Multiple Approaches to Novel GSD Ia Therapies
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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy
in the Department of
Molecular Genetics and Microbiology in the Graduate School
of Duke University

2016
Abstract
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Abstract

Glycogen storage disease type Ia is an autosomal recessive disorder caused by a mutation in the glucose-6-phosphatase (G6Pase) catalytic subunit, encoded in humans by G6PC. G6Pase dephosphorylates glucose-6-phosphate (G6P) in the liver to generate glucose that can be shuttled to the bloodstream to maintain normoglycemia. Patients with GSD Ia typically present at 6 months of age with severe hypoglycemia, which is lethal if untreated. The current treatment is a strict dietary regimen in which children must be fed every 2 hours overnight or given nasogastric tube feeding, and adults must consume uncooked cornstarch around the clock to maintain normal blood sugar levels. This treatment maintains survival but fails to prevent other symptoms related to metabolism of the excess G6P, and patients develop hepatic adenomas that may become hepatocellular carcinoma later in life, in addition to progressive renal complications.

To overcome the problems persisting during dietary therapy, the Koeberl lab has sought to develop gene therapy approaches that use adeno-associated virus (AAV) vectors to replace the G6Pase activity, restoring normoglycemia and normal metabolic processes. However, the vast majority of AAV-delivered genetic material exists as episomes that do not replicate as cells divide, so the effects of AAV gene therapy on GSD Ia mouse and dog models have proven temporary. We hypothesized that driving integration of therapeutic vector genomes into an affected individual's genome would improve beneficial effects' longevity.
We tested several approaches to accomplish this, and have found positive effects using a zinc finger nuclease (ZFN) that targets the mouse safe harbor ROSA26 locus to induce homologous recombination of the \textit{G6PC} donor vector into the mouse genome. We were able to see an improvement in mouse survival to 8 months of age, an increase in G6Pase activity at 3 months of age, and a decrease in glycogen accumulation at 3 months of age, when the ZFN vector is administered alongside the \textit{G6PC} vector, compared with mice that received the \textit{G6PC} vector alone.

We have also taken an alternative approach to overcoming the long-term complications of the current dietary treatment, which would augment rather than replace the current treatment. We have examined several drugs known to induce autophagy in other disease models or cell culture systems, to determine if we could manipulate autophagic activity in \textit{G6PC} knockdown hepatocytes or GSD Ia mice. We have found positive results using rapamycin, a well-studied MTOR inhibitor, in mice and cells, and have screened several other drugs as well, finding positive effects for bezafibrate, mifepristone, carbamazepin, and lithium chloride, in terms of lipid reduction (which accumulates as a symptom of GSD Ia) and/or LC3-II enhancement, which is reduced in GSD Ia due to downregulation of autophagy during G6P accumulation.
Dedication

I dedicate this to my parents, Deborah and James Landau first and foremost, for making sure I survived at least 18 years and for teaching me the mental tools that have allowed me to get to this point. I would also like to thank my grandparents for instilling in me a strong family connection that resides at the root of my drive to improve the lives of those around me, without which I would never have been able to persevere and complete this work.

Finally, my thanks go out to Katie Voss for all the support she has given me over the years, the many consoling words that helped me get through late night vector preps in the lab, and the patience she showed when I had consecutive midnight or pre-dawn animal feedings. For that and more, she has my love.
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Abbreviations

AAV: Adeno-associated virus
AAV-G6P: AAV vector containing human G6PC
AAV-RoG6P: AAV vector containing human G6PC flanked by ROSA26 homology arms
AAV-RoGFP: AAV vector containing eGFP flanked by ROSA26 homology arms
AAV-ZFN: AAV vector containing the gene encoding the 2 ZFN subunits that together target the mouse genomic ROSA26 locus
AMPK: 5’ adenosine monophosphate-activated protein kinase
ATG5: Autophagy protein 5
CRISPR: Clustered regularly interspaced short palindromic repeats
DSB: Double-strand break
GAA: Acid alpha-glucosidase
GAWTS: Genomic amplification with transcript sequencing
G6P: Glucose-6-phosphate
G6Pase: Glucose-6-phosphatase
G6PC: Human glucose-6-phosphatase catalytic subunit gene
G6pc: Mouse or canine glucose-6-phosphatase catalytic subunit gene
GSD Ia: Glycogen storage disease type Ia; AKA, von Gierke’s disease
HDR: Homology-directed repair
HRP: Horseradish peroxidase
iPCR: Inverse polymerase chain reaction
ITR: Inverted terminal repeat
KD: Knockdown
KO: Knockout
L1: Long interspersed nuclear element 1
LAM-PCR: Linear amplification mediated polymerase chain reaction
LM-PCR: Ligation-mediated polymerase chain reaction
LSP: Liver-specific promoter
mTOR: Mechanistic target of rapamycin
mTORC1: Mechanistic target of rapamycin complex 1
NAFLD: Non-alcoholic fatty liver disease
NEHEJ: Non-homologous end joining
OCT: Optimal cutting temperature compound
p70S6K: Ribosomal protein S6 kinase
PAM: Protospacer adjacent motif
PAS: Periodic acid-Schiff
PCR: Polymerase chain reaction
RSE-PCR: Restriction site extension polymerase chain reaction
RT-qPCR: Quantitative reverse transcription polymerase chain reaction
TALEN: Transcription activator-like effector nuclease
VPs: Vector particles
ZFN: Zinc finger nuclease
1. Introduction: GSD Ia, AAV Vectors, Genome Editing, and Autophagy

1.1 GSD Ia background

First described in 1929 by Edgar von Gierke, GSD Ia is a rare disease affecting 1 in 100,000 births, although in the Ashkenazi Jewish population it is far more common, affecting 1 in 20,000. Patients with GSD Ia present around 6 months of age with severe hypoglycemia. In addition to hypoglycemia, patients develop a number of other symptoms including growth retardation, hepatomegaly, hypoglycemia, lactic acidemia, hyperuricemia, and hyperlipidemia. Current treatment for the disease is a lifelong dietary regimen requiring frequent meals to maintain normoglycemia. These frequent meals require either waking up throughout the night to consume uncooked cornstarch for calories, or the use of a nasogastric drip to allow sleeping through the night. The risk of malfunction during use of continuous nasogastric tube feedings could lead to potentially lethal hypoglycemia, especially in infants.

This is one motivation for the ongoing research for GSD Ia gene therapies that could remove the need for constant dietary blood glucose maintenance. With improved dietary therapies, it has become clear that while preventing hypoglycemia prevents mortality and can improve patients’ growth, other issues arise due to the chronic metabolic stress to hepatocytes in these patients, due largely to the toxic levels of substrate and alternate pathways for catabolism of accumulated G6P that is not converted to glucose (Figure 1).
Normally, G6P is processed by G6Pase to generate glucose that can be sent out of hepatocytes into the bloodstream to maintain normoglycemia. Without this activity, G6P accumulates and is processed by several other pathways, leading to many of GSD Ia’s symptoms, including hyperuricemia, lactic acidemia, hyperalaninemia, and hyperlipidemia. It also feeds back to prevent glycogen breakdown, leading to glycogen accumulation in hepatocytes.

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As shown, glycogen is broken down in hepatocytes to produce G6P, which is capable of entering multiple metabolic pathways, but most acutely important is its hydrolysis by G6Pase into glucose and phosphate, which can then be shuttled out of the liver into the bloodstream to maintain critical normoglycemia. The lack of G6Pase activity leads to low levels of glucose, which causes the lifethreatening hypoglycemia that is a trademark of GSD Ia. This reaction blockage also causes accumulation of G6P in hepatocytes, which pressures several other
metabolic pathways into excess activity, leading to toxic "substrate excess" conditions.

G6P that fails to be broken down by G6Pase leads to an imbalance in several reactions, driving those reactions forward, leading to excess end products from those metabolic pathways. G6P can enter the pentose phosphate pathway, leading to excess ribose 5-phosphate, which ultimately accumulates and is broken down into uric acid, which causes hyperuricemia and gout in GSD Ia patients. G6P can also be converted into pyruvate, which can in turn be converted to lactate and alanine, leading to excesses of both of those products, which cause lactic acidemia and hyperalaninemia, respectively.

The most prominent substrate excess symptoms, however, are hyperlipidemia and fatty liver. The accumulated G6P pool can be drawn down into storage molecules in two ways. As the name glycogen storage disease implies, G6P can be pulled into glycogen and stored in hepatocytes, or it can be used into the cholesterol fatty acid synthesis pathway, producing cholesterol along the way as well as newly-synthesized lipids. Some of this is sent to the bloodstream, causing hyperlipidemia, while much of it is stored in hepatocytes, leading to enlarged lipid vacuoles that contribute, along with the excess glycogen deposits, to hepatomegaly in GSD Ia patients.

Under the conditions produced by these pathways' hyperactivity, GSD Ia kidneys and livers exist in a state of chronic stress that may lead to later conditions, including nephropathy, hepatic adenomas, and hepatocellular
cancer. As such, we have sought an all-encompassing treatment that can correct more than just hypoglycemia. To accomplish this, a method must be developed to restore G6P hydrolysis to yield glucose for normoglycemia and to reduce excess G6P so that its other routes do not accumulate toxic products. Our method for doing this uses gene therapy to deliver wild type cDNA of human G6Pc via adeno-associated viral vectors.

1.2 AAV vector background

AAV is a non-enveloped single-stranded DNA virus of the paroviridae family. AAV is incapable of viral replication without concomitant infection with either adenovirus or herpesvirus. The viral structure consists of an approximately 20nm protein capsid encasing about 4.7kb of single-stranded DNA whose ends form secondary structures known as inverted terminal repeats (ITRs) using self-complementarity. The wild AAV genome contains two genes, rep and cap.

The rep gene encodes four different proteins using overlapping reading frames and alternative splicing: Rep78, Rep68, Rep52, and Rep40. These proteins are important for generating proper ITR structures and are necessary for efficient encapsidation. The cap gene encodes the three viral capsid proteins VP1, VP2, and VP3, using a single promoter. These proteins form an icosahedral structure around the DNA strand to generate infectious viral particles.

Once an AAV particle transduces a host cell, its DNA is released through a poorly understood mechanism, and it then forms episomes: circular double-
stranded DNA structures consisting of multiple copies of the AAV genome connected end-to-end by extension of ITRs along the genome. These episomes are used as the DNA templates for viral mRNA production to produce new viral capsids. The episomes are also used as templates for new vector genome synthesis, but they themselves cannot undergo DNA replication to produce new episomes.

Several features of AAV virions makes AAV particularly useful for gene therapy. First, there are many AAV serotype capsids available for packaging therapeutic transgenes, which allows the evasion of pre-existing anti-AAV antibodies against a specific serotype, reduces chances of off-target transduction, and increases tropism for specific target tissues. Second, AAV is naturally replication-deficient due to the requirement of co-infection with helper viruses in order to produce competent viral particles; this further lends itself to usefulness in our ability to manufacture AAV through a highly scalable helper-virus-free system delivering required genes in trans on separate plasmids in a simple system. Finally, AAV is attenuated yet more by the alterations made to its genome in the course of designing gene therapy vectors, removing all but the terminal repeat sequences, and that attenuation allows ample flexibility in packaging size since very little of the wildtype genome needs to be left intact. Combining the flexibility of serotype variation with that of genome manipulation makes AAV therapies adaptable to many different experimental approaches for many different diseases, while its attenuated nature makes it safer and more
controllable than many other gene delivery vehicles.

The availability of multiple AAV capsid serotypes with varied tissue-specific tropisms makes AAV an excellent gene delivery vehicle for gene therapy. Any AAV2 vector genome can be cross-packaged by capsid proteins from any other serotype, thereby providing a toolkit of pseudotyped vectors for experiments. In addition to wildtype AAV serotypes, novel synthetic serotypes have recently been developed, and approaches using methods to mutate existing capsids and to generate recombinant capsids that are crosses between the many already-available serotypes continue, further increasing the diverse pool of serotypes gene therapeutics may tap into.

The availability of multiple serotypes is important for two reasons. First, current AAV gene therapy is frequently transient due to the nature of episomal DNA. Since episomal DNA does not replicate once formed within a cell, any replicative cells targeted with AAV-based gene therapies will dilute out the delivered transgene. The patient's cells continuously increase in number over time, but the number of episomes initially delivered remains fixed, so a smaller proportion of these cells will remain corrected by the gene therapy. Eventually too many cells within the tissues lack vector genomes, and the therapy fails to provide adequate benefits.

The availability of multiple serotypes can help with this problem by allowing multiple AAV treatments to be given to the same patient over time. Following exposure to each serotype, a patient will develop an adaptive immune response
against the viral capsids used. If that same capsid serotype is used again, neutralizing antibodies will largely prevent subsequent gene delivery, thereby rendering the treatment ineffective. However, by simply packaging the same gene of interest in a different serotype capsid, a patient could receive a booster treatment when the initial one wears off.\textsuperscript{20} This is of particular interest when treating GSD Ia since G6Pase-deficient hepatocytes have abnormally high turnover rates, leading to even faster clearance of the AAV-delivered genomes than would normally be expected. However, as there is a limited pool of serotypes available, and that pool is further limited when one requires a specific type of tissue tropism (discussed below), a repeated booster-dose approach to solving the problem with AAV gene therapy transience is an incomplete solution to the problem.

The second reason the availability of multiple AAV serotypes is a boon to gene therapy is that different serotypes have different tropism for which tissues they transduce. This can be a problem, in the case of wanting a large pool of available capsids to repeatedly dose a patient and target the same tissue, but that very property is helpful for limiting the tissues that receive a transgene. This property helps reduce concerns over deleterious effects of viral transduction in certain tissues, especially the gonads, where transduction and subsequent germline modification could lead to gene modifications that could be passed on to future generations of offspring. This ethical dilemma brings into question the rights of the offspring to have their genomes modified prior to ever having been
born, and the use of serotypes with high tissue-specificity helps reduce such concerns. Furthermore, in the patient him/herself, limiting the off-target delivery of vector genomes can reduce the risk of vector integration, however slim they are from AAV, and of dangerous genomic alterations. Rather than transducing all tissues with accompanying risks for toxicity, by delivering an AAV serotype with extreme hepatocyte specificity, like AAV8, there’s far less concern about negative effects or potentially cancer-causing mutations in most of the patient's cells.

A further advantage to having high tissue-specific tropism from a particular serotype, which is relative tissue-specific availability. By using serotypes with high specificity for particular tissues, we can use reduced doses compared with widespread AAV transduction, since the capsids will go mainly to the desired target sites (the liver, in many cases of AAV gene therapy) rather than being spread out across multiple irrelevant tissues.¹⁶ Since AAV does have a tendency to produce dose-related toxicity at very high doses, this tropism for a target tissue is a significant benefit, and one on which ongoing development continues.

Another beneficial feature of AAV vectors is their natural dependency on coinfection with adenovirus or herpesvirus to proliferate. Since safety is a key concern in gene therapy research, it is crucial that delivery vehicles reduce potential harm to patients. One concern when using viral vectors is the risk for a productive infection from wildtype virus that might cause illness or a dangerous immune response to what the body perceives as a true viral infection. Wildtype AAV is incapable of replicating on its own, so using it as a basis for gene therapy
carries with it the low likelihood of generating wildtype AAV that requires co-infection with adeno- or herpesvirus to replicate and induce strong immune responses or cause cell lysis.

The requirement for helper functions from certain genes provided by other viruses, particularly the VA, E2A, and E4, E1A, and E1B proteins of adenovirus, can be provided by other means than co-infection with adenovirus in HEK293 cells. E1A and E1B are already expressed by HEK293 cells, having been used to immortalize the cell line. VA, E2A, and E4 can be provided by a plasmid co-transfected into HEK293 cells along with other plasmids, that together all provide the necessary components for AAV vector production. Using this approach, therapeutic vectors can be made using no live virus capable of human infection, which dramatically enhances the safety of using AAV compared with using live virus. Consequently, different vector components are spread across different plasmids: AAV rep and cap are on one plasmid; adenovirus helper genes are on a second plasmid; and the therapeutic vector genome is on a third plasmid. This makes it simple to perform different vector preparation transfections to produce different serotypes of the same vector genome just by changing the rep/cap plasmid.

