcNAc phosphotransferase leads to ML II alpha/beta, the clinically more severe form. ML III is genetically heterogeneous [32]. In the case of ML III alpha/beta, GlcNAc phosphotransferase activity is reduced rather than absent, resulting in a milder phenotype compared to ML II alpha/beta [8].

Mutations in the gene encoding the γ subunit, *GNPTG*, are responsible for ML III gamma [23,33]. This variant is characterized by normal activity of GlcNAc phosphotransferase towards the small pseudosubstrate α-methyl mannoside but reduced activity towards native lysosomal hydrolases as substrates [34,35]. Studies with knock out mice lacking either *GNPTA* or *GNPTG* have shown that the loss of the α/β subunits completely abolishes formation of mannose 6-

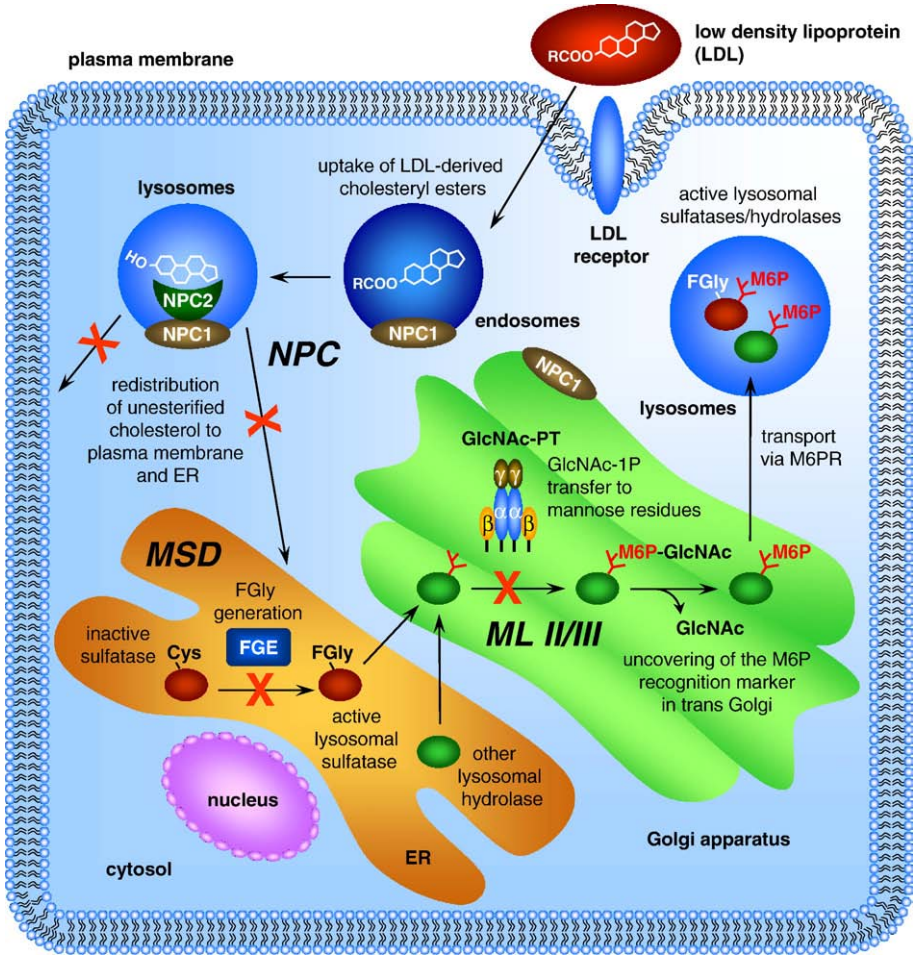

Fig. 1. Lysosomal storage disorders caused by deficiencies of non-lysosomal proteins. The illustration summarizes the molecular bases of Niemann–Pick disease type C (NPC), mucolipidoses II and III (ML II/III) and multiple sulfatase deficiency (MSD) (see text for details). In case of NPC, the intracellular redistribution of LDL-derived cholesterol from lysosomes to the ER and plasma membrane is impaired due to a genetic defect of either NPC1, a transmembrane protein localized mainly in endosomes, but also in the TGN or lysosomes, or in rare cases of NPC2, a soluble lysosomal protein. The loss of either protein leads to lysosomal storage of unesterified cholesterol and gangliosides. ML II and ML III are disorders caused by a deficiency of GlcNAc phosphotransferase (GlcNAc-PT), a Golgi enzyme required for generation of the mannose 6-phosphate recognition marker that directs the targeting of lysosomal proteins via the mannose 6-phosphate receptor pathway. Because lysosomal hydrolases cannot reach their destination, undigested storage material accumulates within lysosomes. Also in MSD lysosomal storage (of sulfated molecules) results from a defect in post-translational modification of hydrolytic enzymes, which is specific for the family of the sulfatases. MSD is caused by a genetic deficiency of FGE, the formylglycine generating enzyme. FGE localizes to the ER and catalyzes the oxidation of a specific cysteine residue of newly synthesized sulfatases to the active site Cα-formylglycine residue, which is essential for enzymatic activity.

phosphate residues, whereas reduced but still significant synthesis occurs in the absence of the γ subunit [36]. Therefore it was proposed that the α/β subunits, in addition to their catalytic function, interact with lysosomal hydrolases and that this recognition is enhanced by the γ subunit. Also a possible role of the γ subunit in facilitating protein folding or maintaining enzyme conformation has been discussed [37]. Intriguingly, there is no direct evidence of interactions between the γ subunit and lysosomal proteins [37], making an understanding of the pathomechanisms of ML III gamma even more difficult.

2.2. Clinical features, diagnosis and therapeutic approaches

The term mucolipidosis refers to the clinically observed combination of the symptoms of mucopolysaccharidoses and sphingolipidoses [38,39]. In most cases of ML II alpha/beta, defects are apparent from early infancy or even prenatally. Patients are typically characterized by dwarfism, coarse facies, stiffness of the joints, skeletal abnormalities, cardio- and hepatomegaly and mental retardation. One of the most characteristic histological features is the presence of enlarged lysosomes filled with undigested compounds in patient fibroblasts, also called inclusion- or I-cells [40]. ML II alpha/beta has a fatal outcome usually between 5 and 8 years of age due to congestive heart failure and/or recurrent pulmonary infections [8]. The variability in clinical symptoms of the mucolipidoses corresponds to differences in residual GlcNAc phosphotransferase activities, resulting in a wide spectrum of severity from prenatally lethal [41] to progressive ML II alpha/beta and mild adult onset forms of ML III alpha/beta [28,42].

ML III was first described as pseudo Hurler polydystrophy due to similarities with the Hurler syndrome (MPS-IH) caused by a genetic deficiency of α-iduronidase [43]. In a series of genetic complementation experiments with patient fibroblasts [44], different complementation groups were identified, corresponding to ML III alpha/beta and ML III gamma [11]. Clinical symptoms of ML III alpha/beta and ML III gamma are indistinguishable and represent a mild and attenuated form of ML II alpha/beta. Both ML III forms are characterized by a later onset, at the age of 2 to 4 years, and a more slowly progressive course, permitting patient survival into the eighth decade [45,46]. About 50% of reported patients have learning disabilities or mental retardation. Skeletal dysplasia and especially destruction of the hip joints are most disabling symptoms of the ML III disorders [8]. Residual GlcNAc phosphotransferase activities in ML III alpha/beta are reported to be 2 to 20% of normal values [8,34,45].

Clinically suspicious patients can be diagnosed by enzymatic and genetic techniques. The enzymatic activities of different lysosomal hydrolases are severely decreased in fibroblasts of affected patients, whereas they are markedly increased in serum or cell culture medium. Sequencing of the impaired genes will confirm the diagnosis and identify the underlying mutations, which are the basis for genetic counseling. Both mucolipidoses ML II and ML III can be diagnosed prenatally [47–49], but no etiological therapies are currently available for their treatment. Studies with a limited number of ML II alpha/beta patients have described bone marrow transplantations as beneficial with regard to improving neurodevelopment and cardiopulmonary complications, although the underlying mechanisms remain unclear [50,51]. In the case of ML III, treatment with bisphosphonate pamidronate has been reported to reduce bone pain and to improve mobility [52].

Since GlcNAc phosphotransferase is a membrane-bound enzyme complex localized in the Golgi apparatus, enzyme replacement therapy as practiced for LSDs caused by deficiencies of soluble lysosomal hydrolases is not likely to be successful. Instead, gene therapy might represent a promising approach as indicated by initial experiments using retroviral transduction [25]. In this study, the *GNPTA* gene was successfully expressed in fibroblasts from an ML II alpha/beta patient, resulting in Golgi localization of GlcNAc phosphotransferase and correction of hypersecretion of lysosomal hydrolases.

