Zebrafish as research model to study Gaucher disease: Insights into molecular mechanisms

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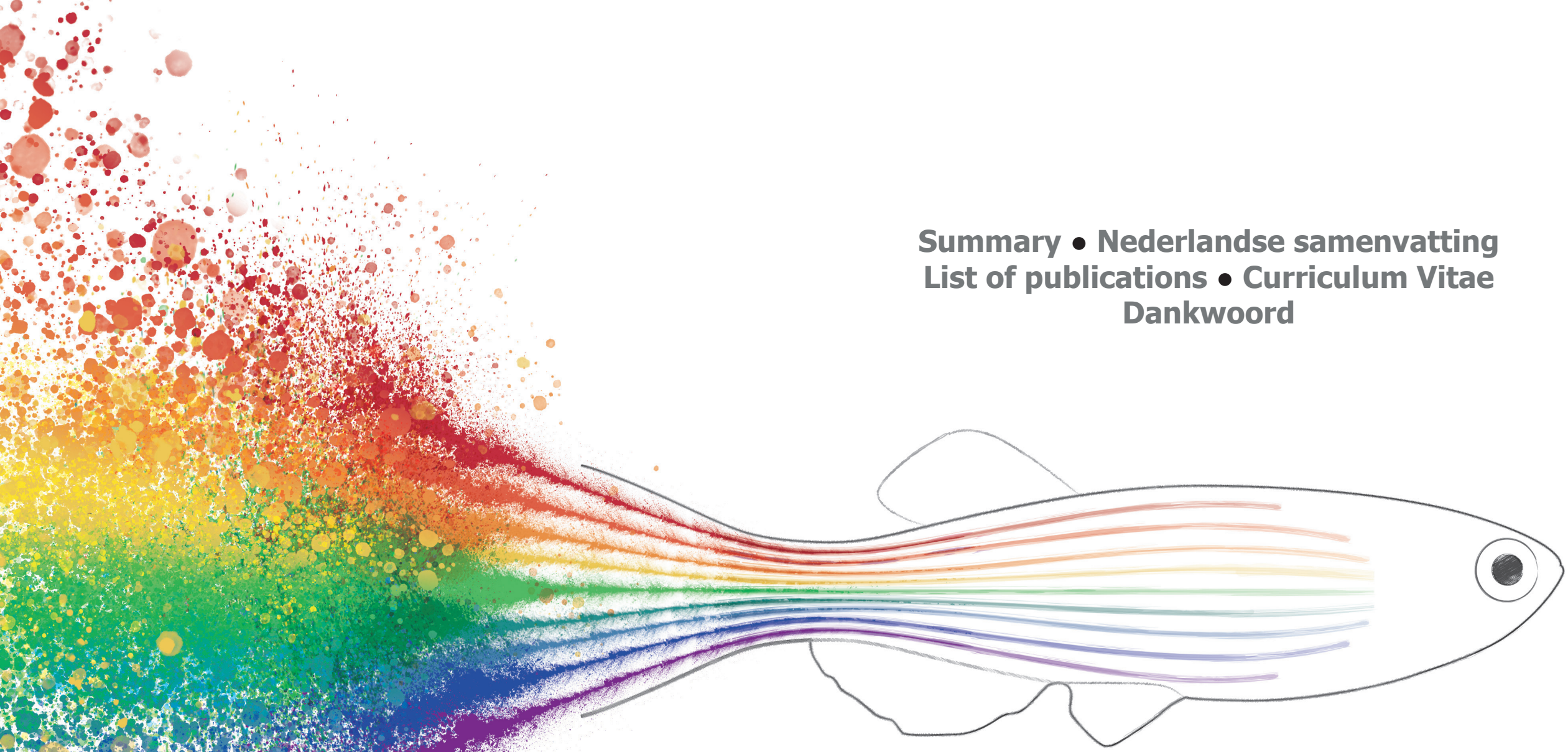