The fact that AAV vector production is performed using HEK293 cells is also of note. This cell line is widely available, easy to grow, amenable to growth in special serum-free media, and amenable to large-scale culture for vector production. The fact that serum-free HEK293 growth media has been developed
greatly simplifies meeting FDA guidelines for use of animal products in the production of gene therapy viral vectors. Of greater value to gene therapy is the scalability of HEK293 AAV vector production. Small scale vector preparations can be performed on cell culture plates to yield between 1E+12-1E+14 AAV particles. This is sufficient to give substantial doses to mice or young canines for experimental procedures, but it would take hundreds of such preparations to treat an adult human. Fortunately, HEK293 cell line variations exist that can be grown in suspension, allowing for much larger numbers of cells to be cultured in bioreactors that could scale to hundreds or thousands of liters, yielding 2E+13 vector particles per liter. This enables AAV gene therapy research to readily translate to clinical settings—a feature critical to any gene therapy approach.

The final feature that makes AAV an optimal delivery vehicle for gene therapy is that only a very small amount of its wildtype genome must be present in trans with the therapeutic gene of interest in order for the gene to be packaged in active viral capsids. This means that all the wildtype genes can be removed, eliminating any possibility of packaging replication-competent AAV particles. It also means that there is ample space in the minimal packaging genome for one to insert the therapeutic gene of interest. The maximum capacity of AAV vector particles is just under 5kb of ssDNA, and the only wildtype structures that need to remain are the two inverted terminal repeats (ITRs) at under 150bp each, so that leaves approximately 4.7kb of space in which one can package any therapeutic transgene. 4.7kb is enough space to package many therapeutic
genes in the form of cDNA, making AAV a highly applicable and flexible system for gene therapy.

While Aav vector-based therapies carry many tangible benefits, concerns have also been raised regarding their safety, in particular their carcinogenic potential. Donsante et al. found an increase in hepatocellular carcinoma incidence in mice treated with an AAV serotype 2-based vector encoding human β-glucuronidase with a β-actin promoter and cytomegalovirus enhancer, in two separate studies. Work by other groups was not able to reproduce these findings, though their work used different vector genome constructs (e.g., different genes, promoters, and enhancers), dosing, and mouse backgrounds. Concerns persist regarding the tumorigenic potential of AAV under certain conditions, but the risk is believed to be highly dependent on the vector structure and dose. More recent work confirmed that enhancer and promoter choice is an important factor in whether AAV treatment could be carcinogenic. As such, promoter selection must play a role in vector design to ensure the safety of potential therapeutics going forward.

One more factor to consider in AAV vector-based treatments is that of immune responses. While AAV serotypes, as discussed above, allow for repeated treatments by changing serotypes between treatments to evade neutralizing antibodies, the immune system may also mount a response against the delivered transgene itself, which could lead to clearance of cells expressing the transgene. This is a known problem that occurs during systemic transgene
expression via constitutively active promoters. However, genes expressed under control of liver-specific promoters or otherwise restricted to liver-specific expression do not trigger such an immune response.\textsuperscript{32-36} 121-125 Regulatory T cells in the liver inhibit immune responses against proteins expressed by hepatocytes, preventing clearance of successfully-transduced hepatocytes and in fact generating tolerance to the protein that can allow it to be expressed elsewhere.\textsuperscript{37, 38}

1.3 Genome editing background

Genome editing has been a major focus for biological research for decades, and its potential for therapeutic effects is just now coming to the forefront of study. In the context of this document, "genome editing" will be used to mean any modification—base pair addition, deletion, or revision—to the genome inherited by a cell during cell division.

Typical genome editing relies on using a eukaryotic cell’s normal DNA repair pathways to to change its genome in a particular way. There are two main pathways that cells use to accomplish this: non-homologous end joining (NHEJ); and homology-directed repair (HDR). NHEJ is the form primarily used in mature nondividing cells, while HDR is primarily used by those in the G2/S phases of the cell cycle.\textsuperscript{39} Of note, hepatocytes routinely divide, especially as part of liver regeneration,\textsuperscript{40} which should therefore lead to HDR acting as the predominant DNA damage repair mechanism.

NHEJ repairs DNA damage by rejoining the two ends of damaged DNA,
which are not homologous to each other. NHEJ typically removes some bases from either end of the double-strand break (DSB), and may also involve DNA synthesis to produce compatible ends for end-joining. These activities generate short insertions or deletions ("indels") that can damage DNA's functionality. For example, an indel of 1-2 bases in an exon will result in a frameshift mutation, likely knocking out the gene's activity. It could also result in nonsense mutations by creating stop codons, or simply perturb gene activity by changing an important amino acid codon. These outcomes are used as part of genome editing to knock out genes or control exon skipping to bypass nonfunctional, deleterious exons, thus correcting negative phenotypes by restoring a gene's functionality.

HDR repairs DNA damage without creating changes at the site of damage or leaving out portions that were deleted during the creation of said damage. HDR relies on the availability of a second copy of the damaged region, typically the sister chromosome during mitosis, to regenerate the damaged region. HDR copies that homologous DNA as a template to fill in the damaged chromosome, resulting in the original DNA strand containing at least some portion of DNA copied directly from the second chromosome. In the context of gene editing, this process can be harnessed to copy some desirable DNA into the target cell's genome by manipulating the HDR machinery to use a particular piece of DNA as the donor template during repair. By doing so, the HDR machinery can copy the provided DNA into the target genome if the donor DNA contains sufficient homology to the damaged site. This can be used for gene insertion or gene
knockouts depending on the cleavage site and what is being inserted. For instance, we can insert a functional transgene to add activity to cells that lacked a functional copy of the inserted gene, or we can induce the break in an active gene and insert a reporter there to identify knockouts or insert resectable sequences to create an inducible gene reactivation system.

The usefulness of HDR alongside NHEJ has dramatically improved over the last 13 years with advances in modular zinc finger nuclease design, Transcription activator-like effector nuclease (TALEN) generation, and now the rapid creation of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nucleases. Genome modification originally relied on adding homologous DNA to cells and relying on baseline levels of HDR to alter the endogenous sequence for a desired outcome. However, the rapid development of nuclease technologies over the last two decades has allowed us to modify eukaryotic genomes with much higher efficiency by inducing DSBs that recruit NHEJ and HDR machinery to create genomic changes at high rates at specific target sites. The three nuclease technologies with the greatest impact have been ZFNs, TALENs, and CRISPR/Cas9.

Zinc finger nucleases (ZFNs) were the first readily customizable nuclease to be harnessed for eukaryotic genome editing. A ZFN consists of a Cys$_2$-His$_2$ zinc finger DNA-binding domain that directs the protein to its target site, linked to the FokI nuclease domain, which has non-sequence-specific endonuclease activity. Once localized by the zinc fingers domain, the nuclease cleaves DNA in the
targeted area.

Individual zinc fingers recognize and bind 3-base-pair DNA sequences.\textsuperscript{51} They consist of 30 amino acids forming an α-helix and two β-strands. The α-helix makes contact with one strand of DNA, providing sequence specificity. Naturally-occurring zinc fingers have been used as platforms for modification to create zinc fingers capable of binding any of the 64 possible 3-base pair DNA codons. The zinc finger domain of a ZFN typically contains 4-6 such fingers, so that it specifically binds a 12-18 base pair DNA sequence. Since FokI nuclease acts as a dimer, two different ZFNs can be provided that require heterodimerization for nuclease activity, thus creating 24-36 base pair sequence specificity—enough to make the recognition sequence unique in the human genome.\textsuperscript{51}

ZFNs do have some drawbacks. First, designing them is not foolproof; one cannot simply pick the zinc fingers that match the desired target site, assemble the plasmid, and generate an enzyme with the desired activity 100% of the time. This is likely because zinc fingers are not entirely independent of one another: they have some binding effect on neighboring codons, enhancing or interfering with neighboring zinc fingers’ activities; and also because sequences of neighboring zinc fingers can alter the orientations of one another, changing their binding abilities due to altered structure.\textsuperscript{54, 55} Advances in computation-based rational design have helped enhance the likelihood of a custom ZFN having proper activity, but it is still imperfect and requires extensive characterization of the final enzyme.\textsuperscript{56} Second, ZFNs tend to have off-target activity, leading to DSBs
at undesirable sites. The zinc fingers used for ZFN construction are not absolutely specific for their targets, and the small amount of flexibility in binding enables ZFNs to bind to and cleave at other genomic locations.\textsuperscript{57, 58} Target site specificity being so important to the safety of therapies relying on nuclease-driven technology, this represents a major concern.

The second major type of nuclease technology applied to genome editing is that of TALENs. TALENs are similar to ZFNs in that they consist of two domains: a DNA-binding domain and the FokI nuclease domain. Use of FokI also means that TALENs, like ZFNs, must function as dimers. The DNA-binding domain of TALENs is derived from bacterially-secreted transcription activator-like proteins that bind to plant genomes to activate expression of desired genes upon infection of the plant. This binding domain consists of repeated sets of typically 34 amino acids with consensus on all but the 12\textsuperscript{th} and 13\textsuperscript{th} amino acids in each repeat. The 12\textsuperscript{th} and 13\textsuperscript{th} amino acids are hypervariable and alone determine DNA binding specificity for each TALE repeat.\textsuperscript{59} The hypervariable amino acids can be changed to target any of the four DNA bases, enabling simple design of custom TALENs for targeting virtually any DNA sequence. This is an advantage over ZFNs, whose fingers bind sets of 3 bases at a time, making them more difficult to design for particular sequences. Rationally-designed TALENs also have a higher success rate following synthesis than do ZFNs.\textsuperscript{60} Thus, the ease with which TALENs can be designed and the reliability of designs producing active targeted nucleases led to TALENs supplanting ZFNs in the forefront of genome editing.
research. The only real downside to TALENs compared with ZFNs is that TALENs genes are about three times the size of those of ZFNs, putting them over the threshold for packaging TALEN heterodimers in AAV vectors.61

The most recent addition to the genome editing nuclease family is Cas9: CRISPR associated protein 9. Cas9 derives from the CRISPR/Cas9 adaptive immune system of bacteria to protect against bacteriophages,62 originally that of Streptococcus thermophilus.63 Cas9 has since been discovered in many different bacteria, providing a variety of traits, some with higher target site specificity, some with shorter gene sizes for superior packaging in small gene therapy vectors such as AAV.

Cas9 relies on short (~20 bp) guide RNA sequences to provide DNA binding, rather than protein-DNA binding as in the case of ZFNs or TALENs.64 In bacteria, two separate pieces of RNA form the guide RNA: the CRISPR RNA and the trans-activating RNA. The guide RNA base pairs with DNA, binding the Cas9 to its target DNA so it can induce cleavage. In bacteria, Cas9 provided adaptive immunity by digesting bacteriophage DNA to stave off viral infection, and the short DNA sequences could be taken into the bacterial genome to later generate guide RNA to allow Cas9 cleavage of similar bacteriophages during future infections.65 Since its functional elucidation in bacteria, Cas9 has been adapted for use in eukaryotic systems for genome editing. The two-part guide RNA system has been simplified and we now have the tools to engineer single RNA strands that serve the function of complete guide RNA.
Cas9 has also been characterized from many different bacteria now, and each bacterial Cas9 has traits that can lend themselves to different applications in several ways.\textsuperscript{66, 67} First, each Cas9 requires a different short consensus motif known as the protospacer adjacent motif (PAM) to be adjacent to the guide RNA.\textsuperscript{68} There is typically some flexibility in these motifs, making them adaptable to a variety of targets. However, not all desirable targets are guaranteed to contain the PAM in a site amenable to nuclease targeting, so the availability of multiple Cas9 proteins with varying PAM recognition capabilities is valuable to ensure that any target site can be cleaved by some Cas9. Additionally, different Cas9 proteins have different levels of activity, and research is ongoing in the discovery and generation of Cas9s with ever-improved efficiency. Lastly, Cas9s have been discovered that are much smaller than the first generation Cas9s, allowing them to be packaged in small vehicles such as AAV for in vivo genome editing.\textsuperscript{69-71}

The CRISPR/Cas9 system has one major advantage and one major disadvantage over ZFNs and TALENs. The advantage that has led to Cas9 becoming wildly popular is that it can be targeted to a genomic site by changing only the short guide RNA to base pair at the site. Doing this requires only a few days of oligonucleotide design, synthesis, and cloning into a backbone such that the new guide RNA replaces the old one in a preexisting plasmid. Furthermore, Cas9 has a very high success rate at new target sites as long as the correct PAM is present, since the enzyme’s structure does not change when the guide RNA is
modified, and Cas9s have rates of cleavage comparable to that of ZFNs and TALENs. Conversely, targeting ZFNs and TALENs to a new site requires a computationally-intensive all-over protein design with relatively low chances of success since the protein structure itself must be altered to change its target site.

The speed and reliability of Cas9 has made it a superior research tool, but it is yet in its infancy in terms of site-specificity and gene therapy applications. Notably, the greatest drawback is that Cas9 has a substantial rate of off-target DNA cleavage, especially when off-target sites exist with only 1-4 mismatched bases. ZFNs have been improved in this regard via modification with the generation of obligate heterodimer FokI mutations that force ZFNs to act as heterodimers instead of homodimers, greatly enhancing ZFN specificity. In a sense, the community is just beginning to use second-generation CRISPR/Cas9 systems with enhanced cleavage activity, simplified guide RNAs, and reduced size for in vivo delivery via AAV. The next critical step before it can be considered for human gene therapy is the design of Cas9 nucleases with higher specificity. Furthermore, there has been little in vivo insight into CRISPR/Cas9 functions, and it is clear that in vitro and in vivo activity is quite different. Mouse studies have been performed, but large animal studies—the next stepping stone on the way to human gene therapy—have yet to be completed.

Ultimately, the CRISPR/Cas9 system is the breakthrough nuclease technology that will most likely be used in multiple gene therapy systems because it can so quickly and easily be adapted to many different targets, while
ZFNs and TALENs require significant time and effort to develop for any given disease, model, or organism. However, ZFNs and TALENs, having been around longer, are more advanced and refined than CRISPR/Cas9s yet are, ZFNs being the most refined compared with their initial designs. This was a key factor in determining which nuclease to go forward with on the nuclease portion of this long-term project. While CRISPRs had just emerged at the time the project began, and were showing promise, they could not yet be packaged in AAV—our well-established effective gene therapy delivery vehicle—and their target specificity was still unclear. The main advantage TALENs had over ZFNs was their relative design ease, but at the time, a ZFN targeting a mouse safe harbor locus had already become available, so making use of the more modern TALEN technology would have been a step backward, and CRISPR was too unreliable. Thus, moving forward at the time of the project's beginnings, we chose to utilize a ZFN targeting the mouse genome.

Using nucleases enables several different types of genome editing. The simplest and most commonly investigated type is gene repair—that is, using genome editing technologies to repair mutant or damaged endogenous genes of the patient or animal model. There are two main forms of gene repair that are commonly studied. First, one can repair missense or nonsense mutations using HDR to provide a homologous sequence to the mutant site, alongside a nuclease that will create a DSB at the site to force DNA repair to use the homologous transgene as the repair template, replacing the mutant base with the correct
The second repair type is specific to Duchenne muscular dystrophy (DMD) in human systems, in which one can repair the endogenous gene by inducing exon skipping of defective nonsense-generating exons.\textsuperscript{44, 83, 84} This type of repair requires that the exon be superfluous to the function of the protein product, as in the case of dystrophin having multiple repeated exons so the skipping of one still yields functional protein.

Exon skipping is significantly constrained in applicability to DMD, since necessitates the target gene have repeated sequences that are not all required for function. Point mutation repair is more widely applicable because it does not carry this restriction. However, it suffers from the requirement that a different nuclease be created specifically for each mutation to be corrected if the target gene is mutated in different locations between different patients. With the emergence of personalized medicine and cost-effective DNA sequencing, it is becoming possible to develop such treatments, but the creation of personalized nucleases has not been realistically possible until the application of CRISPR/Cas9 technology to mammalian systems. Even with the ease of nuclease creation using Cas9, the risk of off-target DNA damage and the requirement for extensive nuclease characterization of each guide RNA means this type of gene therapy still has many years ahead before it is ready for use in humans.