3. Niemann–Pick C1 disease — a disease of subcellular lipid transport

Niemann–Pick type C (NPC) disease is an autosomal-recessive lysosomal lipid storage disorder. According to the most widely accepted model, NPC is caused by an impaired redistribution of LDL-derived cholesterol from the endocytic compartments to the ER and the plasma membrane (extensively reviewed in [53–58]). NPC represents a rare, genetically heterogeneous disease resulting from mutations in either the *NPC1* gene (MIM# 257220, in 95% of the cases) or the *NPC2* gene (MIM# 607625). Biochemically, NPC is not related to the Niemann–Pick type A and B diseases which are due to genetic defects of the lysosomal enzyme sphingomyelinase [59]. Whereas the *NPC2* gene product represents a classical lysosomal protein, NPC1 predominantly localizes to late endosomes [60,61]. NPC1 disease can therefore be considered, although less strictly than in case of MSD and ML II/III, as a lysosomal storage disorder caused by a defect outside of the lysosomes.

3.1. Molecular and cell biological basis of NPC

Under normal physiological conditions, extracellular low-density lipoproteins (LDL) are recognized by LDL receptors on the cell surface, internalized and transported to the lysosomes, where LDL-derived cholesteryl esters are hydrolyzed [62–65]. The released cholesterol is redistributed to the ER and to the plasma membrane, as illustrated in Fig. 1. Although the underlying mechanisms are still unclear, both NPC1 and NPC2 proteins are involved in the egress of cholesterol from late-endosomes/lysosomes and in intracellular cholesterol homeostasis. Deficiency of either the NPC1 or NPC2 protein leads to a massive lysosomal storage of unesterified cholesterol, sphingosine, glycosphingolipids and bis(monoacylglycerol)phosphate mainly in the liver and spleen.

Although no overt cholesterol accumulation is observed in the brain, NPC results in a progressive and finally fatal degeneration of the central nervous system, probably due to lysosomal storage of cholesterol and gangliosides and extensive demyelination of axons [53,58]. Demyelination may also mask an actual increase in neuronal cholesterol content. Although in the brain all cholesterol has to be synthesized endogenously, in NPC it accumulates in the endosomal/lysosomal system, because the plasma membrane constantly (and physiologically) undergoes endocytosis. A disturbed cholesterol (and ganglioside) homeostasis may subsequently result from a defective subcellular redistribution/transport of lipids from the lysosomes to other cellular locations, e.g. axonal membranes [58]. Alternatively, the restricted accumulation of cholesterol in specific cell types of the brain might lead to the observed neurological phenotype. Nevertheless, it remains cryptic why NPC is primarily a neurological disorder.

The *NPC1* gene was identified in 1997 by positional cloning and maps to chromosome 18q11 [66]. It encodes a large integral membrane glycoprotein of 1278 amino acid residues that primarily localizes to late endosomes and transiently also associates with lysosomes and the trans-Golgi network [60,61]. It contains 13 putative transmembrane spans, a sterol sensing domain and a di-leucine motif at its cytoplasmic C-terminal tail which is required for late endosomal/lysosomal targeting [67,68]. NPC1 binds cholesterol and oxysterols at a site located on its luminal loop 1 [69,70]. The precise function of NPC1 is still enigmatic. It has been proposed that NPC1 might act as a fatty acid permease [71], a cholesterol flippase [72], a cholesterol sensor or as part of a multi-protein complex that mediates the egress of cholesterol from lysosomal membranes [56,58,73–75]. Moreover, a possible function of NPC1 in regulating lysosomal concentrations of biogenic amines has been suggested [76]. NPC1 might also assist in partitioning of endocytic and lysosomal compartments and facilitate endocytic transport [72]. Interestingly, a recent study indicates that a loss of NPC1 is associated with decreased lysosomal calcium concentrations, initially caused by storage of sphingosine with subsequent accumulation of cholesterol, sphingomyelin and glycosphingolipids as downstream effects [77]. It is proposed that sphingosine storage is the primary defect, which inhibits calcium entry into the acidic compartment. Reduced calcium levels then might lead to perturbation of vesicular trafficking. In normal cells, the addition of chelating agents that specifically reduce endocytic calcium concentrations rapidly resulted in an NPC disease cellular phenotype. Whether and how NPC1 is involved in sphingosine transport remains a key question for future studies.

The *NPC2* gene is localized on chromosome 14q24.3 [78]. In contrast to NPC1, NPC2 is a small, soluble intralysosomal protein of 151 amino acid residues that contains a mannose 6-phosphate marker [79]. It was originally identified as HE1, a major secretory protein of human epididymis [80]. NPC2 binds cholesterol with submicromolar affinity and a 1:1 stoichiometry [81–83]. The crystal structure demonstrates the presence of a deep hydrophobic binding pocket that expands in the presence of sterols [82,84,85]. It has been proposed that NPC2 functions as a cholesterol transporter in concert with NPC1 [58]. Moreover, NPC2 is able to extract cholesterol from donor membranes and catalyze the intermembrane transfer to acceptor vesicles [86]. This could allow sorting of cholesterol from internal lysosomal vesicles to the limiting membrane of the organelle.

3.2. Clinical features, diagnosis and therapeutic approaches

NPC has a highly variable phenotype regarding the age of onset, severity of symptoms and progression of disease. Defects of either NPC1 or NPC2 are clinically indistinguishable [53]. In most cases, symptoms of NPC become evident in early childhood, usually between 4 and 12 years of age, and include dystonia, ataxia, seizures and vertical gaze palsy [53,58]. Also neonatal jaundice and hepatosplenomegaly are observed, leading to acute liver failure. Adult onset forms are described in about 10% of NPC cases [87]. Histologically, NPC is characterized by a loss of neurons, particularly Purkinje cells in the cerebellum, extensive demyelination, the formation of meganeurites and ectopic dendrites as well as the presence of neurofibrillary tangles similar to Alzheimer's disease [53,58]. Death typically occurs in teenage years.

Once the clinical diagnosis is considered, laboratory tests are required to confirm NPC. Diagnosis is best achieved by both histopathological staining in cultured fibroblasts for abnormal LDL-cholesterol processing and biochemical assays. First, intralysosomal accumulation of unesterified cholesterol, stained with filipin, is detected by a characteristic perinuclear fluorescence pattern after challenging fibroblasts with LDL [88], and second, abnormal intracellular cholesterol homeostasis is proven as impairment of LDL-induced cholesterol esterification [53,88]. However, both methods show deficits in variant forms of NPC. Therefore molecular sequencing of the impaired genes is necessary for confirmation of the diagnosis as well as for genetic counseling and prenatal testing. A continuously updated database of NPC disease gene mutations including information on functional consequences and phenotypes is accessible online (NPC-db, http://npc.fzk.de, [89]).

Two murine models of NPC with different genetic backgrounds are available [90–93]. Analysis of the NPC1 or NPC2 deficient mice did not provide any indication for a possible role of the two proteins in intestinal cholesterol adsorption [94]. In line with these results, cholesterol-lowering agents or a dietary reduction of plasma cholesterol unfortunately fail to slow the progression of the disease [95]. At present, no etiological therapy for NPC is available. Current treatments are largely symptomatic or preventive [53]. In case of NPC1, enzyme replacement therapy is not applicable because of the transmembrane architecture of the NPC1 protein and the neurological phenotype. Accordingly, gene therapy is challenged by the efficient delivery of the *NPC1* gene to its neuronal targets. On the other hand, neuronal replacement by stem cell therapy [96] or substrate reduction therapy [97] open up new therapeutic possibilities. Recent studies with miglustat, a small iminosugar inhibitor of glucosylceramide synthase, the enzyme catalyzing the first committed step in the biosynthesis of most glycosphingolipids, yielded promising results and showed improvement or stabilization of clinical markers [98,99]. In contrast to NPC1, the small intralysosomal NPC2 appears as a suitable candidate for enzyme replacement therapy, but clinical trials are unlikely given the low frequency of occurrence. The recent finding that NPC is characterized by a deregulation of lysosomal calcium opens up new possibilities for therapeutic approaches [77]. Indeed, an elevation of cytosolic calcium concentrations by high doses of curcumin corrected the phenotype of NPC1 deficient cells and led to prolonged survival of NPC1 knock out mice.