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Summary

The primary goal of this thesis has been to evaluate zebrafish as vertebrate animal model for the investigation of lysosomal storage disorders, in particular Gaucher disease (GD). Deficiency of glucocerebrosidase (GCase) degrading glucosylceramide (GlcCer) in lysosomes constitutes the molecular basis of GD. Zebrafish are an appealing model organism to study genetic disorders due to their facile maintenance, their ability to generate hundreds of off-spring which rapidly develop, are transparent and fit in a 96-wells plate. Zebrafish appear to be particularly interesting to study GD since they produce the lipid GlcCer, their genome contains an orthologue of the human *GBA* gene and an active acid β-glucosidase is expressed. Zebrafish also express many of the candidate modifier proteins, such as non-lysosomal Gba2 and lysosomal acid ceramidase (ACase). Research described in this thesis primarily focused on optimizing and using several biochemical and genetic techniques to study the catalytic features of zebrafish GCase and the consequences of its defect in zebrafish larvae and adults. In addition, the impact of two other enzymes, non-lysosomal GBA2 and lysosomal acid ceramidase, on GCase-deficient zebrafish received attention.

Chapter 1 provides an overview of the biological function, synthesis and catabolism of (glyco)sphingolipids (GSLs). Defects in one of the enzymes responsible for degradation of these lipids lead to a group of orphan diseases called lysosomal storage disorders (LSDs). Three LSDs are of particular importance in this thesis: Gaucher disease caused by deficiency of lysosomal GCase, Fabry disease caused by deficiency of lysosomal α-galactosidase A (α-GAL A) and Farber disease caused by deficiency of acid ceramidase (ACase). Zebrafish are introduced as attractive vertebrate research model, for LSDs. The chapter concludes with an overview of tools used to study lysosomal glycosidases in zebrafish larvae and adults, including fluorogenic substrates, activity-based probes (ABPs) and sensitive LC-MS/MS techniques to measure (glyco)sphingolipids.

In **chapter 2**, a combination of biochemical assays and molecular modelling is used to study and compare features of GCase enzymes from different species, including man, zebrafish, frog and turtle. All GCase enzymes showed hydrolysis of the artificial substrate 4-methylumbelliferyl β-glucoside (4MU-β-Glc) at an acidic pH optimum. Although human GCase required either saposin C or sodium taurocholate at pH of 5.2 for optimal activity, zebrafish and frog GCase showed high hydrolysis rates at pH 4.0 without additives. Increased levels of endogenous GlcCer and the deacylated sphingoid base, glucosylsphingosine (GlcSph), in the *GBA*-depleted HEK293T cells were corrected by over-expression of any of the GCase enzymes. In sharp contrast to human and frog GCase, the zebrafish enzyme was unable to perform an *in vitro* transglucosylation reaction with cholesterol as acceptor. It was suggested that cholesterol might not fit as acceptor in the catalytic pocket of zebrafish GCase. *In silico* comparison of modelled structures of the various GCase enzymes, based on the established 3D-structure of the human enzyme, revealed that residues of the catalytic pocket were highly conserved, while divergent residues were observed in the three flexible loops surrounding the catalytic pocket of GCase. Attention was drawn to three residues

with hydrophobic chains positioned close to the catalytic pocket of zebrafish GCase. Site-directed mutagenesis revealed that zebrafish GCase with substitutions of these amino acids did not show improvement in transglucosylation. To further test the hypothesis it should be considered to swap entire loops among enzymes of different species.

Chapter 3 describes the use of zebrafish as convenient organismal model to study *in vivo* target engagement of the mechanism-based irreversible GCase inhibitors. The potency and selectivity of conduritol B epoxide (CBE) and cyclophellitol (CP) were evaluated in developing zebrafish larvae and compared with findings in cultured human cells. Only at high CBE concentrations, non-lysosomal glucosylceramidase (GBA2) and lysosomal α-glucosidase were identified as major off-targets in cells and zebrafish larvae. CP was found to inactivate GCase and GBA2 with equal affinity and is therefore not suitable to generate genuine GD-like models. New CP derivatives, functionalized with a bulky hydrophobic moiety at C8, were validated as potent and selective GCase inhibitors in cultured cells and developing zebrafish larvae. Moreover, these CP analogues selectively inhibit GCase in the brain of adult fish. Overall, **chapter 3** demonstrates the suitability of the zebrafish model to evaluate drug potency, specificity and biodistribution, in particular brain permeability. The investigations in **chapter 2** and **3** illustrate that similar fluorogenic substrates, ABPs and LC-MS/MS techniques can be used to evaluate enzyme characteristics and relevant lipids as those used for human and mouse materials.