To address that problem, an alternative type of genome editing is possible using nucleases with more general targets. Mammalian genomes typically
contain specific loci that serve little or no function in adult animals, such as the ROSA26 locus in mice, and the AAVS1 and ROSA26 ortholog loci in humans. Such sites are referred to as safe harbor loci because they can be disrupted by transgene insertion without disruption of cellular activity, and they allow expression of inserted transgenes. Since these sites are carried by all members of a particular species, one need only design and optimize a single nuclease for the whole species to induce DSBs at these loci in anyone given the treatment. Donor transgenes specific to a given disease, with added homology to the safe harbor loci, can then be provided alongside the nuclease so HDR at the DSB will integrate the donor transgene into the subject's genome, providing a functional copy of a gene that was previously lacking. Care must be taken, however, when designing targeting vectors to ensure that integration at safe harbor loci occurs in orientations and specific sites so as to allow efficient expression.

This is the approach we have taken in the first portion of the project, utilizing a ZFN to cleave the mouse ROSA26 safe harbor locus along with a donor transgene copy of G6PC to replace the lacking gene in GSD Ia mice. A similar approach has also been used to induce integration of functional factor IX genes into the albumin locus of mice without the use of nuclease, but the principle of using HDR to integrate a complete functional transgene at a safe integration site remains the same.

A third type of genome editing is possible through the use of gene-targeting
nucleases: dominant mutation knockouts. Since most cells are out of the G2/S-phase where HDR dominates in DNA repair, most cells use NHEJ as their primary means of repairing DNA damage. NHEJ typically leads to indels that functionally knocks out the gene in which they occur. Thus, use of nucleases targeting particular dominant mutant alleles has the potential to stop such deleterious alleles from causing human diseases like retinitis pigmentosa\textsuperscript{91,92} or Meesmann's epithelial corneal dystrophy.\textsuperscript{93} Genome editing in this context is typically geared more toward the generation of animal models rather than gene therapy applications, but many monogenic diseases are caused by dominant mutations and could therefore be targeted in this way in the future, especially given the new ease and speed with which targeted nucleases can be designed using Cas9.

1.4 Autophagy background

The second half of this project developing improved GSD Ia therapies focuses on the manipulation of mammalian autophagy. Autophagy, specifically macroautophagy, is the process by which a eukaryotic cell engulfs portions of its cellular contents that need to be broken down either because they are defective and hinder the cell's functions, or because the cell requires the components and energy obtained by breaking down its own proteins, lipids, or carbohydrates for other purposes.

Autophagy takes place in multiple stages. First, autophagy \textit{initiation} entails the formation of the phagophore assembly site, mediated by the ULK complex in
mammals—a key complex used in autophagy studies that can induce autophagy simply by overexpression of its namesake protein, ULK1.\(^5\) Initiation is essentially just the localization and activation of the ULK complex, which activates the next critical component, the class III PI3K complex. This complex activates the **nucleation** stage by producing a local pool of phosphatidylinositol 3-phosphate specific to autophagosomes. This pool is used to form the membranes destined to become the autophagosome. The newly-formed membrane can then undergo **expansion**, during which the ATG12–ATG5–ATG16 complex recruits LC3 and attaches phosphatidylethanolamine to form LC3-II, the active form of LC3 that contributes to the growth and complete formation of mature autophagosomes. During expansion, the autophagosomal compartment closes around components destined for degradation and acidifies.\(^9^4\) Finally, the autophagosome fuses with lysosomes and components of the endocytic pathway, forming the autolysosome, exposing the engulfed cellular components to the hydrolases and other breakdown components present in the lysosomes. The autophagocytosed organelles and macromolecules are thus broken down to release components for use in other metabolic pathways such as metabolite synthesis and energy production.\(^9^5\)

In the liver, macroautophagy serves a specialized purpose in addition to those previously mentioned. Since the liver is a major storehouse for both building block and energy-source nutrients (e.g., glycogen and lipid vacuoles), it uses macroautophagy as a means of breaking down its stored resources in order
to generate easily-secretable molecules that it can send into the bloodstream for uptake by other tissues when these nutrients are required. One major role the liver plays in this capacity is the maintenance of normoglycemia, which is accomplished as discussed earlier by the breakdown of glycogen into glucose, which is sent directly into the bloodstream. Much of this activity is accomplished through the glycolytic pathway ending with G6Pase hydrolyzing G6P, but hepatocytes also use lipolysis and autophagy to control blood sugar.\textsuperscript{96} Additionally, lysosomal acid alpha-glucosidase (GAA) can break down glycogen in the liver that can directly yield phosphate-free glucose for bloodstream secretion to control hypoglycemia.\textsuperscript{97} Since autophagy provides lysosomes their degradable contents, and is also involved in lipid breakdown for energy production, autophagy is a key player in the liver's role as an energy depot. As such, autophagy may be targetable as a means of controlling hepatic storage defects and blood sugar regulation.

Autophagy is regulated by multiple cellular conditions (\textbf{Figure 2}), primarily nutrient requirements, and stress, both oxidative and due to pathogen invasion.\textsuperscript{98-100} Autophagy is largely regulated by mTORC1, which represses autophagy and directs the cell toward nutrient conservation. mTORC1 phosphorylates ULK1 and ATG14L, key components of the autophagy pathway, inactivating them, under homeostatic conditions. However, when mTORC is inactivated, it allows ULK1 and ATG14L to be dephosphorylated, which activates them and allows them to progress autophagy as discussed above.\textsuperscript{101} mTORC1 is in turn regulated by a
series of intricate pathways with potential for drug therapy targeting.

Figure 2: Regulation of autophagy by mTORC1.

Activation of mTORC1 by nutrients and growth factors leads to inhibition of autophagy through the phosphorylation of multiple autophagy-related proteins, such as ULK1, ATG13, AMBRA1, and ATG14L, which promote autophagy initiation and autophagosome nucleation. mTORC1 also phosphorylates and prevents nuclear localization of the transcription factor TFEB, a master regulator of lysosomal and autophagy gene expression. Proper lysosome function is essential for autophagy completion.

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Liver autophagy is controlled largely the same as autophagy generally is throughout the body, but metabolic regulation plays a larger role in hepatocytes than in other tissues since the liver must maintain organismal energy homeostasis rather than cell-level-only maintenance. 99,102 In particular, amino acid starvation, glucagon, and insulin stimulate autophagy by signaling various metabolic states that autophagy is capable of balancing. Furthermore, the
presence of G6P itself can downregulate autophagy by inhibiting the downregulation of mTORC1 by hexokinase-II.\textsuperscript{103} Since G6P is typically only present at high levels while a cell is in the fed state, this feedback is normally important for preventing autophagy from staying active when the cell is in a fed state so that it does not continue breaking down nutrients, producing unnecessary energy. Rather, in the fed state, the desirable push is for storage of energy-containing compounds—that is, G6P being converted into glycogen and lipids—rather than autophagy creating more G6P from glycogen and lipids.

Of note, GSD Ia livers have high concentrations of G6P, which deregulates mTORC1 inhibition, allowing mTORC1 to inhibit autophagy. Essentially, GSD Ia hepatocytes behave as if they are always in a fed signaling state, even when the actual metabolic state that the liver should be responding to is a starved state of hypoglycemia in the bloodstream. Due to this, GSD Ia hepatocytes are predicted to be incapable of properly responding to hypoglycemia through autophagic pathways and have chronically downregulated autophagy. \textbf{Figure 3} illustrates this relationship. Therefore, potential therapeutics for GSD Ia may exist in the form of autophagy-inducing drugs. The most well-known of these is rapamycin, but due to its wide array of side effects, it is not ideal for long-term treatment.
Figure 3: Why GSD Ia reduces autophagy by simulating a chronic “fed” state for cells.

(A) In normal hepatocytes, the fed state results in high G6P as cells are actively processing glucose. The high G6P state leads to mTORC1 activation and the inhibition of autophagy, as a means to induce energy storage, e.g. lipogenesis. In the fasted state, hepatocytes are relied upon for energy production, and thus the low G6P state of fasting inhibits mTORC1, which in turn enables autophagy to proceed while inhibiting energy storage pathways. (B) GSD Ia hepatocytes show a similar metabolic state when fed, but during fasting the inability to efficiently process G6P leads to G6P accumulation, which causes cells to exist in a chronically fed state even when starved. Thus GSD Ia hepatocytes exhibit chronically reduced autophagy and increased lipid storage.
Autophagy is a potential target for treatment of many diseases, generally those in which a cellular abnormality is caused by the accumulation of excessive or undesirable substances, such as lipid accumulation, misfolded protein aggregation, or imbalanced neurotransmitters.\textsuperscript{105} One example is non-alcoholic fatty liver disease (NAFLD), in which the liver accumulates excess lipid deposits for a variety of reasons, typically tied to obesity and diabetes.\textsuperscript{106} Chronic NAFLD can lead to cirrhosis, reducing overall organismal liver function. Hepatocyte lipid accumulation is tied to autophagy (increased with autophagy inhibition and decreased with autophagy activation),\textsuperscript{96} and since lipid accumulation is the defining characteristic of NAFLD, research is now being undertaken on ways autophagy could be exploited to benefit NAFLD.\textsuperscript{107,108} Notably, it has been shown that autophagy-inducing drugs can alleviate some of the histopathological NAFLD symptoms in mice, leading to potential future treatments of the growing epidemic.\textsuperscript{107}

Another disease area where autophagy has potential to be exploited for treatment is in neurological disorders, particularly those that occur due to accumulation of aggregate proteins in neurons. Autophagy is known to be impaired in the proteopathies Alzheimer's diseases, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and prion diseases.\textsuperscript{109} Protein aggregate degradation has been accomplished by inducing autophagy using many different drugs, including rapamycin, trehalose, rilmenidine, lithium,
valproate, carbamazepine,\textsuperscript{110} methylene blue, sodium butyrate, suberoylanilide hydroxamic acid,\textsuperscript{111} fluphenazine, and methotrimeprazine.\textsuperscript{112} It has come to light that autophagy downregulation plays a role in many diseases, protein aggregate neuropathies being linked only recently—the discovery of autophagosome accumulation in these diseases was originally interpreted to mean that increased autophagy led to symptoms. The growth of this research has paved a way to adapt autophagy-inducing drugs to other diseases whose symptoms may be ameliorated by enhancing autophagy. In chapter 3, I discuss my own work adapting many such pro-autophagic drug therapies to GSD Ia.

1.5 Background summary

The first critical background point is that GSD Ia is presently incurable, and modern treatments are subpar. They require lifelong dietary management, and despite the survival gained by following the therapy, patients still go on to develop hepatic adenomas, hepatocellular carcinoma, and progressive renal failure. Part of the treatment requires consumption of uncooked cornstarch frequently throughout the night, affecting patients’ sleep patterns. The alternative is to use nasogastric tube feedings, but a malfunction could lead to overnight hypoglycemia that may prove lethal. GSD Ia patients can be kept alive, but at great cost to their quality of life.

Our approach to overcoming the limitations of present GSD Ia treatments is using gene therapy in which AAV vectors deliver a functional \( G6PC \) gene to the hepatocytes of GSD Ia patients. The work is currently being done in mouse and
canine GSD Ia models, and has proven highly effective in reducing hypoglycemia and lipidemia in these animals. Such therapy still has a way to go before reaching human patients.

The greatest limitation we have experienced in this approach is the limited duration during which it has an effect on animals. Both of our animal models have shown that the effects diminish over time in a dose-dependent manner. We have had success repeatedly dosing canines with AAVs using different serotypes each time to avoid the adaptive immune response that would otherwise prevent gene transduction. This provides a way to potentially extend the treatment for several years, but since only a limited number of AAV serotypes efficiently transduce the liver, this approach could not treat human patients indefinitely. As such, we seek a new approach to GSD Ia gene therapy that can provide indefinite, lifelong benefits.

The approach we have pursued and continue to do so, is that of genome editing to insert G6PC into the subject's genome so that the treatment may persist for the lifetime of the individual. Genome editing entails modification of an organism's genome, most commonly via gene deletion or gene correction, in which a target gene is mutated to become nonfunctional or deleted entirely, or in which a mutated gene is restored to a wildtype state, respectively. Gene deletions are typically accomplished using nucleases to cleave genomic DNA so the DNA is repaired imperfectly, leading to a nonfunctional gene. Gene corrections typically involve the use of a homologous transgene segment that
can provide a template for the organism to revise its mutant gene, and may or may not make use of a variety of nucleases to induce DSB repair at the target site in order to increase the likelihood of modification through homology-directed repair.

Alongside genome editing, we have also pursued a novel approach to correcting GSD Ia symptoms that bypasses the need for functional G6Pase entirely, enabling multi-pronged simultaneous approaches to treatment in the future. Autophagy dysregulation plays a role in many diseases, and recent studies have found that it can be modulated therapeutically in many cases. In particular, patients with NAFLD were recently shown to have reduced autophagy, contributing to lipid accumulation in the liver. Autophagy activation was then shown to reduce the lipid accumulation characteristic of NAFLD, indicating its potential for therapeutic exploitation. NAFLD shares many similarities with GSD Ia in terms of symptoms and histopathology, so we sought to investigate whether GSD Ia also shared with it reduced autophagy and the potential for autophagy induction as a therapeutic approach.

To further summarize, current GSD Ia treatments are inadequate because they severely impact patients’ quality of life and fail to remedy long-term complications. AAV gene therapies have shown promise for GSD Ia, but are still imperfect, largely due to their temporary nature and inability to be administered indefinitely. The combination of genome editing and AAV gene therapy could provide long-term treatment for GSD Ia, and recent work on autophagy
manipulation has shown that it likewise may have applications for reducing the long-term symptoms of the disease. There exists further potential to combine these therapies to create treatment regimens more successful than any individual treatments to date.
2. Materials and Methods

2.1 Materials

PCR primers were purchased from Sigma Chemical Co. and Eton Bioscience. ExTaq kits were purchased from Takara Clontech. Taq PCR kits, SYBR green qPCR kits, and PCR Cloning kits were purchased from Qiagen. XL-1 Blue Supercompetent cells were purchased from Agilent Technologies. Wizard Genomic DNA Purification Kit was purchased from Promega. DNA-free DNA Removal Kit was purchased from Ambion. Surveyor mutation detection kit was purchased from Transgenomic. Trizol was purchased from Invitrogen. RevertAid Reverse Transcriptase, phosphorus reagent, and G418 were purchased from Thermo Fisher Scientific. Amyloglucosidase, ALT kits, and GGT kits were purchased from Sigma Chemical Co. OCT was purchased from Sakura Finetek USA, Inc.

α-Lipoic acid, α-Tocopherol, Carbamazepine, Lithium Chloride, Metformin, Methylene Blue, Mifepristone, Trehalose, Verapamil, Resveratrol, DMSO, and Oil Red O reagent were purchased from Sigma Chemical Co. Rapamycin was manufactured by and purchased through the DLAR pharmacy. Ethanol was manufactured by Decon Labs, Inc. and purchased from VWR International. Rapamune was manufactured by Pfizer and purchased through the DLAR pharmacy. Bezafibrate was manufactured by Labochim, provided by Roivant Sciences, Ltd., and purchased from Eastwood Global Trade Inc..

Western blot anti-LC3B antibody was purchased from Cell Signaling
Technology. Glutaraldehyde, paraformaldehyde, Oil Red O, dexamethasone, fetal bovine serum, glucose, and DAPI were purchased from Sigma-Aldrich. Infinity glucose reagent and Pierce BCA Assay Kit were from Thermo Scientific. DMEM, DMEM/F12 1:1, Insulin-Transferin-Selenium (ITS), HBSS, lysotracker red, Penicillin-Streptomycin, l-glutamine, RNAiMax, Bodipy 493/503, sodium pyruvate, AlexaFluor conjugated secondary antibodies recognizing rabbit IgG and siRNAs targeting mouse *G6pc* and human *G6PC* were purchased from Life Technologies. DMEM without glucose, glutamine, or sodium pyruvate was purchased from Corning Life Sciences. Vectashield mounting media was from Vector Laboratories. The triglyceride assay kit and Glucose-6- phosphate assay kit were purchased from Cayman Chemical. Antibodies recognizing LC3, α-Tubulin, phosphorylated p70s6k, total p70s6k, ATG5, and Beclin1 were purchased from Cell Signalling Technologies. Antibodies recognizing G6PC, and LC3 were purchased from Abcam. Antibodies recognizing β-actin as well as HRP conjugated secondary antibodies recognizing mouse and rabbit IgG were purchased from SantaCruz Biotechnologies and Sigma Chemical Co. Hematoxylin and PAS staining kit were from Merck Millipore.