4. Multiple sulfatase deficiency — a disease of post-translational sulfatase modification

Another lysosomal storage disorder caused by a non-lysosomal protein is multiple sulfatase deficiency (MSD, MIM #272200). It is a rare autosomal recessive disorder with a prevalence of 1 in 1.4 million births [100]. MSD was first described by Austin [101,102] as a variant form of metachromatic leukodystrophy (MLD), since the patients analyzed showed prevailing MLD characteristics and decreased arylsulfatase A activity. However, the affected siblings of one family showed additional clinical symptoms reminding of mucopolysaccharidosis as well as low activities of arylsulfatases B and C and various lysosomal storage material. Because of its compound clinical and biochemical appearance the syndrome was later termed MSD [103,104].

Early complementation studies showed that the genetic deficiency underlying MSD must be different from the defect in cells from single sulfatase deficiencies [105–108]. Interestingly, the transduction of MSD fibroblasts with cDNAs encoding wildtype arylsulfatases A, B or C resulted in the expression of sulfatase polypeptides with severely diminished catalytic activity [1]. The sulfatases remained inactive even after purification to homogeneity excluding the possibility of a tightly bound inhibitor. These findings led to the postulation of a post-translational modification that is common to and essential for sulfatases to gain catalytic activity.

4.1. Formylglycine — a novel amino acid allows for a unique hydrolytic mechanism

In 1995 it could be shown that newly synthesized sulfatases contain a crucial cysteine residue, which undergoes oxidation to a Cα-formylglycine (FGly) residue [2] (Fig. 2). This unique and, up to now, sulfatase-specific amino acid is generated by a post-translational modification occurring in the ER (Fig. 1) before the sulfatase polypeptides fold into their native structure [109]. The presence of FGly is obligatory for sulfatases to become catalytically active. It is virtually undetectable in sulfatases isolated from MSD patient fibroblasts [2].

The essential requirement of this unusual amino acid could be explained by functional and structural analyses of several human sulfatases and also type I bacterial sulfatases [110], which were shown to contain FGly as well [111,112]. In particular the crystal structures of arylsulfatase A with and without bound substrate [3,113] and, most convincingly, the high-resolution structure of a sulfatase from *Pseudomonas aeruginosa* [4] demonstrated that FGly forms an aldehyde hydrate with two geminal hydroxyls acting as the catalytic residue at the active site. Both hydroxyl groups are strictly required for catalysis pursuing a unique hydrolytic mechanism. According to this so-called transesterification/elimination mechanism (Fig. 2), one hydroxyl group performs a nucleophilic attack onto the substrate sulfur and covalently binds the sulfate group forming an enzyme sulfate ester. The second hydroxyl is required to induce elimination of the sulfate from the enzyme via an 'intramolecular hydrolysis' in order to regenerate the free aldehyde group of FGly (Fig. 2) [3,4]. A serine mutant (with only one hydroxyl) can catalyze the transsulfation step leading, however, to a dead-end sulfated enzyme, because sulfate elimination is not accomplished due to lack of the second hydroxyl [114].

4.2. FGly generation — a specific post-translational modification during early sulfatase biogenesis

The conversion of the cysteine to FGly is directed by the rather simple linear sequence motif CxPxR, which is part of the so-called sulfatase signature I (**C**-[STACG]-**P**-[STA]-**R**-x(2)-[LIVMFW](2)-[TAR]-G) (PROSITE database) located in the N-terminal catalytic domain of the sulfatases. Within this signature the proline and the arginine in +2 and +4 position, respectively, are the only essential residues apart from the cysteine itself, which determine FGly modification [115,116].

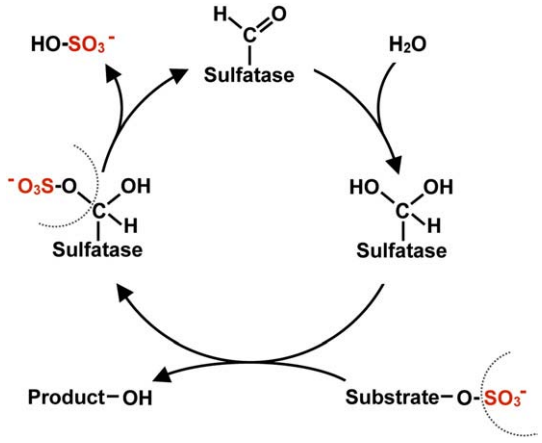

Fig. 2. FGly-mediated sulfate ester hydrolysis. The catalytic form of the FGly residue in the sulfatase active site is the aldehyde hydrate (right), which is formed by addition of a water molecule to the formyl group. Nucleophilic attack of the sulfate ester substrate by one of the geminal hydroxyls of the aldehyde hydrate leads to transesterification (bottom) of the sulfate group onto the enzyme resulting in a sulfated FGly hemiacetal intermediate (left). The second geminal hydroxyl group of this intermediate then reacts to eliminate the sulfate, under cleavage of the C–O bond, and to regenerate the aldehyde (top). For further details see [4].

Table 2
Human sulfatases, their intracellular localization, physiological substrates and associated genetic disorders

Sulfatase	Abbr.	Chromosomal localization	Physiological substrate	Subcellular localization	Associated genetic disorder, OMIM#
Arylsulfatase A	ASA	22q13.31-qter	Cerebroside-3-sulfate	Lysosomal	Metachromatic leukodystrophy (MLD), #250100
Arylsulfatase B	ASB	5q11–q13	CS/DS	Lysosomal	Maroteaux–Lamy syndrome (MPS VI), #253200
Arylsulfatase C (steroid sulfatase)	ASC, STS	Xpter-p22.32	Steroid sulfates	ER/microsomal	X-linked ichthyosis (XLI), #308100
Arylsulfatase D	ASD	Xp22.3	Unknown	ER	Unknown
Arylsulfatase E	ASE	Xp22.3	Unknown	Golgi	Chondrodysplasia punctata 1 (CDPX1), #302950
Arylsulfatase F	ASF	Xp22.3	Unknown	ER	Unknown
Arylsulfatase G	ASG	17q24.2	Unknown	Lysosomal	Unknown
Arylsulfatase H	ASH	Xp22.3	Unknown	Unknown	Unknown
Arylsulfatase I (Sulf5)	ASI	5q32	Unknown	Unknown	Unknown
Arylsulfatase J (Sulf4)	ASJ	4q26	Unknown	Unknown	Unknown
Arylsulfatase K (Sulf3)	ASK	5q15	Unknown	Unknown	Unknown
Galactosamine 6-sulfatase	GalN6S	16q24.3	CS, KS	Lysosomal	Morquio A syndrome (MPS IVA), #253000
Glucosamine 6-sulfatase	GlcN6S	12q14	HS, KS	Lysosomal	Sanfilippo D syndrome (MPS IIID), #252940
Heparan *N*-sulfatase (sulfamidase)	GlcNS, SGSH	17q25.3	HS	Lysosomal	Sanfilippo A syndrome (MPS IIIA), #252900
Iduronate 2-sulfatase	IdoA2S	Xq28	HS, DS	Lysosomal	Hunter syndrome (MPS II), #309900
Sulfatase 1	Sulf1	8q13.3	HS	Cell surface	Unknown
Sulfatase 2	Sulf2	20q13.12	HS	Cell surface	Unknown

(CS/DS, chondroitin sulfate/dermatan sulfate; HS, heparan sulfate; KS, keratan sulfate; MPS, mucopolysaccharidosis; OMIM#, Online Mendelian Inheritance in Man catalog number, John Hopkins Unversity, http://www.ncbi.nlm.nih.gov/).

The further downstream element of the signature, that is also highly conserved in human sulfatases ([LIMW]TG), fulfills an auxiliary function for proper presentation of the CxPxR core motif. In fact, introducing the CxPxR motif into engineered recombinant proteins leads to FGly generation in eu- and prokaryotic expression systems [109,117]. Thus, FGly formation occurs while the protein is still largely unfolded. In mammals it represents a late co- or early post-translational modification occurring in the lumen of the ER, as could be shown by studying import intermediates of de novo synthesized sulfatase polypeptides with and without ribosome (and translocon) association [109,118]. In these in vitro experiments, FGly modification occurred after the critical cysteine had passed the translocon-associated oligosaccharyl transferase responsible for co-translational *N*-glycosylation. Nevertheless, FGly modification was more efficient under co-translational than under post-translational conditions, as held true even in a membrane-free assay [109,118].

4.3. Deficiency of an ER enzyme leads to combined lysosomal storage and other disorders

The ER-resident enzyme catalyzing this modification reaction was discovered in 2003, as described below in further detail, and was termed FGly-Generating Enzyme (FGE). All MSD patients analyzed so far carried mutations in the gene encoding FGE (see below). Thus, multiple sulfatase deficiency may be re-defined as FGE deficiency. The resulting defect of FGly modification affects all sulfatases, as could be verified recently in a MSD mouse model [119] (see below).