A practical advantage of the zebrafish, compared to mice, is the speed and convenience with which new gene knockouts (KOs) can be generated. **Chapter 4** provides a detailed protocol for the generation of gene KOs in zebrafish by means of CRISPR/Cas9 technology. It also describes the already generated mutations in the zebrafish gba*1*, *gba2*, *asah1a*, *asah1b*, *gpnmb*, *cln8* and *npc1* genes. RNA expression and lipid analysis of mutant larvae of Gpnmb, Npc1 and Cln8 confirmed that functional gene knockout have been generated. The impact of defective Gba1, Gba2, Asah1a and/or Asah1b is described in detail in **chapters 5**-**7**. Pilot experiments using the Tol2 transposase technique were undertaken to introduce an exogenous target DNA sequence in the zebrafish genome. The coding sequence of human *GBA* was introduced in the zebrafish *gba1*$^{-/-}$ genetic background, as well as the coding sequence of zebrafish prosaposin with a point mutation in the saposin C region thought to impair a disulfide bridge.
Chapters 5-7 report on GCase deficient "Gaucher" zebrafish, which are viable in contrast to mouse models, and describe the impact of concurrent GBA2 deficiency (**chapters 5** and **7**) and the impact of excessive GlcSph (**chapter 6**).

Chapter 5 focused on the role of zebrafish Gba2 in glycosphingolipid metabolism during GCase deficiency. Genetic and pharmacological modulation has been used to study GCase- and/or Gba2 deficiency in individual zebrafish larvae of 5 days post-fertilization (dpf) by evaluating their enzyme status using specific ABPs and their GlcCer metabolism using sensitive LC-MS/MS methods. Prominent formation of GlcSph was apparent in *gba1* KO and *gba1:gba2* KO larvae as well as during pharmacologic inactivation of zebrafish GCase. Accumulation of GlcCer in GCase-deficient fish is relatively limited, likely due to the

alternative metabolism of the lipid to its sphingoid base and the presence of active GCase derived from the heterozygous mother. Significantly higher levels of GlcCer and lower levels of GlcChol were measured in Gba2 deficient zebrafish larvae, underlining the importance of Gba2 for generation of GlcChol. Iminosugar inhibitors for GlcCer synthase and Gba2 were used to assess and correct abnormalities of GlcCer metabolism during GCase deficiency. In addition, overexpression of human GCase and injection of recombinant GCase both corrected the increased GlcSph levels.

In **chapter 6** the potential toxicity of excessive GlcSph during GCase deficiency is examined. Zebrafish have two orthologues of human acid ceramidase (ACase): Asah1a and Asah1b. From both single ACase KO fish only the *asah1b* KO fish fail to produce excessive GlcSph during GCase deficiency, while combined deficiency of both Asah1a and Asah1b is required to accumulate ceramide. A detailed comparison of *gba1* KO fish with excessive GlcSph and *gba1:asah1b* KO zebrafish without GlcSph revealed several differences but also interesting similarities. A significant amelioration of phenotype was observed in the *gba1:asah1b* KO fish. These fish did not show the same abnormalities in posture and swimming behaviour as *gba1* KO fish at 12 weeks post-fertilization (wpf). Double deficient *gba1:asah1b* KO fish showed an increased lifespan, however at 15-17 wpf abnormal swimming was apparent. Both *gba1* and *gba1:asah1b* KO fish showed comparable GlcCer accumulation in tissues and increased expression of the storage-cell biomarkers chitinase (*chia.6)* and *gpnmb*. The infiltration of microglia and storage cells in the periventricular grey zone of the optic tectum also appeared comparable. In their brains the two mutant fish showed similar autophagy, indicated by increased protein levels of p62, and inflammation, reflected by increased mRNA levels of *il1-β*, *tnfβ* and *apoeb*, as well as indications for similar activation of the complement cascade. Thus, abolishing Asah1b-ACase results in some phenotypic improvements but it does not prevent all abnormalities such as storage cell formation, neuroinflammation and complement activation.