### 2.2 Genome editing methods

#### 2.2.1 Preparation of AAV vectors

The AAV vector plasmid, pAAV-rDG6P, contained the vector genome comprised of a terminal repeat (TR) at each end flanking a transgene comprised of the human G6Pase minimal promoter to drive a human G6Pase cDNA
followed by a human growth hormone polyadenylation signal, which was flanked by sequences from the mouse 28S ribosomal DNA gene. The G6Pase-encoding transgene was previously described.\textsuperscript{113} The AAV vector plasmid, pAAV-L1SVneo, contained the vector genome comprised the SV40 early promoter driving a neomycin/G418 resistance gene, which was flanked by ~1300bp of human LINE1 sequence 5’, and ~1400bp of human LINE1 sequence 3’ of the neo/G418 resistance gene, all flanked by a terminal repeat (TR) at each end.

The AAV vector plasmid, pAAV-RoG6P, contained the vector genome comprised of a TR at each end flanking a transgene comprised of the human G6Pase minimal promoter to drive a human G6Pase cDNA followed by a human growth hormone polyadenylation signal, which was flanked by sequences from exon 1 of the mouse ROSA26 locus.\textsuperscript{114} The G6Pase-encoding transgene was previously described.\textsuperscript{113} The AAV vector plasmid, pAAV-ZFN, contained the transgene for the two subunits of the ROSA26-targeting “R4L6 eZFN”\textsuperscript{79} separated by a T2A self-cleavage peptide and expressed from the LSP and flanked by ITRs. The LSP in AAV2/8-ZFN and AAV2/9-ZFN contained a thyroid hormone-binding globulin promoter sequence downstream from 2 copies of an α1-microglobulin/bikunin enhancer sequence. The LSP previously achieved long-term efficacy in hemophilia B mice within an AAV vector encoding coagulation factor IX.\textsuperscript{115} The AAV vector plasmid, pAAV-RoGFP, resembled pAAV-RoG6P except it contained eGFP expressed by the same LSP used for pAAV-ZFN, instead of a human G6Pase promoter driving G6Pase expression. The AAV
vectors were pseudotyped with AAV capsid proteins as described (packaging plasmids courtesy of Dr. James M. Wilson, University of Pennsylvania, Philadelphia, PA), and the helper plasmid was pAdHelper (Stratagene).113

2.2.2 AAV vector administration and eGFP quantification in wild type and carrier mice

Wild type and carrier mice used for the eGFP experiment were bred using heterozygous G6pc+-/ mice. They were not given subcutaneous dextrose, and vector administration was intraperitoneal (10uL/g volume of AAV2/8-ZFN for 1E+13 VP/kg, and 5uL/g volume of AAV2/8-RoGFP for 1E+13 VP/kg), which has been shown to have comparable transduction to veinous routes.116

Mouse livers were collected at 1 or 6 months of age and fixed in 4% paraformaldehyde overnight. Tissue was then transferred to 30% sucrose for 2 days prior to being flash-frozen in OCT and stored at -80°C. Frozen 30 µm sections were imaged for eGFP fluorescence on a Zeiss LSM 510 inverted confocal microscope at 10x magnification and eGFP positive cells were quantified by counting bright cells from two representative sections per mouse.

2.2.3 AAV vector administration to G6pc-/- mice

Carrier G6Pase (+/-) mice were housed in the Duke Vivarium and bred to produce homozygous, affected G6pc-/- offspring. Affected genotype was confirmed by PCR analysis of tail DNA with primers within and flanking the neo gene insertion in the G6Pase gene as described.117 Heterozygous G6pc+/- mice were pooled with homozygous wildtype mice throughout the experiments, as G6pc+/- heterozygotes are biochemically equivalent to wildtype mice and are
routinely treated as such in the literature. G6pc-/- mice were injected with vectors via the retro-orbital sinus at 12 +/- 1 days of age without regard to sex, and both males and females were included in all groups. Injection was performed following isoflurane anesthesia with a 28 gauge insulin syringe (10 ul/g volume; 2E+13 VP/kg each vector for mice later collected at 8 months of age; 1.3E+13 VP/kg AAV2/9-RoG6P and 4.8E+12 VP/kg AAV2/9-ZFN for mice later collected at 3 months of age), and hemostasis was achieved by brief manual pressure. Daily injection of 0.1 to 0.2 mL 10% dextrose subcutaneously was initiated at 3 days of age and continued for 2-3 weeks. Mice used in the 3-month time course were given such dextrose injections twice-daily for 3-4 weeks. Mice were fasted periodically for 2 hours beginning at 10 am, or for 8 hours beginning at 8 am. All mouse procedures were done in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines.

2.2.4 Quantification of DNA repair at the ROSA26 locus in the liver

Liver DNA was extracted using the Wizard Genomic DNA Purification Kit. The ROSA26 locus was PCR amplified by Takara ExTaq with the following reagents: 2.5uL ExTaq buffer; 2uL 2.5mM dNTP mix; 1uL 10uM primer SrvF1 (5'-AAGGGAGCTGCAGTGGAGTA-3')79; 1uL 10uM primer SrvR1 (5'-GCGGGAGAAATGGATATGAA-3')79; 17.3 uL water, 1uL (100ng) genomic DNA; 0.2uL HotStart ExTaq polymerase. Cycling conditions were: 20 cycles of melting at 98° for 10s, annealing at temperatures reduced each cycle by 0.5° from 70-60.5° for 10s, extension at 72° for 30s; then 20 cycles of melting at 98° for 10s,
annealing at 60° for 10s, extension at 72° for 30s; and holding at 4°. First-round PCR products were diluted 1:100 and 1uL used in a nested reaction with the same conditions except primers were SrvFnst (5’-GGGAGGTGTGGGAGGTTT-3’) and SrvRnst (5’-TGGCCACTCGTTTAACCTC-3’). Second-round PCR products were self-hybridized by incubation in a thermocycler with the following conditions at a -0.1°/s ramp rate: 95° for 3 minutes; 85° for 20s; 75° for 20s; 65° for 20s; 55° for 20s; 45° for 20s; 35° for 20s; 25° for 20s; hold at 4°. 17.8uL of the hybridized second-round products were then incubated with 2.2uL of 150mM MgCl₂, 1 uL of Surveyor Nuclease, and 1uL of Surveyor Enhancer S at 42° for 1 hour prior to loading on a 10% PAGE-TBE gel. The gel was stained with ethidium bromide and analyzed with densitometry to quantify the prevalence of NHEJ DNA repair at the ROSA26 locus.

2.2.5 Identification of transgene/mouse-genome junctions

Genomic DNA was extracted from mouse liver as described above. The first round of PCR was set up as follows: 500ng genomic DNA; 2.5uL of 10x standard Taq buffer with 15mM MgCl₂; 1uL of 25mM MgCl₂; 3uL of 2.5mM dNTP mix; Q-Solution; 1.25uL of 10uM Primer 1 (5’-GTAATCAATACCATGTGGCTC-3’); 1.25uL of 10uM Primer 2 (5’-TCGAGCTGGTCTTCTACGTC-3’); 0.25uL Taq; and water to 25uL. Cycling conditions were: 95° for 3 minutes; then 35 cycles of 95° for 30s, 58.1° for 30s, 72° for 2m20s; then 72° for 5 minutes and hold at 4°. 2uL of a 1:200 dilution of the first-round PCR was used in the second round, which was the same as the first round save that extra MgCl₂ was not added, the primers
were Primer 3 (5’-GACATCCACCTGGAAACCATT-3’) and Primer 4 (5’- CGTCAGTGTCATCCCCTACT-3’), and annealing was done at 54.1° instead of 58.1°. Second-round PCR products were subcloned using a PCR cloning kit (Qiagen) per the manufacturer’s instructions. Resulting plasmids were grown in XL1-Blue Supercompetent Cells and Sanger sequenced with the in-house provided T7 Promoter and SP6 primers from Eton Bioscience.

2.2.6 Quantification of vector DNA in the liver
Quantitative realtime PCR was performed using SYBR green in a Roche LightCycler 480II following the manufacturer’s instructions. Gene-specific primers for the human G6PC promoter (sense 5’- CAAAGATCAGGGCTGGGTTGA-3’, and antisense 5’- CTTGGTGTTGATTGCTCTGCT-3’), and for mouse β-actin (sense 5’-AGAGGGAAATCGTGCGTGAC-3’ and antisense 5’- CAATAGTGATGACCTGGCCGT-3’) were used for each reaction. Plasmid DNA corresponding to 0.01 copy to 100 copies of human G6PC gene (in 500 ng genomic DNA) was used in a standard curve. To determine the vector genome copy number, the Cp values of samples were compared to the standard curve. Cycling conditions were 5 minutes at 95°C, followed by 45 cycles of 95°C for 10s, 60°C for 10s, and 72°C for 20s followed by acquisition.

2.2.7 Evaluation of biochemical correction
Enzyme analysis was performed as previously described. Briefly, tissues were flash-frozen and stored at -80°C. Glycogen content was measured by complete digestion of polysaccharide using amyloglucosidase. The structure of
the polysaccharide was inferred by using phosphorylase free of the debranching enzyme to measure the yield of glucose-1-phosphate. Specific G6Pase activity was measured by using glucose-6-phosphate as substrate after subtraction of nonspecific phosphatase activity as estimated by β-glycerophosphate. Glucose was analyzed with a Kodak Biolyzer (Eastman Kodak Company) according to the manufacturer’s recommendations.

2.2.8 Histochemical staining for G6Pase activity in the liver
G6Pase was detected qualitatively in frozen sections (6 μm) mouse liver by an optimized cerium-diaminobenzidine method as described\(^\text{120}\). G6Pase-expressing cells were counted from ten 20x fields were counted for each mouse and averaged, then counts were averaged across treatment groups. Counting was performed blinded to sample ID.

2.2.9 Quantification of human G6Pase mRNA expression in the liver
Quantitative realtime PCR was performed on cDNA reverse transcribed from total RNA collected from mouse liver tissue. RNA purification was done using Trizol (Invitrogen, Waltham, MA) following the manufacturer’s instructions, and RNA was dissolved in 25uL of water. 10uL of RNA was treated to remove DNA using the DNA-free DNA Removal Kit in half-reactions of the manufacturer’s routine DNase treatment instructions, with an extended DNase treatment time of 1 hour. RevertAid Reverse Transcriptase was used with random hexamers to generate cDNA from 5uL of DNA-free RNA, per the manufacturer’s instructions. Quantitative realtime PCR was performed using SYBR green in a Roche
LightCycler 480II following the manufacturer’s instructions. Input cDNA was 1uL of cDNA diluted 1:10 with water. Gene-specific primers for human G6PC cDNA (sense 5’- CTGTTCAGCTTCGCCATC-3’, and antisense 5’- GGGAGGCTACAATAGAGCT-3’), and for mouse β-actin (sense 5’- AGAGGGAAATCGTGCGTGAC-3’ and antisense 5’- CAATAGTGATGACCTGGCCGT-3’) were used for each reaction. Cycling conditions were 5 minutes at 95°C, followed by 45 cycles of 95°C for 10s, 60°C for 10s, and 72°C for 20s followed by acquisition. Relative expression was calculated using the ΔΔCt method.\textsuperscript{121}

2.2.10 Statistical analysis

The heteroscedastic two-tailed T-test was used to compare groups unless otherwise noted. Survival analysis included production of Kaplan-Meier curves. P values of less than 0.05 were considered significant.

2.3 Autophagy manipulation methods

2.3.1 Cell culture

AML-12 (CRL-2254) cells were purchased from ATCC and maintained at 37 °C in DMEM/F12 1:1 containing 10% FBS, 1x ITS, 10 nM dexamethasone and 1x penicillin/streptomycin in a 5% CO2 atmosphere. For siRNA transfection, cells were transfected using RNAiMAX, following manufacturer's reverse-transfection protocol. Unless otherwise noted, assays were performed 96 hrs after siRNA transfection.
2.3.2. Western blotting

With the exception of the drug screening LC3 Western assay, cultured cells were scraped and lysed in mammalian lysis buffer (SigmaAldrich) and tissues were homogenized by MagNA Lyser beads (Roche) in the same buffer, containing protease and phosphatase inhibitors. Concentration of protein was determined by the BCA Kit (Bio-Rad). Protein was stored at -80 °C. Immediately prior to western blotting, Laemmli sample buffer was added to the samples (250 mmol/l Tris, pH 7.4, 2% w/v SDS, 25% v/v glycerol, 10% v/v 2-mercaptoethanol, and 0.01% w/v bromophenol blue), and the samples were then heated to 105 °C for 5 minutes, kept at 4 °C for 10 minutes, and ran on an SDS-polyacrylamide gel. For the drug screening Western blots, cells were scraped directly into 2x SDS-PAGE loading buffer (100 mM Tris-Cl [pH 6.8], 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol), boiled in 1.5mL tubes for 10 minutes, and loaded on a 20% SDS-PAGE gel. Proteins were next transferred to a polyvinylidine difluoride membrane (Bio-Rad) in transfer buffer with 25 mmol/l Tris, pH 8.8, 192 mmol/l glycine, and 10% v/v methanol. All washing, blocking and antibody solutions were prepared in PBS with 0.1% Tween-20 (PBST). Membranes were blocked for one hour in 5% milk (1% BSA for drug screen blots and bezafibrate-treated mice), followed by incubation overnight at 4 °C with primary antibodies in 1% bovine serum albumin. Membranes were washed thrice, followed by secondary antibody incubation at room temperature for 1 hr in 1% bovine serum albumin, followed by 3 more washes in PBST, and placed in PBS. Blots were probed using an enhanced chemiluminescence system (GE
Healthcare) on a GelDoc imager (Bio-Rad), except in the case of drug screen and bezafibrate treatment Westerns, which were exposed on X-ray film (Kodak). Densitometry was performed using ImageJ software (NIH).

2.3.3 Oil Red O staining

At day 0, AML-12 cells were transfected with siRNA targeting G6pc. 16 hours before assay (after 72 hours of cell growth), cells were switched to DMEM containing l-glutamine but no pyruvate or glucose and only 5% FBS (referred to henceforth as "ketogenic media"). On the day of the assay, Oil Red O working solution was prepared by diluting Oil Red O stock solution (.35 g Oil Red O in 100 mL isopropanol) 3:2 with dH₂O. All assay steps were performed at room temperature. At time of assay, cells were washed once in PBS, fixed in 10% formaldehyde in dH₂O for 15 min, the solution taken off, and then placed in the same solution for 1 hour. Cells were washed twice with dH₂O, and then incubated for 5 min in 60% isopropanol. Cells were dried, and then incubated for 10 min with Oil Red O working solution. Cells were washed 4 times with dH₂O, and images acquired. Cells were again dried, and for Oil Red O stain quantification, Oil Red O was eluted with 100% isopropanol. The optical density at 500 nm of 100 μL of the eluted Oil Red O solution was measured, and results were expressed as absorbance relative to control cells.

2.3.4 Mouse experiments

Carrier G6pc+/- mice were housed in the Duke Vivarium and bred to produce homozygous, affected G6pc-/- offspring. Affected (-/-) genotype was
confirmed by PCR analysis of tail DNA with primers within and flanking the neo
gene insertion in the G6Pase gene as previously described\textsuperscript{117}. Daily injection of
0.1 to 0.2 mL 10% dextrose subcutaneously was initiated at 3 days of age and
continued for 2-3 weeks. Vector-injected $G_{6}pc^{-/-}$ mice were injected
intravenously with AAV2/9-G6Pase (1E+13 VP/kg) vectors via the retroorbital
sinus at 12 ± 1 days of age with outcomes previously described\textsuperscript{120}. Rapamycin-
injected $G_{6}pc^{-/-}$ mice were injected intraperitoneally with rapamycin (Rapamune
1 mg tablet, Pfizer Inc.) suspended in a 1:10 DMSO:PBS solution (4 mg/kg) daily
for 6 days starting at day 4 (n=4) and untreated 4 day old $G_{6}pc^{-/-}$ mice received
vehicle of 1:10 DMSO:PBS only (n=5). All $G_{6}pc^{-/-}$ mice continued to receive daily
dextrose injections during this time. Mice were sacrificed and tissues collected at
10 days of age.