Sulfatases represent a family of hydrolytic enzymes essential for the degradation and remodeling of sulfate esters. In mammals, sulfatases are involved in the turnover of various sulfated substrates such as glycosaminoglycans (heparin, heparan sulfate, chondroitin/dermatan sulfate, keratan sulfate), steroid hormones (e.g. dehydroepiandrosteron 3-sulfate) and sulfolipids (e.g. cerebroside-3-sulfate) [100,120] (Table 2). Furthermore, they have important regulatory functions in modulating heparan sulfate dependent cell signaling pathways [121–127] as well as in the activation of sulfated hormones during biosynthesis [128,129]. In vivo, sulfatases display stringent specificities towards their individual substrates and have low functional redundancy.

Thirteen of the 17 sulfatases encoded in the human genome have been characterized biochemically [130]. Based on their subcellular localization they can be divided into lysosomal and non-lysosomal enzymes (Table 2). The latter are found either at the cell surface (Sulf1, Sulf2), in the endoplasmic reticulum (arylsulfatases C, D and F) or in the Golgi apparatus (arylsulfatase E) and act at neutral pH. In contrast, all lysosomal sulfatases (arylsulfatases A, B and G, iduronate 2-sulfatase, heparan *N*-sulfatase, glucosamine 6-sulfatase and galactosamine 6-sulfatase) share an acidic pH optimum [131]. Arylsulfatase G has been discovered only recently as a lysosomal sulfatase [132]; so far no disorder has been associated with this sulfatase. The genetic deficiency of each of the other six lysosomal sulfatases causes specific and severe lysosomal storage disorders, namely metachromatic leukodystrophy (MLD) and mucopolysaccharidoses type II, IIIA, IIID, IVA and VI (Table 2), which highlights the essential and non-redundant function of these enzymes [120]. In affected patients, the degradation of a specific sulfated compound is blocked, leading to its accumulation in lysosomes and extracellular fluids. Lysosomal storage impairs autophagic delivery of bulk cytosolic contents to lysosomes, finally resulting in accumulation of toxic proteins, cellular damage and apoptosis [133]. Two non-lysosomal human sulfatases could be related to single sulfatase deficiencies, namely arylsulfatase C (also termed steroid sulfatase) to X-linked ichthyosis and arylsulfatase E to chondrodysplasia punctata type 1 [120] (Table 2).

4.4. Clinical features and diagnosis

Patients suffering from MSD combine clinical symptoms of the different single sulfatase defects. As a major sign they show a neurodegenerative course of disease with loss of sensoric and motoric abilities similar to MLD and neurological deterioration. Mental retardation, hepatosplenomegaly, shortening of stature and corneal clouding appear like in different mucopolysaccharidoses. In addition, ichthyosis and skeletal changes similar to chondrodysplasia punctata type I could be found [100,134,135].

The combination of different clinical symptoms and also the time of appearance differ among MSD patients. These differences allow for a classification of disease types. MSD can be subdivided into neonatal very severe, late infantile severe, late infantile mild and juvenile cases [100,134,136]. A variant infantile form of MSD was published as the Saudi-variant of MSD [137]. Patients can be classified by comparing ten different clinical symptoms appearing in MSD and their time of onset (Table 3). The few described neonatal very severe cases were all diagnosed shortly after birth because of the typical combination of different symptoms resembling several of the mucopolysaccharidoses. The affected children died within the first year [138–141]. In contrast, all other described clinical cases showed a slower progression of disease with a neurodegenerative course comparable to late infantile metachromatic leukodystrophy, clearly and obviously differing from neonatal very severe cases [142–144]. Severe infantile cases of MSD could be distinguished from mild cases by the earlier onset of

Table 3
Classification of MSD patients and associated clinical phenotype

Symptoms/disease type	Neonatal very severe	Late infantile severe	Late infantile mild	Juvenile mild
Retardation	+++	+++	+++	++
Neurodegeneration/ neurological symptoms	+++	+++	+++	++
Ichthyosis	+++	+++	++	+
Dysmorphism	+++	+++	−	+
Organomegaly	+++	+++	−	−
Skeletal changes	+++	++	+	−
Intrauterine manifestation	+++	+++	−	−
Heart disease	+++	−	−	−
Corneal clouding	+++	−	−	−
Hydrocephalus	+++	−	−	−

Four different MSD subtypes, subdivided by the appearance of ten different symptoms present in MSD and their time of onset, can be distinguished. The severity of cases ranges from neonatal very severe forms with the onset of all of the ten symptoms shortly after birth to mild cases with selected symptoms appearing later in life (age at onset: +++ <2 years, ++ 2–4 years, + >4 years, − not observed).

symptoms. Further, mild infantile cases display a serial appearance of symptoms, most of them being recognized at two to four years of age and beyond (Table 3). Only rare cases of juvenile MSD were published. The affected patients showed selected symptoms of MSD, appearing in early childhood [100, 134, 135, 137,145,146].

Also biochemically, MSD is distinct and classifiable. Patients with MSD show a mixed excretion of glycosaminoglycans and storage of the different sulfatase substrates [147,148]. The residual activities of different sulfatases measured in leukocytes and fibroblasts of patients can be sub-grouped into two classes with less than 15% compared to normal activities into group I and more than 15%, sometimes reaching normal values, into group II [149–152]. Clinically, group I reflects severe cases of MSD, whereas group II activities correspond to milder cases [138,153]. The reason for differences in residual activities of the individual sulfatases in the same patient between very low and nearly normal values is still unclear. A possible explanation could be a difference in the affinity of mutant FGE towards different sulfatase polypeptides. One important observation in terms of MSD diagnosis is the fact that residual sulfatase activities can vary due to different cell culture conditions and time points of measurement [151,154,155]. Group II fibroblasts seem to be more sensitive to this kind of change than group I fibroblasts [136].

The combination of the most important clinical symptoms neurodegeneration, dysmorphic signs reminding one of the mucopolysaccharidoses, and ichthyosis validates the clinical diagnosis of MSD. The presence of demyelination similar to MLD, shown by hyperintense magnetic resonance imaging signals in the posterior periventricular and subcortical white matter, hepatosplenomegaly, pathological X-ray examinations suggestive of chondrodysplasia and bone deformation, lysosomal storage in different tissues and excretion of different glycosaminoglycans are additional paraclinical signs of MSD [100,134,135]. MSD is verified by the measurement of decreased activities of different sulfatases in leukocytes, in addition to cultured fibroblasts, as sulfatase activities can differ due to cell culture conditions [103,142,145,148,151,154,156], and by sequencing of the *SUMF1* gene [144,157–159]. Pitfalls in the diagnosis of MSD are a sequential appearance of leading clinical features, which delays the diagnosis [142]. Prenatal testing for MSD is possible.

5. FGE — the MSD enzyme

5.1. Initial characterization of enzymatic activity

Based on the key finding that the generation of formylglycine (FGly) in the active site of sulfatases is defective in sulfatases from MSD patients [2], it became the pivotal aim to identify the enzyme, which catalyzes this unique amino acid modification. Soon after the discovery of FGly we could show by in vitro translation/translocation experiments that newly synthesized sulfatases receive their FGly modification during or soon after translocation into the ER [109,160] and that the FGly generating machinery recognizes a short linear CxPxR motif conserved among sulfatases (see above) [115]. FGly formation was found to be an enzyme-mediated reaction that occurs in the reticuloplasm, the soluble matrix of the ER [118]. Synthetic arylsulfatase A-derived peptides containing the CxPxR motif competitively inhibited sulfatase modification. In vitro, the enzyme is active at a broad pH range with an unusual alkaline optimum at pH 9–10, and depends on the presence of reducing agents such as glutathione or dithiothreitol [118] (see below). Measuring FGly formation in a membrane-free assay paved the way to develop and apply simple purification protocols. This assay employed in vitro synthesized sulfatase substrates that, after incubation with ER-derived fractions, were analyzed at the level of its tryptic peptides by a combination of reversed-phase HPLC, chemical derivatization of the formyl group and mass spectrometry [118,161]. Each single sample had to be subjected to a laborious one-week procedure that, despite the low throughput, for many years formed the basis to follow the modification activity after chromatographic separation of reticuloplasm proteins.