Chapter 7 gives an overview of findings using zebrafish with complete KOs of the genes encoding GCase (*gba1)*, Gba2 (*gba2*), ACase (*asah1b*) and combinations thereof. These fish are raised to 12 wpf and their phenotype as well as biochemical and pathological parameters are described. Carrier *gba1*$^{+/-}$ fish show no apparent phenotype nor any biochemical abnormalities, while brain of Gba2 KO fish only reveal significant increased levels of specific GlcCer species. No clear abnormal phenotype is observed for any of the GCase deficient fish at 8 wpf, even though increases in GlcCer, neuroinflammation and autophagy are already demonstrable. Phenotypic abnormalities of the single *gba1* KO zebrafish have an early onset (± 9 wpf), starting with a drop of the tail and progressing into a change in swimming behaviour. In contrast, many of the double *gba1:gba2* KO develop severe phenotypical abnormalities in a matter of days, without the preceding characteristics such as the drop of the tail. Additionally, they have a significantly shorter lifespan than single *gba1* KO fish. Pilot experiments indicate a related earlier onset of dopaminergic neuronal loss in the *gba1:gba2* KO fish, however the underlying pathophysiological mechanism remains elusive.

ACase-mediated deacylation of accumulating glycosphingolipids to its sphingolipid bases is not unique to GD. **Chapter 8** evaluates the potential of using zebrafish to study another common lysosomal storage disorder Fabry disease (FD). Deficiency of α-GAL A constitutes the molecular basis of FD, characterized by accumulating globotriaosylceramide substrate in lysosomes which is partly converted by ACase into globotriaosylsphingosine (lysoGb3). **Chapter 8** shows that the zebrafish genome annotates one α-Gal A and one orthologue of the highly homologous α-N-acetylgalactosaminidase (α-Nagal). The presence of α-Gal A and α-Nagal proteins in zebrafish cells and larvae was confirmed by chemical proteomics using an α-Gal configured ABP. Fluorogenic α-galactoside and α-N-acetylgalactosaminide substrates reveal considerable α-Nagal activity in zebrafish larvae and adult organs. α-Gal A activity is typically lower than α-Nagal activity, but relatively high in fertilized eggs, reproductive organs, liver and kidney, compared to other tissues. Interestingly, no Gb3 could be detected using sensitive LC-MS/MS methods in any of the studied zebrafish materials such as cells, larvae, brain, liver, kidney and testis. The absence of the lipid Gb3 is consistent with the absence of a gene encoding lactosylceramide 4-α-galactosyltransferase (A4galt; Gb3 synthase) in zebrafish or any other teleost species. HEK293T cells lacking endogenous α-GAL A were generated using CRISPR/Cas9 technology. The increase in endogenous Gb3 levels in these cells could be comparably corrected by over-expression of either human α-GAL A or zebrafish α-Gal A. These findings indicate that zebrafish α-Gal A can hydrolyse the endogenous substrate Gb3 in the cellular setting, however it showed no *in vitro* hydrolysis of the artificial substrate NBD-Gb3. The role of the conserved α-galactosidase encoded by the *gla* gene in the zebrafish remains unknown. It is envisioned that *gla* knockout zebrafish expressing human A4GALT might render a useful Fabry disease model. The comparison of such fish with those with a concomitant *asha1b* KO might assist the investigation of the specific impact of excessive lyso-Gb3 in Fabry disease pathology.

Chapter 9 discusses the obtained results of the undertaken investigations and describes future prospects of research. In addition, established and novel techniques are highlighted which could be useful to study other common clinical manifestations of GD patients, such as bone defects and blood abnormalities. Comparison of the different *gba1* KO zebrafish models might reveal underlying molecular mechanisms of common clinical manifestations upon concomitant GBA2 or ACase deficiency. Anatomical and physiological similarities and differences of the zebrafish compared to mammals are also discussed, in order to put the use of the zebrafish GD model in perspective.

In conclusion, investigations described in this thesis show that zebrafish offer exciting new possibilities to study molecular mechanisms underlying pathological processes during lysosomal hydrolase deficiencies. Due to differences, information obtained from *gba1* KO fish might not directly be translated to GD patients, however important mechanistic insights may be revealed by studying these teleost models. In particular, the unique *asah1b* KO fish, not able to generate the presumed toxic lyso-lipid, could give valuable insights into the role of GlcSph, psychosine, lyso-Gb3 or other lyso-lipids in accelerating disease manifestations.