Bezafibrate-injected $G_{6}pc^{-/-}$ mice were injected intraperitoneally with
bezafibrate (powder, Labochim) suspended in a 1:10 DMSO:PBS solution (25
mg/kg) daily for 3 days starting on day 4 (n=4) and “untreated” 4 day old $G_{6}pc^{-/-}$
mice received vehicle of 1:10 DMSO:PBS only (n=3). WT mice were also given
25 mg/kg bezafibrate daily for 3 days starting on day 4 (n=3). All $G_{6}pc^{-/-}$ mice
continued to receive daily dextrose injections during this time. Mice were
sacrificed and tissues collected at 7 days of age.

\textbf{2.3.5 Canine experiments}

Canine weights were measured weekly beginning at birth prior to vector
administration. All animals were monitored every three to four hours for at least
the first week of life to ensure that they were gaining weight and nursing appropriately. If they were not nursing, bottle or tube feeding was employed as well as dextrose therapy to control hypoglycemia. GSD Ia affected canines were identified by methods previously described and administered AAV-G6Pase vector at 2-3 days of age as described. Rapamycin (Rapamune oral solution, Pfizer Inc.) was administered orally to 4 adult canines from this study to induce autophagy (1 mg/kg/day for 1 week).

2.3.6 Triglyceride measurement
Triglycerides were measured using Cayman Chemical's triglyceride assay kit according to the manufacturer's instructions. Protein concentration was determined in an un-assayed portion of tissue lysate, for normalization.

2.3.7 Glucose-6-phosphate measurement
AML-12 cells were treated with siG6PC or negative control siRNA, and grown for 96 hours, reaching 100% confluency at this time. After 96 hours, cells were rinsed with PBS, and glucose-6-phosphate was measured using Cayman's glucose-6-phosphate fluorometric kit according to the manufacturer's instructions.

2.3.8 Glycogen measurement
Enzyme analysis was performed as previously described. Briefly, tissues were flash-frozen and stored at -80°C. Glycogen content was measured by complete digestion of polysaccharide using amyloglucosidase. The structure of the polysaccharide was inferred by using phosphorylase free of the debranching enzyme to measure the yield of glucose-1-phosphate.
2.3.9 Histology
Formalin fixed liver sections from G6pc-/- and G6pc+/+ mice treated with DMSO vehicle or rapamycin were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissue processing and staining was performed by the Duke-NUS histopathology core. Images were acquired at 20x.

2.3.10 Periodic acid-schiff base staining of tissue sections
Paraffin-embedded tissue sections on slides were deparaffinized according to Abcam's general IHC protocol, and tissue sections stained according to manufacturer's instructions from Merck PAS stain kit. Images were acquired at 20x.

2.3.11 Liver size determination
Abdominal radiographs were performed 1 week before and after treatment with rapamycin of GSD Ia canines using an InnoVet select E7242x x-ray machine (Summit Industry), and processed using an IDEXX-CR140 Model 145 digital imaging system (IDEXX Laboratories Inc.). Right lateral radiographs were analyzed using ClearCanvas Workstation 2.0. Ventral measurement of liver size was approximated from the ventral aspect of the diaphragm to the caudal-most aspect of the ventral aspect of the liver, and dorsal measurement was approximated from the dorsal aspect of the diaphragm to the caudal most aspect of the dorsal silhouette of the liver.

2.3.12 Dog liver enzyme measurement
Blood was collected from dogs during routine health exams 2 months prior to
rapamycin treatment and GGT and ALT analyzed using a Heska Dri-Chem 7000 at the Duke University Division of Laboratory Animal Resources Veterinary Diagnostics Lab. GGT and ALT was measured from blood drawn 1 week after rapamycin treatment and analyzed at Antech Diagnostics Laboratories (Cary, NC, USA).

2.3.13 Immunofluorescence
Prior to treatment, cells were seeded on glass coverslips. Following treatment, cells were washed in PBS, fixed for 15 minutes in 4% formaldehyde, and washed again. Cells were then permeabilized in 100% methanol at -20 °C for 10 minutes, washed, and blocked in TBST containing 1% normal goat serum for 1 hour. Cells were then incubated with primary antibody overnight at 4 °C, washed thrice with PBS, and then incubated for 2 hours at room temperature with Alexa Fluor secondary antibodies (Invitrogen). Cells were washed once, and then treated with DAPI at 1:3000 dilution in PBS for 15 minutes, followed by three washes with PBS. For experiments involving lipid staining, Bodipy 493/503 at a 1:1000 dilution was also added with DAPI. Coverslips were mounted using Vectashield mounting media (Invitrogen), and visualized using an LSM710 Carl Zeiss confocal microscope.

2.3.14 Statistical analysis
Cell culture experiments were performed in triplicates and repeated three independent times using matched controls except for the drug screen experiments, which were not performed with replicates and were performed two
independent times. Results were expressed as mean ± SEM. The heteroscedastic two-tailed T-test was used to compare groups unless otherwise noted.
3. Genome Editing Shows Potential for Enhanced Therapeutic Benefits in GSD Ia

The work in this section was performed by the Dwight Koeberl laboratory staff, with special recognition given to the Gersbach lab, particularly Pablo Perez-Pinera for creating the ROSA26-targeting ZFN and providing the plasmid encoding it. I provided animal husbandry, tissue collection, Surveyor analysis, PCR design and analysis, sequencing analysis, GFP image analysis, G6Pase activity, and glycogen content data.

3.1 Early approaches: editing at the ribosomal DNA

Therapeutic genome editing is on the cutting edge of gene therapy approaches. As such, we approached our objective, to improve the longevity of AAV-based GSD Ia gene therapy, from multiple angles before settling on the one with most promise.

Our first approach was based on early work presented by the Grompe research group that demonstrated the effectiveness of nuclease-free gene integration at the mammalian ribosomal DNA genomic region. This region of DNA allows AAV integration more readily than other sites, which helps improve the rate of successful therapeutic integration, and it is repetitious, so disruption of one or more copies by gene insertion is unlikely to cause cellular abnormalities.

We received a sample of the AAV genomic plasmid used by the Grompe group, and modified it using standard plasmid cloning techniques to contain our
established human $G6PC$ flanked by the Grompe group’s rDNA-targeting homology arms. The homology arms are included to increase the likelihood of DNA repair using homologous recombination to integrate the transgene at the homologous locus, to further improve the integration rate beyond the naturally-occurring above-random integration rate for AAV at the site. The vector was pseudotyped as AAV2/8 to produce "AAV-rDG6P" and injected in 11-day-old GSD Ia mice. Livers from these mice were collected at 6 months of age and analyzed for vector DNA integration at the target site.

11-day-old GSD Ia mice were given $3.8E+11$ VP of this vector per gram of body weight. Mice receiving this dose of vector survived beyond their untreated 2-week lifespan, indicating the vector delivered the therapeutic transgene, and the transgene was expressed at levels sufficient for therapeutic effect. The mice were sacrificed and their tissues harvested after 6 months. DNA from the livers was extracted and PCR performed using forward primers against genomic rDNA and reverse primers against the human $G6PC$ cassette in a cross-junction PCR. **Figure 4** shows that several primer combinations yield PCR products that hybridize with a $G6PC$ probe in a Southern blot. Since integration is necessary for successful PCR, this indicates that integration must have occurred to some extent, but the high level of background PCR amplification compared with positive signal called for significant refinement of the approach to confirm the events.
Multiple primer pairs were tested to optimize the assay. Each lane (1-4) represents one primer pair. Liver DNA from mice 343 and 344 was used as the PCR template. Southern blots (left) were probed with radiolabeled human G6PC cassette. The corresponding gel image is to the right of each blot. Primer combinations yielding no signal are not shown.

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To refine the approach, we looked to the many different variations of PCR designed to amplify unknown genomic DNA. As shown in figure 5A, we first used inverse PCR to amplify the junctions between the integrated transgene and the mouse genome. Inverse PCR (iPCR) works by digesting with a known restriction enzyme within the transgene, then creating conditions amenable to self-ligation to circularize the digested genome-transgene DNA junctions, which could then be sequenced using primers targeted to the transgene that would progress through the junction. However, iPCR proved to be a fairly nonspecific technique that enabled many off-target ligation and PCR products to form, and even the most optimized reactions, resulting from months of refinement, yielded very little primary species amplification (Figure 5A).
Figure 5: iPCR and GAWTS-PCR amplifies products from AAV-rDG6P-treated mice.

(A) Second round (nested) inverse PCR reactions on genomic liver DNA from two AAV-rDG6P-treated mice. Each lane is one mouse. (B) Second round (nested) GAWTS PCR reactions on genomic liver DNA from multiple AAV-rDG6P-treated mice. Each lane represents a reaction from a different mouse, under optimized conditions. “NTC” indicates a no-template control (e.g., water as the template) reaction.

We discontinued the rDNA-based integration project for a time based on new results published by another group, but returned to it when we had another technique that showed promise: genomic amplification with transcript
sequencing (GAWTS). This technique ligates a short promoter sequence onto a region of digested DNA, then uses *in vitro* transcription to generate an RNA strand copy of the digested and ligated DNA. That RNA is then reverse-transcribed and used for PCR to generate DNA including the transgene-mouse genome junction, which can be sequenced either directly or cloned into a backbone and transformed to grow adequate quantities for sequencing. This technique has improved specificity over traditional ligation-mediated PCR (LM-PCR) because the transcription reaction can only proceed on a successful ligation product, and the PCR reaction can only occur on transcribed then reverse-transcribed DNA strands that contain the transgene. We observed some amplification with the technique (*Figure 5B*), but ultimately found that the amplification was due to the presence of contaminating original DNA strands rather than the transcribed-then-reverse-transcribed specific DNA products, which ultimately led to nonspecific reactions in the case of the rDNA vector-injected mice. The large amount of off-target amplification made it impossible to obtain the strong specific reactions necessary for sequencing junctions, as seen in the smeary reaction lanes of figure 6b. We moved on from the rDNA project since much of the available data in the field indicated the treatment, relying on only ~300bp of rDNA homology, was unlikely to have worked. Our own attempts to demonstrate positive effects also proved futile up to that point, so we decided to reallocate resources to other projects with a greater likelihood of success.
Before ending the rDNA project branch entirely, we treated one canine with the vector, but failed to provide sufficient therapeutic benefit early in life, possibly due to the approach being flawed, but more likely due to a recent change in a newly-implemented vector preparation technique that unexpectedly generated non-transductive particles. Shortly thereafter we changed back to the previous vector preparation technique and left behind the ribosomal DNA-targeted gene insertion approach due to data published at that time by the Grompe research group.\textsuperscript{126,127} The single canine that was treated with the ribosomal DNA vector did survive, thanks to emergency administration of a second vector consisting of just the therapeutic $G6PC$ transgene.

The second exploratory approach we took, simultaneously with the ribosomal DNA-targeted approach, was targeting long interspersed nuclear elements (LINEs). LINEs are repeated regions of DNA in many eukaryotic genomes that each span about 6kb and comprise 20% of the human genome.\textsuperscript{128,129} A complete, active LINE will encode an RNA-binding protein and an endonuclease/retrotranscriptase complex that together serve to generate additional LINEs in the genome. In this regard, they resemble proretroviruses and behave similarly, though they are an endogenous part of the eukaryotic genome rather exogenous invaders. They do not encode critical genes, one reason the LINE family is an excellent target for gene insertion research. Since it is nonessential DNA, it can be freely disrupted without damaging important gene-encoding tracts of DNA. Furthermore, the abundance of LINE copies (500,000 in
humans, 100,000 in mice) helps increase the likelihood of integration via HDR. With so many potential sites of integration, the odds are higher that DNA damage may occur at an integration-amenable site with or without the presence of targeted nucleases, which increases the chances that HDR may integrate the therapeutic transgene.

It had previously been shown that targeting LINEs for gene integration using HDR results in a greater than 10-fold increase in genome editing.\textsuperscript{130} We sought to couple this increased targeting rate with the up-to-1,000-fold increased genome editing rate provided by AAVs over plasmids\textsuperscript{131,132} to obtain an exceptional rate of transgene integration. Specifically, we used the largest LINE family in mammals, LINE1 (L1).\textsuperscript{133}

Four different treatments of HEK293 cells evaluated the possibility of integration at LINEs: (i) transfection with pAAV-L1SVneo; (ii) transfection with pMALAPSN, the plasmid containing the SVneo and some stuffer sequence; (iii) transduction with AAV containing the L1SVneo vector genome; and (iv) transduction with the AAV vector containing the MALAPSN vector genome. All four groups were subjected to selection in G418, resistance to which is provided by the neoR gene in each of the plasmids and AAV vector genomes. These four groups would allow us to determine: (i) the integration rate enhancement of LINE sequence homology flanking a transgene; (ii) the rate of enhancement provided by using AAV delivery vehicles over non-viral, plasmid-based techniques; and (iii) the combined efficiency increase by using both the LINE homology and AAV
This experiment yielded a number of G418-resistant cell lines, which were successfully maintained for 15 days in G418 media to ensure permanent genome-level modification had occurred. The number of colonies formed by AAV transduction were quantified at several cell-plating dilution factors to determine the relative integration efficiency of each AAV treatment (Table 1). We found that the AAV-MALAPSN vector consistently generated colonies with greater frequency than did AAV-L1SVneo. This ran contrary to our expectation that the L1 homology arms would increase the likelihood of G418-resistance integration-induced colonies. The observed effect may be due to L1 genomic regions tending to be silenced, in which case there may have been many more integration events than the number of colonies produced, but many of those events were silenced by local LINE1 methylation.134
Table 1: HEK293 LINE-integration colony formation.

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<thead>
<tr>
<th>Dilution Factor</th>
<th>AAV-MALAPSN</th>
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<th>AAV-L1SVneo</th>
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<td>2.5</td>
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HEK 293 cells were transduced with AAV virions containing LINE1-flanked SVneo genomes or SVneo genomes lacking the LINE1 homology arms, at an MOI of 1000. Cells were split after 96 hours and subjected to G418 selection for 2 weeks to select for cells with permanent neo expression.

Despite failure to observe the predicted difference in integration efficiency, we went forward with another aspect of the experiment, which was to determine whether there were preferred genomic LINE sites, or sequences that increased the probability of integration at particular LINEs. This was pursued using PCR methods similar to those used for the rDNA integration analyses, and further PCR methods were added to overcome technical problems. The greatest difficulty with identifying LINE integration sites was that, since LINEs are so repetitive and long, sequencing results had to be very long; at least 3kb on the 5’ side of the integrated transgene, and 5kb on the other side, to guarantee we could identify where in the genome the LINE was located that took in the G418-resistance transgene.

Ultimately, four PCR techniques were used to analyze the modified LINE
sites in the Hek293 cell lines. In chronological order, they were: iPCR (Figure 6A); restriction-site extension PCR (RSE-PCR) (Figure 6B); modified LM-PCR (Figure 6C); and linear amplification-mediated PCR (LAM-PCR) (Figure 6D). After sufficient analysis and attempts at optimizing each method to reduce background and improve sensitivity individually, I utilized each subsequent method in the above order. iPCR was discussed previously. RSE-PCR functions as traditional LM-PCR but without a ligation step: it uses complementarity between the 3' end of the adapter to anneal the adapter to the digested genomic DNA, and the first round of PCR extends the bottom genomic DNA strand across the annealed adapter to add the primer sequence to the genomic fragment, allowing future cycles to amplify using an adapter primer specific to the added sequence. The modified LM-PCR we used was based on the work that later became the proprietary "Genome Walker" LM-PCR method. Traditional LM-PCR simply attaches a double-stranded oligonucleotide to the ends of restriction enzyme-digested genomic DNA to put a known sequence outside of the unknown genomic region, enabling PCR amplification across the unknown region of interest. This modified method ligates a full-sized top-strand adapter oligonucleotide to the 5' end of the genomic DNA, and a truncated bottom-strand oligonucleotide with a non-extensible 3' end amine modification to the 3' end. Inability to extend the 3' end ensures that amplification can only occur if the first PCR cycle using the adapter-ligated DNA successfully copies the top strand adapter sequence into the bottom strand product, so that the adapter-specific 5'
primer can pair with the augmented bottom strand and generate top-strand copies in subsequent PCR cycles.