5.2. Discovery of the gene and protein underlying MSD

In 2003, the gene encoding the FGly-modifying factor was identified independently by our group [157] and by the group of Andrea Ballabio [158]. The two approaches to find the gene, however, were rather different. In our biochemical approach, we first had to develop a fast, straightforward method to assay FGly-generating activity. This was achieved by using a 23mer peptide substrate (P23), containing the dodecamer sulfatase signature of arylsulfatase A (**CTPSR**AALLTGR), and MALDI-TOF mass spectrometry to quantify substrate and product after the reaction [157]. The assay was used to detect enzyme activities during purification starting from reticuloplasm of bovine testis microsomes. The key chromatographic step during a four-column procedure was the affinity chromatography with the P23-related peptide ASA65-80-C69S as immobilized ligand. It proved to be essential to substitute the critical cysteine 69 of the ASA65-80 peptide by serine, because under the applied conditions the enzyme is inactivated by the wild-type peptide (B. Schmidt, unpublished). The eluate after SDS-PAGE showed two bands of about 39 and 41 kDa, which were identified by peptide mass fingerprint analysis as full-length and N-terminally truncated versions of the same gene product that matched with a bovine EST database entry. The human gene ortholog (92% identity between the corresponding mature proteins) is located on chromosome 3p26.

Cosma et al. identified the same gene by means of functional complementation of an immortalized human MSD cell line using microcell-mediated chromosome transfer [158]. A panel of human/mouse hybrid lines containing individual human chromosomes was fused to the MSD cells and the result of complementation was analyzed by measuring sulfatase activities in cell lysates. Complementation with the complete chromosome 3 was shown to rescue sulfatase activities. To narrow down the chromosomal localization of the responsible gene, irradiation microcell-mediated chromosome transfer was performed. Many chromosome 3 irradiated hybrids were tested for sulfatase activities and genotyped until the complementing gene could be mapped to a 2.4 Mb chromosomal region at position 3p26. When the genes located in the complementing region were scanned for mutations in MSD patients, one gene was found to contain mutations in all patients tested [158]. The two groups agreed to name the gene sulfatase modifying factor 1 (*SUMF1*) and the encoded protein was denoted formylglycine-generating enzyme (FGE). To verify the sulfatase-activating function of FGE, a number of mammalian cell types were co-transfected with FGE and sulfatase encoding cDNAs.

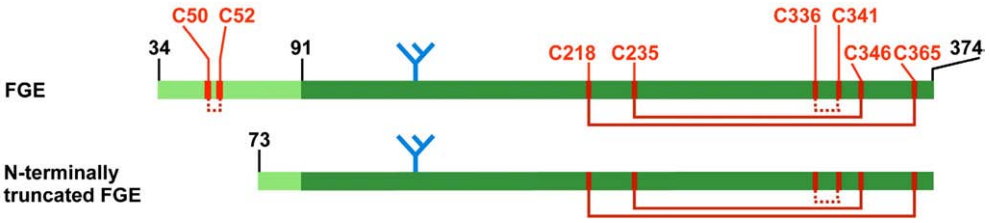

Fig. 3. Basic architecture of FGE. Full-length FGE (top) and N-terminally truncated FGE (bottom), which results from furin-like processing in the Golgi, are largely made up of the globular DUF 323 domain (dark green). This domain is stabilized by two permanent disulfide bridges, as indicated (with positions of the involved cysteines given in red). C336/C341 is the redox-active cysteine pair of the catalytic site. Furthermore, truncated FGE lacks the N-terminal extension present in full-length FGE with the conserved cysteine pair C50/C52, which forms intermolecular disulfides and is required for FGly generation in vivo [162, 164] (see text). This N-terminal extension further confers ER retention of FGE through interaction with the RDEL-protein ERp44 (see text). The N-linked glycan, indicated as a tri-antennar sugar tree (in blue), belongs to the high-mannose type, but is processed further upon trafficking past the ER [162].

Depending on the cell type and the individual sulfatase, enzyme activities were increased up to 50-fold. Most importantly, endogenous sulfatase activities in cells from MSD patients could be restored by retroviral transfer of the *SUMF1* cDNA. These findings emphasize the essential and limiting role of FGE in the activation of sulfatases as well as the monogenetic nature of MSD [157, 158].

5.3. Biochemistry and cell biology of FGE

The *SUMF1* gene has a size of about 106 kb and contains 9 exons coding for a protein of 374 amino acids. FGE is expressed ubiquitously, with the highest mRNA levels in pancreas and kidney. Intracellularly, FGE is localized in the ER where it fulfills its enzymatic function [157,158,162]. In cultured cells, however, a variable fraction of FGE is also secreted into the medium, as can be detected even at endogenous expression levels [6,162]. ER targeting is accomplished through an N-terminal signal sequence of 33 residues. After signal peptide cleavage, mature intracellular FGE has a size of 41 kDa consisting of two domains. The core domain, forming the largest part of FGE (residues 91–374), is highly conserved in both pro- and eukaryotes [157,163] (Fig. 3). The N-terminal extension (residues 34–90), which probably forms a separate domain, is only found in eukaryotes, where it serves essential functions in vivo (see below). Asparagine 141 carries a carbohydrate side chain of the high mannose type. Eight cysteines form four disulfide bridges; the N-terminal cysteine pair C50/C52 and the more C-terminally localized pair C336/C341, however, were also found in reduced form (Fig. 3).

When FGE is secreted, the carbohydrate chain is modified to the complex-type and in the majority of molecules a stretch of 39 amino acids (residues 34–72) is cleaved off from the N-terminus by a furin-like protease in the Golgi [162, M. Mariappan, unpublished data] (Fig. 3). Both, full length and the secreted N-terminally truncated FGE were shown to modify sulfatase-derived peptides in vitro, indicating that the globular domain of FGE contains the active site of the enzyme [162]. In vivo, remarkably, the N-terminal extension of FGE and its fully conserved pair of cysteines (C50/C52, Fig. 3) are required for sulfatase activation, indicating a cooperation between the N-terminal extension and the globular domain of FGE to perform its physiological function [164]. Additionally, we found that the N-terminal extension is also required for the retention of FGE in the ER [164]. In subsequent studies it was shown that the retention of FGE is mediated by ERp44 [165,166], an ER protein known to retain other prominent ER proteins by forming mixed disulfide bridges through its cysteine 29 residue. The interaction of FGE and ERp44 involves the N-terminal extension of FGE but is primarily non-covalent. Although a disulfide bridge between Cys-29 of ERp44 and Cys-50 or 52 of the FGE N-terminal extension can form to stabilize the complex, this mixed disulfide bond turned out not to be required for retention [165].

Other ER proteins, namely ERp57, protein disulfide isomerase (PDI) and pFGE, a paralog of FGE, as well as ERGIC-53, located at ER exit sites, were also described to interact with FGE as detected by co-immunoprecipitation from FGE overexpressing HeLa cells [166]. However, their function is not fully understood as yet. Whereas ERp57 and PDI also seem to contribute to retention of FGE, ERGIC-53 was shown to bind FGE at the oligosaccharide side chain, thereby preventing proteasomal degradation of FGE — possibly by promoting its progression along the secretory route. If true, this would indicate that ERGIC-53 and ERp44 in functioning as ER export and retrieval factors, respectively, contribute to a balanced control of FGE localization [166]. Beside its function in retention of FGE, PDI seems to participate, directly or indirectly, in the activation of sulfatases, as suggested by co-overexpression of PDI and reporter sulfatases and also by PDI silencing experiments [166].

The role of pFGE in sulfatase activation is also still unclear. This protein shows a high homology with FGE (47% identity) but misses the N-terminal extension and 6 of the 8 cysteines of FGE. It is coexpressed together with FGE in many tissues, binds sulfatases, although with a lower affinity than FGE, but does not possess FGly generating activity [167]. This inactivity can be explained merely by the fact that pFGE lacks the catalytic cysteines 336 and 341 (see below). Overexpression of pFGE together with FGE leads to a partial decrease of FGE-mediated sulfatase activation, indicating a possible role in regulation of FGE activity [167,168]. The hypothesis that pFGE, which is retained in the ER via a non-canonical KDEL-type signal (PGEL), serves FGE retention through pFGE/FGE complex formation turned out to be wrong [169].

As mentioned above, a low and variable amount of endogenous FGE is secreted from cells into the medium. Further, it was shown for different cell lines that secreted FGE can be taken up via mannose receptor-mediated endocytosis [6]. Following uptake into MSD fibroblasts, FGE was found to reach the ER, leading to an increase of sulfatase activity. Apart from the surprising paracrine function and unusual trafficking of FGE, this result is remarkable, as it suggests that enzyme replacement therapy in principle is applicable for treatment of MSD. Also gene therapy should be effective because secreted FGE produced by transduced cells could allow metabolic cross-correction [6] (see Section 8).

6. Crystal structure of FGE — towards an understanding of FGE function and dysfunction in MSD

In 2005, we solved the crystal structures of secreted FGE (Fig. 4A) [170] and its paralog pFGE [171]. We found both molecules to form a single globular domain with a novel fold that is stabilized by two calcium ions and has a remarkable low content of secondary structures (20% β-sheets and 13% α-helices in FGE). In the crystal of secreted FGE, lacking residues 34–72, the first visible N-terminal amino acid is Leu-86, from where the globular domain extends to the C-terminus. It contains six cysteines, four of which were disulfide-bridged and two (Cys-336/Cys-341) partially in the reduced form (Fig. 4A). Cys-336 and Cys-341 are both fully conserved and essential for catalytic activity. They are localized in an oval-shaped groove, which could be proven to form the active site of FGE, as shown by

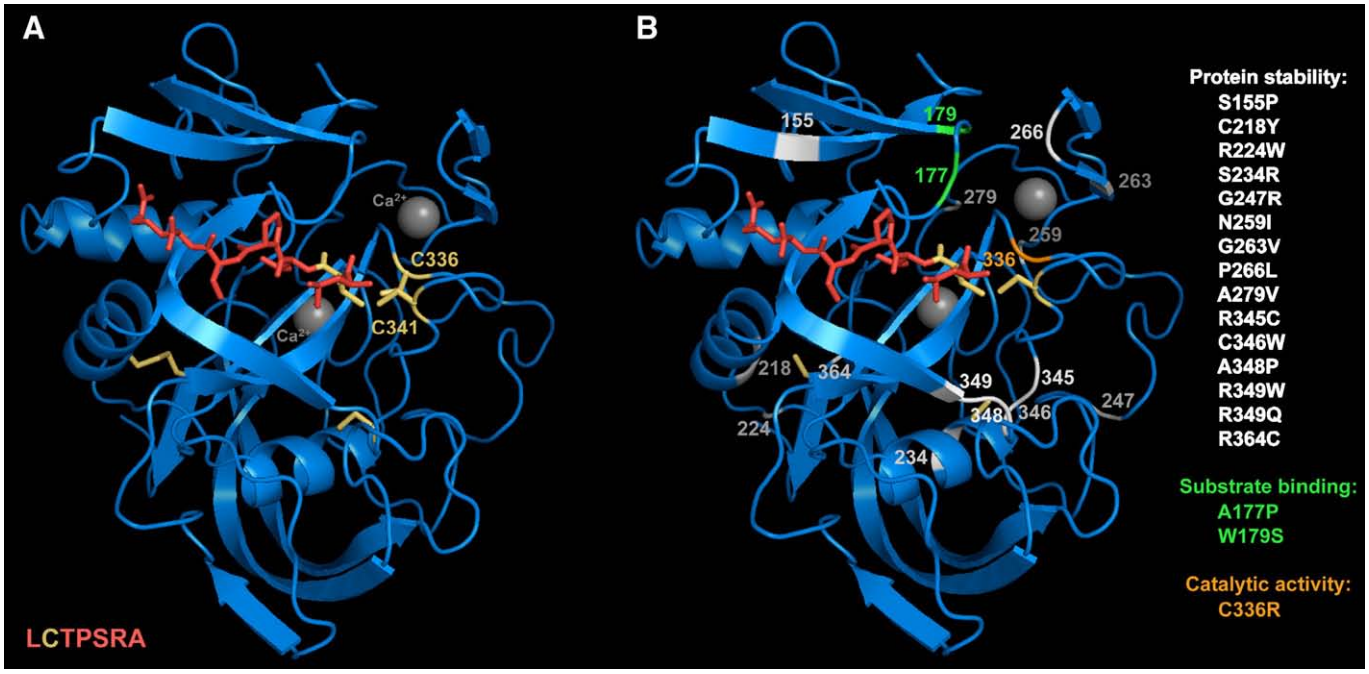

Fig. 4. Crystal structure of FGE and molecular basis of MSD. (A) The globular domain of FGE (ribbon presentation) is shown in complex with the sulfatase-derived peptide LCTPSRA (stick presentation in red and, for the critical cysteine, yellow). The stabilizing disulfide bridges of FGE (in yellow) and Ca^{2+} ions (gray spheres) are indicated as well as the redox-active catalytic cysteines C336 and C341. (B) MSD-causing missense mutations are mapped onto the FGE structure and are colored according to the predicted or in some cases directly proven effect [170,136] on FGE structure and function (white, destabilizing; green, inhibition of substrate binding; orange, catalytically inactivating). The majority of the mutations will prevent folding of FGE into its native conformation by destabilizing the hydrophobic core. Further destabilizing mutations are located at a tight surface loop (263 and 266) and at Ca^{2+}-site 1 (259). For further details see [170].

crosslinking with a photoreactive substrate peptide [162] and by co-crystallization of the FGE mutant C336S with a LCTPSRA substrate peptide (Fig. 4A) [172].

6.1. FGE — a metal-independent mono-oxygenase

The catalytic mechanism of FGly generation still bears unresolved questions, but some aspects have been uncovered. Molecular oxygen (O_2) was found to be consumed equimolarly during generation of FGly (J. Peng et al., unpublished data). The present data suggest that FGE is a mono-oxygenase [170]. Strikingly, redox active metal ions or other redox cofactors have not been detected — neither in the crystal structure nor by plasma mass spectrometry of the active enzyme. Apart from the artificial reductant dithiothreitol (see below), no such factors need to be supplied to sustain multi-turnover FGly formation activity in vitro. Consequently, FGE must employ a unique mechanism to utilize molecular oxygen that is very different from the redox chemistry seen in conventional oxygenases and dehydrogenases. This mechanism obviously relies merely on cysteine redox chemistry of the catalytic cysteine pair in the FGE active site, serving to transfer oxidizing equivalents to the sulfatase substrate cysteine (Fig. 5).

In fact the two active site cysteines turned out to behave very differently. On the one hand, Cys-336 is extremely prone to oxidation by molecular oxygen, yielding sulfenic or, in vitro, even sulfonic acid. On the other hand, Cys-341 forms a disulfide bridge to the substrate cysteine, as seen in the FGE-C336S/peptide co-crystal [172] or in trapped catalytic intermediates (B. Schmidt, unpublished data). Thus, it appears likely that Cys-341 binds the substrate and Cys-336 oxidizes it [170].

After substrate binding, a small cavity is formed in the active site surrounded by the three critical cysteines, i.e. the active site cysteine pair of FGE and the substrate cysteine. The size and location of this cavity accommodates the essential oxygen molecule in a way that it is close to all three cysteine residues. The cavity is shielded from bulk solvent, excluding the entry of further oxygen to avoid overoxidation of Cys-336 [172,173].

A simplified mechanism for FGly generation, focusing only on the chemistry at the sulfatase substrate cysteine, is proposed in Fig. 5. The oxygen molecule is cleaved by FGE and one oxygen atom is reduced to

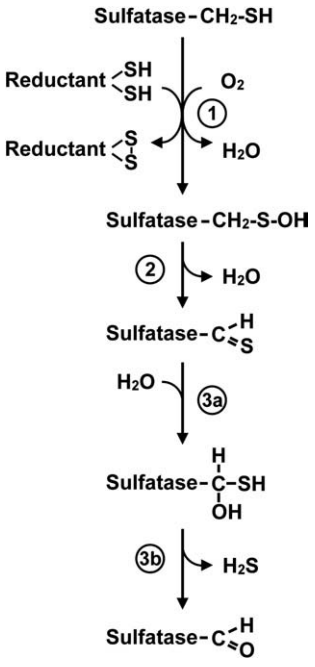

Fig. 5. Proposed mono-oxygenase mechanism of FGly generation in sulfatases. (1) Upon activation of molecular oxygen by FGE cysteine sulfenic acid (–CH_2S–OH) is generated first at cysteine C336 of FGE (not shown) and then, by hydroxyl transfer, at the critical sulfatase cysteine residue. An external reductant, such as glutathione, is required for this step to reduce the second oxygen of O_2 to water. (2) A water molecule is eliminated from the cysteine sulfenic acid leading to the formation of an instable thioaldehyde intermediate (–CHS). (3) This spontaneously hydrolyzes to the FGly aldehyde (–CHO). For further details see [116,170].

water, whereas the other oxygen is used to form a sulfenic acid (–CH_2S–OH) first at Cys-336 of FGE, from where the OH-group is then transferred onto the critical cysteine of the sulfatase (Fig. 5, step 1). O_2 reduction requires the supply of four electrons, two from the sulfatase cysteine and two from an external reductant. Elimination of a water molecule from the cysteine sulfenic acid forms a thioaldehyde (step 2), which undergoes spontaneous hydrolysis to the aldehyde via a thioaldehyde hydrate intermediate (step 3). FGE catalyzes the reaction (i) by binding the unfolded substrate polypeptide in the active site, possibly involving formation of a disulfide bridge between Cys-341 and the substrate cysteine, (ii) by activating molecular oxygen independent of redox-active metal ions, probably via Cys-336 sulfenic acid (step 1) and (iii) by β-elimination of a water molecule from the cysteine sulfenic acid (step 2) most likely through Ser-333 mediated proton abstraction from the β-carbon (for details see [170,172]). As a reductant in step 1 dithiothreitol can be used in in vitro assays. The physiological reductant, however, is still unknown.