**Figure 6:** Multiple PCR variations on LINE-targeted cell lines yield a mixture of specific and non-specific products.

(A) Second round (nested) iPCR on transfected and transduced HEK293 colonies. Each lane represents a colony used in the PCR reaction. Labels indicate the AAV vector or plasmid applied to the cells to generate G418-resistant colonies; H₂O indicates no-template control; gDNA indicates untreated HEK 293 genomic DNA as the PCR template. (B) Second round (nested) RSE-PCR using multiple types of polymerase to minimize background and increase amplification. Each lane represents PCR on a G418-resistant colony produced by AAV-L1neo transduction, except the last 2 lanes from each enzyme section, which were non-transduced HEK 293 gDNA and water as a no-template control, respectively. (C) Second round (nested) modified LM-PCR using a non-extensible adapter to reduce off-target amplification. Each lane represents a different HEK-293 clone derived either from plasmid-transfected or AAV-transduced cells. (D) LAM-PCR optimization showing both PCR stages, and similar results coming from both
positive and negative controls. AAV-L1 indicates the DNA template came from an HEK 293 clonal cell line transduced with AAV-L1SVneo. AAV-MALAPSN indicates the cell line had been transduced with AAV-MALAPSN. pL1 indicates the cells were transfected with pL1SVneo. pMALAPSN indicates the cells were transfected with pMALAPSN.

The last method that was used was the most intricate, but also the most specific, and had the track record of being capable of identifying viral insertion sites. LAM-PCR is also a type of specialized LM-PCR, which uses biotinylated oligonucleotide primers. The method entails extending vector-specific biotinylated primers for several cycles in a linear PCR—that is, the PCR lacks reverse primers—then adhering the biotin-labeled single-stranded DNA to streptavidin-coated beads, which can be collected and used as the template for exponential PCR following second-strand synthesis. The specificity of this method comes from the enrichment step that preserves only amplified DNA (that is, DNA strands with biotinylated ends, allowing them to bind to beads and be collected), removing any genomic DNA that failed to amplify from the vector-specific primer. In principle, the only DNA going into the exponential PCR is copied genomic DNA containing the vector genome, which should dramatically reduce the chances of nonspecific amplification occurring during the exponential stage. However, even this method proved not specific enough for our needs. Each of the methods yielded nonspecific PCR products that made sequencing the junctions impossible. We ultimately decided our efforts were best spent on other work until technical advancements became available in the future.

Both the rDNA- and LINE-integrating approaches have merit, though
technical limitations proved their downfall at this time. I anticipate that in the future, when whole-genome sequencing becomes more robust and capable of sequencing long segments of contiguous DNA, the complicated PCR approaches will no longer be necessary, and research using similar approaches may yield huge advances in gene therapy. But at this time, the technology is not available to properly analyze these approaches in an efficient high-throughput manner.

3.2 ROSA26 ZFN AAV administration provides therapeutic benefits in GSD Ia mice

This project was begun as a collaboration between the Koeberl lab and the Gersbach lab, who had then just published their paper describing a ZFN targeted to the mouse ROSA26 safe harbor locus. The ZFN they had developed showed strong *in vitro* activity and was small enough to be packaged in a single AAV using a 2A self-cleavage domain that allowed the genome to encode both ZFN subunits and express them off a single promoter. This would allow us to deliver a single AAV dose to generate the complete ZFN, alongside a second vector that delivered the donor transgene capable of recombination at the ROSA26 locus. It is worth noting that as we completed these experiments, other nucleases were emerging such as TALENs that were easier to create, and CRISPR/Cas9 technology that was faster to re-target. But since those nuclease technologies' main advantage is that they can be designed to target specific sequences more quickly and easily than can ZFNs, their advantage was negated by the existence of this new ZFN. Thus, we continued to pursue this approach using a ZFN rather than either of the newer technologies.
This approach uses a ZFN that cleaves near the center of the mouse locus, ROSA26. ROSA26 does not encode any essential genes and can be freely disrupted without harming the mice, making it a "safe harbor" for gene insertion. Alongside the AAV encoding the ROSA26 ZFN, dosing with an AAV delivering a gene of interest flanked by ROSA26 sequences enables homologous recombination to proceed at cleaved genomic ROSA26 sites, so the gene of interest may integrate into the genome and be expressed in all daughter cells derived from cells that undergo the event.

### 3.2.1 ROSA26 ZFN AAV administration enhances GFP retention in mice

As a pilot for this experimental design, wildtype mice were injected with AAV containing the RoGFP genome (AAV-RoGFP), with or without AAV encoding a ROSA26-targeted ZFN (AAV-ZFN). Negative control mice received PBS alone. The vector AAV-ZFN contained an expression cassette consisting of the thyroid hormone-binding globulin liver-specific promoter (LSP)\textsuperscript{115} driving high-level expression of the ROSA26-specific ZFN\textsuperscript{79} in the liver. AAV-RoGFP contained eGFP under control of the same liver-specific promoter used in AAV-ZFN, surrounded by ~850bp of ROSA26 homology sequences on each side.

We observed that at 1 month post-injection, mice receiving the RoGFP had strong expression (Figure 7a), as expected given that AAV8 efficiently transduces hepatocytes and maintains expression for 1-2 months post-injection.\textsuperscript{15} This confirmed the efficiency of our RoGFP vector at inducing GFP expression in hepatocytes. We analyzed experimental mice at 6 months post-
injection to determine whether addition of AAV-ZFN caused GFP expression to persist better than it did without ZFN activity. We found that virtually all GFP activity was lost after 6 months in mice that received AAV-RoGFP alone, whereas GFP expression was still noticeable in mice that received the dual-vector treatment (Figure 7b-c). Quantification of GFP-positive hepatocytes in each group showed a statistically significant difference, confirming that use of AAV-ZFN can enhance the longevity of donor transgene expression (Figure 8).
Figure 7: AAV-ZFN treatment increases GFP expression persistence in AAV-RoGFP-treated mouse livers.

(A) Liver section one month following administration of AAV2/8-RoGFP or PBS.
(B) Three representative liver sections 6 months following AAV2/8-RoGFP administration. (C) Three representative liver sections 6 months following AAV2/8-RoGFP and AAV2/8-ZFN administration.

Magnification 100x; bar is 100 µm. Mice received 1E+13 VP/g IP at 2 weeks of age. Liver sections are 30 µm.
Figure 8: GFP-positive cell quantification reveals an increase in GFP expression following AAV-ZFN coadministration.

Quantification of GFP+ cells per high magnification field using a Zeiss LSM 510 inverted confocal microscope; GFP+ cells counted from two representative fields of view (10× objective) of four mouse livers per each group and average number of cells calculated. Error bars: mean ± SD.

3.2.2 ZFN is active in mice following AAV delivery

Following the success of the GFP treatment in wildtype mice, we transitioned the approach to G6PC transgene delivery in GSD Ia mice. The vector AAV-RoG6P contained the cDNA for human G6PC under control of the minimal human G6PC promoter\(^{113}\) and a bovine growth hormone polyA signal, flanked on each side by ~850 bp sequences with homology to the ROSA26 target site. These vectors were initially cross-packaged with AAV8 capsid for the 8-month long time course, and AAV9 capsid for the 3-month long time course (Table 2) to accomplish high-level liver transduction.

To assess the activity of the ROSA26-targeted ZFN at its target site in vivo, 2-week-old G6pc-/− mice were injected with AAV2/8-ZFN and AAV2/8-RoG6P,
each at a dose of 2E+13 viral particles per kilogram (Table 2). Genomic DNA from the livers of these mice at 8 months of age was used to determine ZFN activity at the target site by the Surveyor assay. DNA cleavage sites are typically repaired using error-prone NHEJ, which creates indels at genomic sites where repair takes place. The rate of indel production was quantified using the Surveyor Nuclease assay. The average allele modification rate measured in knockout mice (n=4) was ~2% whereas in wild type mice (n=3) it was ~0.44% (Figure 9). This estimate does not include alleles in which targeted donor vector integration occurred, nor does it include instances of homologous recombination that used the other chromosome to repair ROSA26, producing no indel. There was no evidence of ROSA26 cleavage in mice that did not receive AAV-ZFN (n=3) (Figure 9). These experiments demonstrated that the ROSA26-targeted ZFN is active in mouse liver when delivered in vivo as an AAV2/8 vector, with slightly higher levels of allele modification in G6pc-/− mice.
### Table 2: ZFN experiment mouse treatment groups

#### Mice collected at 8 months of age

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vector</th>
<th>Dose (VP/kg, each vector)</th>
<th>Number of mice treated</th>
<th>Number surviving for analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G6Pase (-/-) KO</strong></td>
<td>AAV2/8-RoG6P</td>
<td>2E13</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>AAV2/8-RoG6P + AAV2/8-ZFN</td>
<td>2E13</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Wild type</strong></td>
<td>AAV2/8-RoG6P</td>
<td>2.00E+013</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>AAV2/8-RoG6P + AAV2/8-ZFN</td>
<td>2E13</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

#### Mice collected at 3 months of age

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vector</th>
<th>Dose (VP/kg, each vector)</th>
<th>Number of mice treated</th>
<th>Number surviving for analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G6Pase (-/-) KO</strong></td>
<td>AAV2/9-RoG6P</td>
<td>1.3E13</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>AAV2/9-RoG6P + AAV2/9-ZFN</td>
<td>1.3E13 + 4.8E12</td>
<td>13</td>
<td>11</td>
</tr>
</tbody>
</table>
Figure 9: Gene editing at the ROSA26 locus occurs specifically in mice administered AAV2/8-ZFN.

(A) The Surveyor assay demonstrated that indels indicative of non-homologous end joining occurred only in mice that received AAV2/8-ZFN. G6pc-/- knockout mice had significantly more ZFN target site-specific DNA repair events than wild-type mice. (B) Representative gel images illustrating the difference between knockout and wild-type mice. Surveyor Nuclease-digested product bands are indicated by black arrows.

“WT, UT”: untreated wild-type mouse control, representative of the control mice that did not receive AAV-ZFN. “-n”: no-Surveyor nuclease control of wild-type mouse PCR product. * indicates P<0.05. Error bars: mean ± SD.
3.2.3 Therapeutic transgene genomic integration is enhanced by homology arms and AAV-ZFN administration

This experiment's goal is to extend the persistence of a therapeutic transgene by integration into the mouse genome at a target locus. As such, we sought to confirm the presence of the G6PC transgene at the targeted ROSA26 locus in the above-mentioned 8-month-old mice injected at 2 weeks of age. Two rounds of PCR were required to amplify junctions between the ROSA26 locus and human G6PC transgene from the AAV2/8-RoG6P vector (Figure 10). We confirmed the identity of the anticipated junction by subcloning the PCR products and Sanger sequencing. The predicted junction was detected in 7 of 10 mice treated with both AAV2/8-RoG6P and AAV2/8-ZFN vectors. Only one of 8 non-ZFN-treated mice yielded significant PCR amplification, but the yield was too low to sequence. Assuming that this single mouse is a true nuclease-independent targeted integration event, perhaps a result of low level ZFN-independent homologous recombination, there is still a significant difference between the two groups with p<0.03 (Fisher’s 2-tailed test). One double-vector-treated mouse that contained a homology-directed repair junction also contained a separate NHEJ integration event consisting of the entire donor vector at the ROSA26 site. This amplicon, larger than that of HR, is indicated by the asterisk in figure 10.
Figure 10: Vector transgenes are integrated into the ROSA26 locus.

G6pc−/− knockout and wild type mice were treated with both AAV2/8-ZFN and AAV2/8-RoG6P or only AAV2/8-RoG6P prior to analysis at 8 months of age. (A) Illustration of the predicted integration structure following homology-directed repair between the mouse genomic ROSA26 cleavage site and the AAV2/8-RoG6P viral genome donor homology arms. Primer locations are denoted P1-P4. (B) Representative gel of PCR products from all mice following the nested round of PCR. Predicted product size from an HDR event is 1335 bp. Ladder is 1 kb Plus (Invitrogen). White arrow indicates the 1kb position. “PBS”: PCR reaction was run on DNA from an affected mouse injected with PBS instead of vector and collected at 2 weeks of age. “NTC”: No template control PCR reaction, run using water instead of DNA as template. Asterisk indicates the amplicon whose sequence demonstrated an NHEJ event.

To determine whether integration had the desired effect of increasing transgene longevity, hepatic transgene levels in 8-month-old knockout mice were quantified by qPCR. Mouse samples were compared to a standard curve as
previously described\textsuperscript{120} to calculate the number of AAV2/8-RoG6P and AAV2/8-ZFN genomes present per cell (Figure 11). We found no significant difference in 

\textit{G6PC} transgene copy number between the dual-vector and single-vector groups. However, there was a significant difference of $p<0.02$ between the \textit{G6PC} and \textit{ZFN} copy numbers within the dual-vector group, showing 13-fold lower retention of the AAV2/8-ZFN genomes. A lower level of retention of the \textit{ZFN} vector may reduce the risk of potential off-target genome modification that could result from long-term \textit{ZFN} expression. It also indicates that inclusion of homology arms results in an increased retention rate, as the AAV-ZFN genome lacked homology arms the AAV-RoG6P genomes had.
Figure 11: Donor vector genomes persist better than ZFN genomes in hepatocytes.

AAV2/8-RoG6P and AAV2/8-ZFN genomes were quantified by qPCR of G6pc-/- mouse liver DNA at 8 months of age. The ZFN genome was found to exist at much lower levels than the RoG6P genome when both were delivered in equal amounts by AAV vectors (p<0.014). Error bars: Mean ±SD.
3.2.4 AAV-ZFN coadministration with AAV-RoG6P enhances therapeutic metrics in a GSD Ia mouse model

Untreated G6pc-/- mice typically fail to survive longer than 3 weeks. We have previously established that treatment with a G6Pase cDNA transgene under control of the minimal human G6Pase promoter can increase mouse survival to over 12 months. To determine whether co-administration of a ZFN cleaving a safe harbor locus and a targeted donor transgene could improve the therapeutic transgene duration in mice, we compared the survival rates of G6pc-/- mice that were co-administered AAV2/8-ZFN and AAV2/8-RoG6P (n=6) with those of mice that received the AAV2/8-RoG6P donor vector alone (n=7). Dual-vector mice significantly outlived single-vector mice (p<0.04), with 100% versus 43% survival to 8 months, respectively, and the difference was particularly noticeable early in life (Figure 12). This suggests improved therapeutic duration when co-administering an AAV-ZFN that catalyzes targeted transgene integration. Furthermore, survival analysis of female mice, which typically respond more poorly to AAV gene therapy, demonstrated uniform 100% survival in the co-administration group, in contrast with 33% survival in the AAV2/8-RoG6P alone group (Figure 13; p<0.05). Mortality among the whole population and also among female mice was thus prevented by administration of AAV-ZFN. The especially high mortality among female mice receiving only the donor vector might be attributed to decreased transduction with AAV vector, in comparison with male mice—an established trend that is not yet fully characterized.
Figure 12: Prolonged survival of G6pc-/- mice depends on administration of AAV2/8-ZFN.

All mice that received AAV2/8-ZFN in addition to AAV2/8-RoG6P survived for 8 months. In contrast, only 43% of mice that received AAV2/8-RoG6P alone survived for the same duration, showing a significant difference (P < 0.04 using the log-rank test).
Female mice showed the same requirement for ZFN as the population as a whole, with 100% survival among mice receiving both vectors and only 33% among those receiving the donor vector alone (P < 0.05 using the log-rank test). Only postweaning mice are shown, because sex cannot be determined prior to weaning.