6.2. A general mechanism for sulfatase binding and specificity

The co-crystallized FGE–peptide complexes delineate the general substrate-binding mode used by FGE for all human sulfatases during their maturation. The high conservation of the CxPxR motif in eukaryotic and most prokaryotic sulfatases indicates that all sulfatases are bound to and modified by FGE using the same mechanism. The binding of the key proline and arginine residues, in central and terminal position of the CxPxR consensus, respectively, involves pockets of high hydrophobic surface or electrostatic complementarity, respectively [172,174]. Accordingly, the FGE substrate binding site displays exquisite sensitivity to alterations in the sulfatase sequence as seen by scanning mutagenesis [115] and by natural mutations in human arylsulfatase B, heparan *N*-sulfatase, galactosamine 6-sulfatase and iduronate 2-sulfatase, all of which lead to mucopolysaccharidoses (summarized in [172]). As the mutated sulfatases most likely fail to bind to FGE and are therefore not modified, manifestation of these diseases in the affected patients further corroborates that the five residues of the human C-[TSAC]-PSR motif are necessary and sufficient for specific FGE/sulfatase interaction.

6.3. Molecular basis of MSD

Until today, 30 mutations were found in the *SUMF1* gene, which lead to different MSD phenotypes. 21 of these mutations are missense mutations appearing in homozygosity or compound heterozygosity. The remaining mutations are deletions of single nucleotides leading to frameshifts and premature stops of the FGE protein, larger deletions, splice site mutations and stop mutations (p.R327X, p.S359X). Two of the missense mutations affect the N-terminal methionine, leading to failure of translation initiation or, in case that the downstream in-frame methionine-91 codon would be used, to production of FGE lacking the N-terminal 90 residues. Another mutation (L20F) affects the hydrophobic core of the signal sequence. The remaining 18 missense mutations affecting residues visible in the crystal structure were mapped onto the FGE fold to predict structural consequences leading to impaired function of the enzyme (Fig. 4B) [170].

According to their predicted effects on FGE the missense mutations could be divided into three groups: i) Most of the mutations destabilize the protein structure by hampering efficient packaging of the protein core or by loss of important side chain interactions (Fig. 4B). Two missense mutations with high allele frequency belong to this group: c.1045C>T (p.R349W) appearing in patients of Turkish origin and c.836C>T (p.A279V) appearing in patients of French and French-Canadian origin. Another mutant, p.N259I, abolishes the binding of one of the calcium ions, which contribute to the stability of the protein fold. ii) A second group of mutations lead to interference with substrate binding. This prediction is verified by two MSD-causing FGE mutations, namely p.A177P and p.W179S, which both are located in a loop region near the substrate binding site (Fig. 4B). The FGE–peptide complex structure confirmed that positions 177 and 179 are indeed close to the bound peptide [172]. iii) The third group of mutations leads to impaired catalytic activity. This group is represented by the mutant p.C336R, which is a null mutant [170,175] because arginine replaces one of the essential active site cysteines directly involved in the catalysis of FGly generation. These predictions on the basis of molecular modeling were proven for a number of mutants by expression and analysis of the mutant proteins (see below).

7. Genotype–phenotype correlation in MSD — potential and limitations

The discovery of the *SUMF1* gene, the determination of the FGE crystal structure and, most recently, the creation of a *SUMF1* gene trap (*SUMF1*^{Gt}) mouse model allowed one to look closer into the molecular basis underlying MSD and further, to define and test molecular parameters with possible impact on disease severity. The basic observations are summarized as follows. Transduction of MSD fibroblasts with wild-type *SUMF1* cDNA more or less fully restored the activities of different sulfatases in these cells [157,158]. Further, overexpression of different MSD-causing FGE mutants in patient cells or murine *SUMF1*^{Gt} cells led to a correct or at least partially correct localization in the ER for all mutant FGEs analyzed so far [136,175,176]. Importantly, expression of FGE missense mutants in *SUMF1*^{Gt} cells directly influenced endogenous sulfatases leading to an increase of sulfatase activities, albeit to a much lower extent as compared to expression of wild-type FGE. In contrast, untransduced *SUMF1*^{Gt} cells (or those transduced with the null mutant p.C336R) showed no sulfatase activities [175]. Furthermore, it is a common finding that MSD patients exhibit detectable, albeit low or even very low residual sulfatase activities. Thus, MSD-causing mutations, at least in non-neonatal cases, are hypomorphic. A complete loss of FGE function is supposed to lead to embryonic or neonatal lethality in human [175]. Accordingly, *SUMF1*^{Gt} mice show high mortality in the first weeks of life, though 10% reach three months of age [119]. High embryonic/neonatal lethality is also observed in mice with a knock-out of only two sulfatases, namely the non-lysosomal sulfatases Sulf1 and Sulf2 [121,177]. These cell surface endo-sulfatases (Table 2) modulate important cell signaling pathways underlying differentiation and development via specific 6*O*-desulfation of cell surface heparan sulfate proteoglycans [121,178]. Thus, developmental problems seem to be a contributing, if not the primary cause for early fatality in neonatal MSD.

Given the hypomorphic nature of the majority of MSD-causing mutations, resulting in residual functionality of the expressed mutant versions of FGE, the question arises whether a genotype-phenotype correlation exists in MSD. For prognosis of the outcome of the disease, until today only the clinical as well as the biochemical classification of different types of MSD (see Section 4.4) can be taken into consideration. A predictable genotype–phenotype correlation is complicated by the fact that 17 defective sulfatases contribute to the complex clinical phenotype in MSD, each of which most likely gives rise to a compensatory response. When attempting to explain the differences in MSD manifestation from the molecular findings, the key approach must be to define the residual functionality of the relevant FGE mutants expressed in patients.

Indeed, one can deduce some principle mechanisms in MSD that influence the clinical phenotype. *SUMF1* mutations in patients with a neonatal very severe course of disease were either nonsense mutations with large deletions, frameshift mutations or missense mutations directly affecting the active site of FGE (like p.C336R, see above). Further, a functional characterization of different MSD causing FGE missense mutations found in homozygosity (or in combination with a frame-shift null allele) in MSD patients (p.A177P, p.W179S, p.

A279V, p.R349W) revealed drastically reduced residual FGE activities for three mutants (A177P, W179S, R349W) and also impaired protein stability for two of the mutants (A279V, R349W) [136], thereby confirming the functional consequences of missense mutations on the FGE protein, as predicted from the crystal structure (see Fig. 4B) [170]. Interestingly, residual sulfatase activities in fibroblasts from patients carrying the investigated mutations in homozygosity were extremely reduced only in case of mutation R349W leading to both protein instability and very low residual FGE activity (group I fibroblasts). Patients with this homozygous mutation all show a late infantile severe phenotype. On the other hand, fibroblasts with either stable proteins and low residual FGE activity (A177P, W179S) or instable proteins with high residual FGE activity (A279V) exhibited just slightly reduced sulfatase activities (group II fibroblasts). Accordingly, patients with these mutations show a milder course of disease [136]. Taken together, both residual FGE activity and protein stability seem to determine the severity of disease manifestation in MSD. More data on further mutations are necessary to validate this approach and to obtain a better understanding of genotype/phenotype correlations in MSD. The available data, however, already now suggest that the direct analysis of mutant FGE activity and stability might allow a more reliable biochemical classification, as compared to the more indirect sulfatase activity determinations in patient cells, which in group II fibroblasts have proven to depend on cell culture conditions (see Section 4.3). The findings, however, do not exclude that secondary factors or modifier genes, unspecified as yet, play a role in determining disease manifestations. In particular for milder mutations these factors may obscure reliable prognosis.