To investigate whether AAV2/8-ZFN co-administration also improved the magnitude of therapeutic effect at a late time point, blood glucose, hepatic G6Pase activity, and hepatic glycogen content were analyzed. These are standard means of assaying the efficacy of treatment for GSD Ia. We found that there was no significant difference between the two treatment groups when comparing their plasma glucose after 2 hours of fasting at 5 months of age (Figure 14). However, both vector treated groups had significantly elevated glucose during fasting, when compared with untreated 2-week-old G6pc-/- mice.
Liver G6Pase activity for either treatment group at 8 months of age did not differ from 2-week-old untreated affected controls, indicating that the G6Pase activity at 8 months was below the threshold of detection for the G6Pase activity assay (Figure 15). Hepatic glycogen content at 8 months of age corroborated the G6Pase data, showing no significant difference between the two treatment groups (Figure 16), but both vector treated groups had significantly lower glycogen content in the liver than did untreated 2-week-old G6pc-/- mice. Finally, quantitative reverse transcription PCR confirmed that the vector-treated groups did not differ in human G6PC mRNA expression at 8 months of age, but the signal for each vector-treated group was highly elevated above the background observed for untreated 2-week-old knockout mice (Figure 17).
Figure 14: Dual-vector treatment does not improve blood glucose levels compared with single vector treatment after a 2-hour fast at 5 months following treatment.

Both treatment groups showed similar blood glucose. Both were significantly different than previously published values for untreated G6pc-/- knockout mice. Two-week-old untreated mice were used because knockout mice do not survive past 3 weeks. Error bars: mean ± SD.
Figure 15: Hepatic G6Pase activity at 8 months of age in G6pc-/- knockout mice does not reveal a difference between single- and dual-vector AAV treatment.

No difference was observed between the two treatment groups or between either group and untreated knockout mice. Six-month-old WT mice are shown for positive reference. Error bars: mean ± SD.

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Figure 16: Hepatic glycogen accumulation in treated and untreated knockout mice, and wild-type mice, reveals no difference between single- and dual-vector treated groups.

Both treatment groups had comparable glycogen accumulation in their livers, though both groups had significantly more glycogen than wild-type controls. Glycogen in treated mice was also reduced compared with historical data on hepatic glycogen in knockout mice. An untreated control used to verify day-to-day experimental consistency was consistent with historical results. Error bars: mean ± SD.
Relative human *G6PC* expression (vector-specific) normalized to mouse β-actin, as determined by RT-qPCR, was equivalent whether or not mice received AAV2/8-ZFN. However, both groups showed highly elevated expression compared with the background signal for untreated mice.

It is therefore clear that treatment with AAV2/8-RoG6P results in therapeutic biochemical effects with or without the AAV2/8-ZFN vector, but we were not able to show that the AAV2/8-ZFN vector improves biochemical measures of GSD Ia. The lack of difference was likely due to the high mortality observed in the group that did not receive AAV2/8-ZFN, leading to selection for mice that responded especially well to gene therapy with AAV2/8-RoG6P alone. That is, we believe the mice in the single-vector group that did have low G6Pase and high glycogen died due to the consequential illness, and were never included in endpoint assays because their tissues were not able to be collected. Additionally, the high mortality created a problem with statistics by reducing the available samples in the single-vector group to a small number, necessitating extremely tight values to
obtain statistical significance.

To overcome the problem posed by the high mortality rate leading to low sample sizes selected for similar potency, a short-term experiment was performed in which mice received the AAV2/9 vectors, because an AAV2/9 vector encoding G6Pase achieved higher biochemical correction than the equivalent AAV2/8 vector in G6pc-/- mice in previous work, and enhancing the effect was likely to enhance any differences between the two groups. Furthermore, the ratio of donor to ZFN vector was adjusted to improve the efficiency of gene editing. Mice were assayed at 3 months of age, rather than 8 months, to avoid the loss of data from longer-term therapeutic decline and mortality.

Plasma glucose after 8 hours of fasting was not significantly different between the two AAV2/9 vector-treated groups at 12 weeks (Figure 18). However, significant differences were observed in both hepatic G6Pase activity (p<0.03) (Figure 19) and hepatic glycogen content (p<0.02) (Figure 20) between single- and dual-vector treated groups at 3 months of age. This confirmed that co-administration of AAV2/9-ZFN with AAV2/9-RoG6P improved the therapeutic efficacy of the human G6PC transgene. Finally, vector genome quantification of mouse livers at 3 months of age that had been administered either one or both vectors cross-packaged as AAV9 at adjusted dosages (Table 2) did not reveal a difference in the G6PC transgene copy number between groups (Figure 21).
Groups of mice with GSD Ia were treated with AAV2/9-RoG6P+ AAV2/9-ZFN (n = 11), or AAV2/9-RoG6P alone (n = 7). No difference was observed between the two groups in this assay, but both were improved compared with untreated knockout mice. Groups of “no vector” G6pc-/- mice (n = 7); and untreated wild-type mice (WT; n = 4) were controls. Error bars: mean ± SD.
Figure 19: G6Pase activity in livers from 3-month-old mice is enhanced in the dual-vector group compared with the single-vector treatment group.

Groups of mice with GSD Ia were treated with AAV2/9-RoG6P+ AAV2/9-ZFN (n = 11), or AAV2/9-RoG6P alone (n = 7). G6Pase activity in the liver was significantly higher in dual-vector-treated mice, in comparison with either “no vector” G6pc-/- mice or single-vector-treated mice. Groups of “no vector” G6pc-/- mice (n = 3); and untreated wild-type mice (WT; n = 6) were controls. Error bars: mean ± SD.
Figure 20: Dual-vector treatment reduces glycogen content in livers from 3-month-old mice compared with single-vector treatment.

Groups of mice with GSD Ia were treated with AAV2/9-RoG6P+ AAV2/9-ZFN (n = 11), or AAV2/9-RoG6P alone (n = 7). Hepatic glycogen accumulation was reduced in dual-vector treated mice, in comparison with either “no vector” G6pc/-/ mice or single-vector-treated mice. No significant difference was observed between double-vector mice and wild-type mice. Error bars: mean ± SD.
Figure 21: Vector genomes did not persist at higher numbers in dual-vector treated mice than single-vector treated mice.

AAV2/9-RoG6P genomes were quantified by qPCR for human G6PC in knockout mouse liver DNA at 3 months of age. The G6PC transgene levels did not differ between treatment groups. Error bars: Mean ±SD.

In addition to in vitro assays, livers from mice at both 8 months and 3 months of age were sectioned and subjected to histochemical staining for G6Pase activity. Consistent with the data from biochemical assays for 8-month mice, staining for G6Pase showed no benefit from AAV2/8-ZFN administration in those mice (Figure 22). However, 3-month-old mice showed marked improvement in the dual-vector treated group, in comparison with the single-vector group (Figure 23). Quantifying the number of G6Pase-expressing cells visible demonstrated a significant difference between the groups, indicating that ZFN activity increased the number of cells with detectable G6Pase activity at 3 months of age (Figure
Figure 22: G6Pase activity staining of livers from 8-month-old mice shows no difference between dual- and single-vector treated mice.

(A) Representative G6Pase staining of an untreated knockout mouse at 2 weeks of age. Note the absence of expression throughout the liver. (B) Representative staining section of the liver from an 8-month-old wild-type mouse that had been treated with AAV-RoG6P. Note the uniform brown stain throughout all hepatocytes. (C,D) Representative sections from mice that received the AAV2/8-RoG6P donor vector only, or the donor vector as well as AAV2/8-ZFN, respectively. Note that G6pase-positivity was highly variable across mice in both groups at this age. (E) Quantification of G6Pase-positive cells from mice in both groups. No significant difference was found to exist between treatment groups due to the high variability between the two. Error bars: SD.
Figure 23: G6Pase histochemical staining in the livers of 3-month-old mice reveals an improvement in G6Pase-expressing cells in mice receiving dual-vector treatment.

(A) Representative G6Pase staining of an untreated knockout mouse at 10 days of age. Note the absence of expression throughout the liver. (B) Representative staining section of a wild-type mouse liver. Note the uniform brown stain throughout all hepatocytes. (C,D) Representative sections from mice that received the AAV2/9-RoG6P donor vector only, or the donor vector as well as AAV2/9-ZFN, respectively.
Figure 24: Quantified G6Pase-positive hepatocytes in 3-month-old AAV-treated mice.

G6Pase-positive cell counts from both treatment groups (n = 7 each) demonstrated a significant enhancement in positive cell counts when both vectors were administered (P < 0.03). Error bars: mean ± SD.

3.3 ZFN Genome Editing Discussion

Treatment of GSD Ia currently entails a lifelong dietary regimen designed to prevent hypoglycemia, the primary concern that results in early death. This approach greatly extends the lifespan of patients, but fails to prevent many of the long-term complications of the disease like hepatic adenomas that may progress to hepatocellular carcinoma, and progressive renal failure. These complications likely result from downstream metabolic anomalies caused by the inability to release hepatic glucose. As such, approaches to improving disease prognosis as well as quality of life must target the root cause of the disease.

AAV-based gene therapies seek to treat GSD Ia at this root by supplementing the missing human G6PC gene with a therapeutic transgene.
encoding G6Pase. However, targeting the developing liver of young patients would require multiple doses due to rapidly-expanding hepatocytes diluting out the episomal transgene delivered by AAV. As a result of this, multiple repeat doses are required to maintain a therapeutic effect over the course of many years. Since each dose requires a different AAV capsid to evade a recipient’s antibodies against previous vectors, and each capsid preferentially targets different tissues, it is currently infeasible to treat a human patient in this manner. In order to overcome this temporal limitation, a permanent gene therapy for GSD Ia is required.

This method describes a novel gene-integration approach using two AAV vectors, one encoding a ROSA26-targeting ZFN, and the other encoding human G6Pase with homologous donor arms for ROSA26 surrounding the transgene. This dual-vector system is designed to cleave the mouse ROSA26 safe harbor locus to induce homology-directed repair at this site so the donor vector is copied into the mouse genome. Once in the mouse genome, the transgene should provide a lifelong source of G6Pase activity from that hepatocyte and its subsequent daughter cells. Though this is expected to occur in only a small subset of hepatocytes, it is known that as little as 3% of wild type G6Pase activity can prevent the symptoms of hypoglycemia. Of note, hepatic correction with gene editing will not reverse the progressive renal involvement in GSD Ia, and further development of methods to correct G6Pase deficiency in the kidney will still be needed.
We found that the ROSA26-targeting ZFN gene is active in hepatocytes when delivered by AAV, ensuring that DNA damage-induced repair mechanisms are triggered to enable G6PC transgene integration. Moreover, we observed targeted integration events dependent on AAV2/8-ZFN administration, confirming that the ZFN induces integration as hypothesized. Achieving integration of a fully-functional transgene at a safe harbor locus in vivo provides proof-of-principle for future work on similar applications not only for GSD Ia, but in a number of other monogenic diseases as well. That ability to adapt safe harbor loci-targeting ZFNs to multiple therapies is what makes this approach particularly valuable.

Prior to this work, an in vivo AAV-delivered ZFN therapy was found effective in a mouse model of hemophilia B using a ZFN specific to the deficient blood coagulation factor IX locus to drive gene correction.\textsuperscript{138,142} Such approaches to genome editing require development and refinement of nucleases specific to each therapeutically relevant gene, as well as downstream animal models and clinical trials for each. Our approach would circumvent at least part of the process by establishing a universal safe harbor-targeting nuclease deliverable by AAV vectors that could be paired with various therapeutic transgenes. In particular, we anticipate that this approach could be developed for human therapies by targeting the AAVS1 safe harbor locus, which has already been used similarly in vitro.\textsuperscript{143-145} Another potential target for AAV-based human monogenic gene therapy is the albumin locus, which is currently being investigated in murine models.\textsuperscript{90,146}
Blood glucose following fasting, hepatic G6Pase activity, and hepatic glycogen content are standard metrics of gene therapy efficacy in GSD Ia mice.\textsuperscript{117} G6Pase activity for both AAV-RoG6P-treated mouse groups is below the threshold of detection by 8 months, but their hepatic glycogen content was improved (reduced) compared with untreated mice. The reduced glycogen content indicates that G6Pase activity must have taken place prior to tissue collection, and was strong enough to prevent life-threatening glycogen accumulation. A shorter time course confirmed that at 3 months of age, mice that received the ZFN vector had greater G6Pase activity and reduced glycogen, in comparison with mice that did not receive the ZFN vector. Similarly, qPCR shows that both groups contain similar levels of the transgene in their livers by 3 months, with the trend holding at 8 months. Furthermore, RT-qPCR demonstrated similar expression levels of the transgene between both groups at 8 months, highly elevated above background levels in untreated mice. Most importantly as an overall metric of therapeutic effect, we observe a significant increase in lifespan when mice were given AAV2/8-ZFN, bringing the 8-month survival rate up from 43\% to 100\%. The trend held in female mice alone, bringing their survival from 33\% to 100\%. Since females are known to take up AAV vectors less efficiently into hepatocytes than males, leading to decreased gene therapy efficaciousness, the striking improvement observed in females is noteworthy.\textsuperscript{137} Furthermore, mice highly expressing the ZFN early in life showed no signs of mortality specific to receipt of AAV-ZFN, which argues against toxicity
induced by the ZFN’s presence.

An interesting phenomenon also emerged that was not initially predicted: an increase in observed allele modification in knockout mice compared with their normal littermates. We believe it occurred due to a selective advantage for stably transduced G6Pase-containing hepatocytes in GSD Ia. More vector-containing hepatocytes were present in ZFN-treated mice, based upon increased G6Pase staining in dual-vector mice compared with single-vector treated mice at 3 months of age (Figures 23-24). A subpopulation of those G6Pase-positive cells had integrated the vector due to ZFN activity in dual-vector treated mice. The same ZFN activity that leads to allele modification therefore also correlates with an increased likelihood of a cell stably expressing G6pase. It is possible that a selective advantage for G6Pase-positive cells would be present in the GSD Ia liver due to the cytotoxic effects of G6Pase deficiency, as postulated by Grompe and others.\textsuperscript{147} Such a selective advantage would be absent in the wildtype mouse liver. Therefore, we expect that a selective advantage in the GSD Ia liver for G6Pase-positive hepatocytes containing the ZFN could explain the higher rate of allele modification in $G6pc^{-/-}$ mice, in comparison with their normal littermates.

The low survival of non-ZFN-treated mice could explain why biochemical differences between the two treatment groups were undetectable by 8 months of age. The high mortality in mice that did not receive AAV2/8-ZFN selected for mice that responded strongly to gene therapy by 8 months of age, while those mice with a poor therapeutic response died prior to tissue collection and analysis. This
is an inevitable outcome for the disease model due to near-universal mortality by 2-3 weeks of age in absence of gene therapy. We overcame this limitation by improving the ZFN treatment and performing an abbreviated experiment to ensure data collection occurred prior to late-onset mortality, and we observed enhanced biochemical correction of the liver in mice that received the dual-vector treatment, compared with those mice that received the donor vector alone.

The improvements in mouse survival and integrated transgene-encoded enzyme activity points to this approach as a strong contender for future human gene therapy. AAV is currently being tested in several clinical trials, paving the way for its use in additional human treatments, and this work on a small, single-vector-packageable ZFN opens the avenue to AAV as a delivery vehicle for integrating gene therapy. Specifically as it pertains to GSD Ia, this approach holds great promise because present treatments fail to prevent long-term complications, necessitating a new treatment route to reduce the risks and quality of life impacts on both children and adults living with GSD Ia.
4. Manipulation of Autophagy in GSD Ia Model Systems Produces Effects with Potential Therapeutic Benefits

The work in this section was performed as a collaboration between the groups run by Dwight Koeberl at Duke and Paul Yen at Duke NUS, particularly Benjamin Farah. My contributions to this work include the majority of mouse animal husbandry and tissue collection including tissue weight data collection, drug administration to mice, participation in life-prolonging and drug administration duties for canines, development of the Oil Red O and Western blot drug screens.

4.1 GSD Ia includes an autophagy-deficient phenotype

The basis for endeavoring to adapt autophagic drug therapies to GSD Ia came from the symptomatic similarities between NAFLD and GSD Ia. NAFLD is characterized by lipid accumulation and hepatomegaly, and in its more severe forms also comprises fibrosis, cirrhosis, and hepatocellular carcinoma. GSD Ia livers likewise present lipid accumulation, hepatomegaly, fibrosis, and eventual hepatocellular carcinoma. Due to the striking similarities of some of the most common symptoms for both diseases and the chronic outcomes, we investigated whether recent advances in autophagy manipulation for NAFLD could be applied to our GSD Ia experimental models.