8. The *SUMF1* gene-trap as a mouse model for therapeutic approaches

Recently, Settembre et al. described a mouse model for MSD that was generated from an ES cell clone with a gene-trapped *SUMF1* allele [119]. The homozygous *SUMF1*^{Gt} mice show a complete loss of sulfatase activity, as tested for eight different sulfatases in various tissues, thus proving that FGE represents the only system for FGly generation in mammals. As mentioned, total loss of sulfatase activity in *SUMF1*^{Gt} mice further indicates that MSD-causing *SUMF1* mutations in human are hypomorphic, since all MSD patients exhibit at least very low residual sulfatase activities [119].

The *SUMF1*^{Gt} mice display congenital growth retardation and frequent early mortality, with 10% reaching the age of 3 months. A flat facial appearance as well as skeletal abnormalities was visible. The mice manifested head tremor and seizures due to defects of the central nervous system. Histologically, glycosaminoglycan staining was detected in liver, kidney and, as a prime site, in macrophages which were massively present in all tissues. These macrophages and activated microglia in cerebellum and cortex, accompanied by neuronal cell loss and astroglyosis, indicate systemic and neuroinflammation, which are thought to be key pathophysiological processes in MSD [119].

The *SUMF1*^{Gt} mouse model not only allows a systematic study of the pathophysiology of MSD; importantly, it also provides a model to test therapeutic approaches to the treatment of MSD, similar to other mouse models of individual lysosomal storage disorders. A first approach of gene-therapy in this mouse model was highly interesting. When Flag-tagged FGE was expressed in the *SUMF1*^{Gt} mouse using liver-specific expression via an adeno-associated viral vector, Flag-tagged FGE protein and also increased sulfatase activity could be determined in the liver and, surprisingly, also in other organs like lung and kidney [6]. The transduced liver cells expressed and, in addition, secreted functional FGE, which obviously was taken up by other tissues leading, most remarkably, to partial restoration of sulfatase activities in the recipient cells. Zito et al. could show in vitro that secreted FGE obviously is taken up by other cells, where it is correctly targeted back to the ER [6]. Importantly, also in this case FGE was able to enhance sulfatase activities significantly.

Thus, enzyme replacement therapy (ERT) with purified recombinant FGE could be one therapeutical option. Nevertheless, like in other LSDs, the blood-brain barrier poses an important difficulty for ERT and makes intrahecal application necessary [179]. Alternatively, ERT with a mixture of lysosomal sulfatases could, at least theoretically, represent another therapeutic option. In this case, however, only lysosomal sulfatases could be substituted, since ER or Golgi resident sulfatases would probably not reach their intracellular place of action if administered in ERT.

In contrast, gene therapy might serve to distribute functional *SUMF1* cDNA within affected patients and to restore all sulfatase activities. However, despite encouraging results for other LSDs and for MSD in the *SUMF1*^{Gt} mice (see above), a major therapeutical breakthrough in human is still to be achieved [180]. Bone marrow transplantation as well as influencing protein folding by the use of small molecules as pharmaceutical chaperones, such as isofagomine in case of Gaucher disease [181], are other yet unproven methods of MSD treatment. Whatever therapy is considered, developmental defects caused by Sulf1 and Sulf2 deficiency already during embryonic stages cannot be reversed by any postnatal treatment. Nevertheless, the *SUMF1*^{Gt} mouse will provide important information about the feasibility and efficacy of any MSD therapy to be developed in the future.

9. FGE as a tool for therapy of single sulfatase deficiencies

For a number of LSDs, enzyme-, cell- and gene-based therapy protocols have been developed, among them those LSDs caused by single sulfatase deficiencies, namely MLD, MPS-II, -IIIA, -IIID, -IVA and -VI. While various studies have been carried out using patient cells or mouse models for all these diseases, clinical trials have been performed only for MPS-II and -VI, [182–186], for which ERT has been successful in both cases. FGE as the essential sulfatase activator plays a fundamental role for industrial production of recombinant sulfatases to be used in ERT but also when attempting cross-correction using gene therapy strategies. For both approaches the beneficial role of *SUMF1* co-expression with the sulfatase structural gene or cDNA have recently been demonstrated [7,187–190].

In proof-of-principle experiments Fraldi et al. have used AAV and lentiviral vectors for the co-delivery of sulfatase and FGE-encoding cDNAs into cells from patients or from murine models with MLD, MPS-II, MPS-IIIA or MPS-VI [189]. In all cases FGE enhanced sulfatase activity resulting in improved clearance of sulfolipid or glycosaminoglycan accumulation. Thus, co-delivery of FGE-encoding cDNA with the respective sulfatase cDNA seems demanding in gene therapy of single sulfatase deficiency disorders.

Two papers verifying this concept for treatment of the MLD mouse [188] and the MPS-IIIA mouse [190] were published recently demonstrating that co-expression of FGE clearly improves the efficacy of gene therapy using either injection into the tail vein [187] or, most important for diseases mainly affecting the central nervous system, into the brain [188,190]. In the MLD mouse, FGE co-expression not only improved ASA activity in the targeted tissue but also, and even more pronounced, in the serum after intravenous injection. Furthermore, after intracerebral injection in adult MLD-mice the ASA activity was shown to be improved in the non-injected hemisphere of the brain as well [188]. High serum or cerebrospinal activities are of particular relevance when attempting to cross-correct an enzyme deficiency in all tissues. To this end, transduced producer cells have to secrete high activities of lysosomal sulfatases that can be taken up via the mannose 6-phosphate receptor into non-transduced cells causing correction also there. As a consequence, after treatment of MLD mice marked reduction of sulfatide storage was observed throughout the entire brain leading to improvement of neurological functions [188].

Promising results were also obtained upon AAV-mediated intracerebral co-delivery of both heparan *N*-sulfatase and FGE-encoding cDNAs into the lateral ventricles of newborn MPS-IIIA mice, a model for another LSD mainly involving the brain and in fact the most common of the MPS disorders. Although FGE co-expression improved heparan *N*-sulfatase activity only 1.5-fold in the brain, as compared to delivery of heparan *N*-sulfatase cDNA alone, a reduction of brain pathology and an improvement of the behavioral phenotype, affecting motor and cognitive functions, were observed [190]. It should be emphasized, however, that in these studies pathological storage reduction was achieved only in the brain areas which were efficiently transduced by the AAV vector.

Despite a number of remaining problems, also for the treatment of MLD mice [191], which will be potentiated in case of treating the much larger human brain, sulfatase plus FGE co-expression from AAV vectors represents a highly promising approach on the way towards clinical trials. On the other hand, ERT protocols already now have been applied successfully in patients of MPS-II and -VI [182–186]. Given the finding that FGE massively enhances sulfatase activities in a number of overexpressing sulfatase producer cell lines, it is our hope that large-scale production of highly active sulfatases will be much facilitated, so as to allow for more efficient and also much broader applications in the near future. Indeed, encouraging results were obtained recently for MPS-IVA, an LSD without involvement of the brain, for which initial ERT studies in a mouse model have been successful after administration of galactosamine 6-sulfatase produced by FGE-coexpressing cells [7].

10. Conclusions

MSD, ML II/III and NPC1 show complex disease manifestations with lysosomal storage as the prime cellular defect that initiates manifold organic dysfunctions. The non-lysosomal localization of the affected proteins and, in the case of MSD and ML II/III, the pleiotropic consequences on many lysosomal enzymes resulting from defective post-translational modifications has intrigued cell biologists and biochemists for many decades. The identification and characterization of the affected gene product not in all cases paved the way to understand the basic causal relationships leading to the manifestation of the disorder. This becomes particularly obvious in the case of NPC1 disease, where the true function of the NPC1 membrane protein is not understood. For GlcNAc phosphotransferase the enzymatic function is clear, but the mechanism of how it recognizes newly synthesized proteins with a lysosomal destination remains to be unraveled at the molecular level. For both issues, detailed functional studies using reconstituted in vitro systems with defined substrates, and also structural analyses of apo- and substrate-bound proteins are ambitious perspectives that certainly would bring major advancements, as had been the case for understanding FGE and its role in MSD. For the latter disorder, the most demanding questions relate to the metal-free mono-oxygenase mechanism of FGE function and the involvement of other components and redox factors of the ER in sulfatase activation as well as the unusual trafficking pathways employed by FGE. Clarification of these aspects may each provide hints for therapeutic approaches in order to either enhance residual FGE activity, which is detectable in all MSD patients, or to effectively envisage enzyme replacement therapy. For all three syndromes it must be an ultimate goal to develop gene therapy towards clinical application in order to permanently equip patients with functional copies of their defective genes.

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