First, since LC3-II, a marker of autophagy, is known to be diminished in NAFLD, we sought to confirm that it is likewise downregulated in GSD Ia. We found that GSD Ia mice had reduced levels of LC3-II as well as reduced levels of
the pro-autophagic proteins ATG5 and Beclin 1 (Figure 25). LC3-II is also reduced in GSD Ia mouse kidneys, the kidney being the secondary organ affected by GSD Ia (Figure 26). Finally, we examined whether AAV treatment with G6PC transgene-carrying vectors prevents the autophagy deficiency. We found that providing the therapeutic benefits of a G6PC transgene does indeed reduce development of GSD Ia autophagy-related symptoms from developing (Figure 27). This demonstrates that autophagy is indeed reduced in GSD Ia mice and is directly caused by G6pc deficiency, showing its relation to NAFLD symptoms and providing more support for our hypothesis that autophagy manipulation, which has benefits in NAFLD models, may provide new treatment routes for GSD Ia models and, eventually, patients.

Figure 25: Loss of G6pc leads to decreased levels of ATG proteins in liver, and decreased levels of autophagosomes in kidney.

(A) Western blotting G6pc/- mouse livers showed decreased LC3-II levels versus WT mice. (B) ATG5 protein level is reduced in the livers G6pc KO mice.
(C) Beclin 1 protein level is reduced in the livers of the same mice. For all experiments shown, n=3, except (A), where n=7, and * represents p<0.05 between experimental groups being compared. Error bars: SEM.
Autophagosome number as indicated by LC3-II/actin ratio is decreased in the kidneys of \(G6pc\) KO mice. N=3, and * represents p<0.05 between experimental groups. Error bars: SEM.

Treatment of KO mice with AAV2/9-G6Pase ("+G6Pc") restores autophagy as indicated by the restoration of LC3-II levels quantified by Western blot beyond those of WT mice. N = 3 for each group, and * indicates p <0.05. Error bars: SEM.

Since we found that the low-autophagy phenotype of NAFLD occurs in GSD Ia, we moved forward from multiple angles on researching the potential for autophagy manipulation in GSD Ia. One of these approaches was to recapitulate the GSD Ia phenotype from mouse livers in the AML-12 mouse hepatocyte cell line by knocking down \(G6pc\) using siRNA ("siG6P"). The symptoms characteristic of GSD Ia were confirmed in several ways. First, we confirmed the expected
reduction in total LC3-II quantity by Western blotting (Figure 28). To further support this finding, we performed LC3 puncta staining and quantified the puncta—which represent autophagosome formation—per nucleus, and found a corroborative reduction in puncta in knockdown cells (Figure 29). Since a deficiency in G6PC in humans and animals results in G6P accumulation that feeds into other metabolic pathways and causes disease symptoms, we also analyzed the G6P accumulation in this AML-12 knockdown model, and found that G6P does in fact accrue when G6pc is knocked down by siRNA in AML-12 cells (Figure 30).
Figure 28: LC3-II is reduced in G6pc siRNA-treated AML-12 cells.

Treatment of AML-12 mouse hepatocyte cells with siG6pc reduces autophagosome number as indicated by the LC3-II/tubulin ratio determined by Western blots. N = 3 for each group, and * indicates p <0.05. Error bars: SEM. 

---
Figure 29: G6pc knockdown reduces endogenous LC3 puncta in AML-12 cells stained with α-LC3 antibody.

LC3 brightness was quantified and compared to the number of nuclei within the same visual field and was found to be decreased in G6pc siRNA-treated AML-12 cells, indicating a reduction in autophagosomes when G6pc is knocked down. N=3 for each treatment and * indicates p <0.05. Error bars: SEM.

Figure 30: Glucose-6-phosphate levels are increased in G6pc knockdown AML-12 cells.

G6P accumulates in AML-12 cells knocked down for G6pc using siRNA, showing similarity to GSD Ia hepatocytes. N=3 for each treatment, and * represents p<0.05. Error bars: SEM.

4.2 Rapamycin enhances autophagy in GSD Ia model systems and reduces toxic levels of metabolite accumulation

Having confirmed the autophagy-reduction phenotype in both mice and
G6pc siRNA AML-12 cells, we explored the use of rapamycin to induce autophagy in these models. Rapamycin is the prototypical mTOR inhibitor, and since mTOR downregulates autophagy, inhibiting it via rapamycin results in an increase in autophagic activity. This was first tested in the AML-12 model to conserve difficult-to-breed GSD Ia mice. We found that application of rapamycin to G6pc knockdown AML-12 cells enhanced autophagy and reduced lipid accumulation, as shown in autophagic marker Western blotting and Oil Red O staining (Figures 31-32). Phosphorylated p70s6k (p-p70s6k) is indicative of active mTORC, and we observed that rapamycin reduces the amount of p70s6k that is phosphorylated in siG6P-treated AML-12 cells. More importantly, application of rapamycin to knockdown cells results in an increase in LC3-II, which directly indicates an increase in autophagic activity. Similarly, we expected a reduction in lipid accumulation from pro-autophagic rapamycin treatment, and we observe such a lipid reduction, of both visual staining and quantification of Oil Red O adherence intensity.
Figure 31: Rapamycin treatment increases autophagy activity markers in G6pc knockdown AML-12 cells.

Western blotting for several autophagy-related proteins indicates that rapamycin (Rap) treatment restores autophagy in G6pc KD AML-12 cells. N = 3 for each treatment, * indicates p <0.05 between groups being compared, and ** indicates p <0.01 between groups. Error bars: SEM.

Figure 32: Rapamycin treatment reduces lipid accumualtion in G6pc knockdown AML-12 cells.

Oil Red O staining shows that lipid accumulation is restored to low levels by rapamycin application to G6pc knockdown AML-12 cells. N = 3, * indicates p <0.05 between groups being compared, and ** indicates p <0.01 between groups. Error bars: SEM.

G6pc-/- mice were given intraperitoneal injections of 5mg/kg rapamycin suspended in 10% DMSO/90% PBS daily for 7 days starting on day 5 of life. We
observed an increase in LC3-II in rapamycin-treated mouse livers via Western blotting as predicted (Figure 33). Electron microscopy was also performed on mouse livers to quantify autophagic vesicles, and mice treated with rapamycin showed an increase in this indication of autophagy (Figure 34).

**Figure 33: Rapamycin increases autophagosome levels in GSD Ia mouse livers.**

Western blotting for LC3-II in mouse livers indicates that treatment of *G6pc*-/- mice with rapamycin (Rap) increases autophagosome number in the liver as determined by the LC3-II/actin quantified by Western blots. N = 3 for each group, and * indicates p <0.05. Error bars: SEM.
Figure 34: Ultrastructural electron microscope analysis indicates rapamycin-treated GSD Ia mice showed more hepatic autophagic vesicles than untreated mice.

Electron microscope images were analyzed for the presence of autophagic vesicles in GSD Ia mouse hepatocytes without or without rapamycin treatment. Rapamycin administration was found to increase the number of autophagic vesicles compared with untreated G6pc-/- mice. N = 3 for each group, and *** indicates p <0.001. Error bars: SEM.

Liver triglyceride content was quantified to determine whether enhancing autophagy reduced lipid accumulation as in AML-12 cells, and we found that liver triglyceride content was reduced by half in G6pc-/- mice following rapamycin administration (Figure 35). Treatment was sufficient to normalize G6pc-/- mice to the naturally low WT triglyceride levels. That is, the treated group had no significant difference in hepatic triglycerides when compared to vehicle-injected WT mice. Furthermore, electron microscopy revealed a visible reduction in lipid vacuole size and number in GSD Ia mouse livers that received rapamycin (Figure 36).
Rapamycin reduced hepatic triglycerides by 50% in GSD Ia mouse livers, compared with untreated knockout mice that had 7 times the hepatic triglyceride levels of wildtype livers. N = 4 per group, * indicates p <0.05, and ** indicates p <0.01. Error bars: SEM.

Figure 35: Rapamycin reduces hepatic triglyceride content in GSD Ia mice.

Electron microscopy reveals that rapamycin-treated GSD Ia mouse livers have

Figure 36: Lipid vacuoles are diminished in rapamycin-treated GSD Ia mouse livers.

Electron microscopy reveals that rapamycin-treated GSD Ia mouse livers have
fewer lipid vacuoles in hepatocytes compared with untreated GSD Ia mouse livers, indicating clearance of lipids that accumulate in untreated knockout mice. The white arrowhead indicates intralysosomal lipid accumulation.

In addition to autophagy and lipids, we also quantified hepatic glycogen content, the elevation of which is characteristic of GSD Ia, and found a substantial reduction for GSD Ia mice that received rapamycin (Figure 37). Hepatic glycogen content was further analyzed using PAS staining to stain for polysaccharides, including glycogen, in liver sections. We found that GSD Ia mice undergoing rapamycin treatment had visibly reduced glycogen-laden vacuoles compared with untreated affected mice (Figure 38). This confirms our hypothesis that manipulating autophagy can reduce glycogen accumulation by breaking it down through alternate routes from the traditional one relying on G6Pase, bypassing the need for G6Pase in GSD Ia hepatocytes.
Glycogen assays revealed a reduction in glycogen (expressed as glucose released during the reaction) in GSD Ia hepatocytes of mice treated with rapamycin compared with those that go untreated, indicating that rapamycin treatment induces glycogen clearance from knockout mouse hepatocytes. N = 5 per group, * indicates p < 0.05, and ** indicates p < 0.01. Error bars: SEM.

Figure 38: Histologic analysis (20x magnification) reveals a decrease in lipid and glycogen accumulation in rapamycin-treated mouse livers.

Mouse liver sections were stained with H&E and PAS. PAS staining showed reduced hepatic glycogen accumulation in rapamycin-treated mice. H&E stain revealed necrotic cells and lipid vacuoles. In PAS stained samples, insets are
In addition to the AML-12 and mouse GSD Ia models, we also examined the GSD Ia canine model. The model shows similar symptoms to humans, primarily lethal hypoglycemia, and later in life the canines develop hepatic adenomas and kidney failure like adult human patients. Injections with AAV delivering \( G6PC \) to canines are effective for a time, but do not restore 100% of the phenotype.\(^{113} \) Because treatments are only partially effective, these canines were excellent for examining the efficacy of rapamycin in a large animal model. GSD Ia canines were given 1mg/kg rapamycin orally daily for 10 days. This was not an endpoint for the canines, so we could not perform the same assays that were done for mice, but we were able to examine liver health using ultrasound to determine the status of the canine’s hepatomegaly, and ALT and GGT serum-level assays to determine liver damage. We found that while there was no significant dorsal liver length change, ventral liver length was reduced following rapamycin treatment (Figure 39). This indicates reduction in hepatomegaly documented prior to drug administration.
Figure 39: Treatment of GSD Ia canines with rapamycin reduces hepatic size, and lowers circulating hepatic enzymes.

(A) Representative abdominal radiographs of GSD Ia canine before and after 1 week of rapamycin administration. Measurements indicate ventral liver length. (B) Ventral and dorsal lengths of livers from GSD Ia canines as measured from radiographs pre- and post-rapamycin treatment; values on graphs indicate means. Dorsal liver length was significantly reduced in rapamycin-treated canines compared with their starting sizes on day 0. N = 4, and * indicates p <0.05.

Likewise, liver health as indicated by serum levels of the liver enzymes GGT and ALT improved with rapamycin administration. GGT levels went down significantly after the 10-day treatment when expressed as either absolute values or fold reduction from the starting point (Figure 40), and ALT levels went down with significance when expressed as fold reduction from starting values (Figure
These indicate a reduction in liver damage following a 10-day course of oral rapamycin treatment in the canine GSD Ia model.

**Figure 40:** GSD Ia canine circulating GGT enzyme levels are reduced following rapamycin treatment.

Serum gamma-glutamyl transferase (GGT) levels expressed as absolute and fold-change in rapamycin-treated canines showed significant reductions between starting values and final values following rapamycin treatment. N = 4, and * indicates p <0.05.
Figure 41: GSD Ia canine circulating ALT enzyme levels are reduced following rapamycin treatment.

Serum alanine aminotransferase (ALT) levels expressed as absolute and fold-change in rapamycin-treated canines showed significant reductions between starting values and final values following rapamycin treatment. N = 4, and ** indicates p <0.01. Error bars: SEM.

4.3 Autophagy-enhancing drug discovery for GSD Ia has revealed many potential therapeutic compounds

The success of the rapamycin experiments at enhancing autophagy and reducing lipid and glycogen accumulation in cells and mice, and improving liver health in canines, showed that autophagy modulation could be a powerful new avenue for GSD Ia therapies. However, rapamycin is a potent, nonspecific mTOR inhibitor, causing it to carry many undesirable side effects.\textsuperscript{149-151} We therefore examined several other drugs for their potential in enhancing autophagy in GSD Ia. A literature review identified 11 drugs that showed promise in other models where autophagy enhancement ameliorates disease symptoms (Table 3). In brief, a wide array of drugs was selected, including those that modulate autophagy via the inositol-3-phosphate (IP3) pathway, those that do so through AMPK modulation to act through mTORC1, and those using yet-undetermined
pathways. Analysis of additional literature led to the selection of three concentrations of each compound to be tested in cell culture using the G6pc siRNA knockdown AML-12 mouse hepatocyte cell line model of GSD Ia as an initial screen for the drugs.

Table 3: Autophagy-enhancing drugs.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Published Concentration</th>
<th>Low Conc.</th>
<th>Med Conc.</th>
<th>High Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-lipoic acid</td>
<td>25, 50, 100, 200 uM</td>
<td>50 uM</td>
<td>100 uM</td>
<td>250 uM</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>100, 200, 400, 800 uM</td>
<td>100 uM</td>
<td>250 uM</td>
<td>400 uM</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>100 uM</td>
<td>25 uM</td>
<td>100 uM</td>
<td>250 uM</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>Cerebrospinal fluid: 0.7-1.5 ug/mL</td>
<td>1 ug/mL</td>
<td>5 ug/mL</td>
<td>10 ug/mL</td>
</tr>
<tr>
<td>Lithium</td>
<td>2, 4, 6, 8, 10, 12 mM</td>
<td>0.5 mM</td>
<td>1 mM</td>
<td>2 mM</td>
</tr>
<tr>
<td>Metformin</td>
<td>50 uM, 0.25 mM, 0.5 mM, 2 mM, 2.5 mM</td>
<td>0.25 mM</td>
<td>1.5 mM</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>10, 100, 1000 nM</td>
<td>10 nM</td>
<td>100 nM</td>
<td>1000 nM</td>
</tr>
<tr>
<td>Mifepristone</td>
<td>0.1, 1, 10 uM</td>
<td>0.1 uM</td>
<td>1 uM</td>
<td>10 uM</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>50, 100 uM</td>
<td>25 uM</td>
<td>50 uM</td>
<td>100 uM</td>
</tr>
<tr>
<td>Trehalose</td>
<td>100 mM</td>
<td>50 mM</td>
<td>100 mM</td>
<td>200 mM</td>
</tr>
<tr>
<td>Verapamil</td>
<td>70 uM</td>
<td>50 uM</td>
<td>100 uM</td>
<td>250 uM</td>
</tr>
</tbody>
</table>

Drug concentrations were chosen based on similarity to published concentrations in either cell culture models resulting in target effects or autophagy, or in vivo extracellular fluid concentrations following animal treatments. The latter case is noted with the fluid type. Concentrations used in our in vitro AML-12 cell culture treatments are listed as the low, medium, and high concentrations that were screened.

The first test using AML-12 cells was the Oil Red O stain to examine the
presence of lipid vacuoles in cells given the treatments (Figure 42). Cell culture wells were each treated with one of the selected doses for each drug and were scored for both reduced lipid accumulation compared with controls, and improved cell survival, since the siRNA knockdown model has high lethality in the AML-12 cells line (Table 4). The screen's results pointed to several drugs as being the best contenders for further research: bezafibrate, carbamazepine, lithium chloride, and mifepristone.
G6pc siRNA knockdown (or random siRNA as a control, as indicated in the top-left image as “siNeg”) AML-12 cells were given three different doses of each drug treatment. siNeg cells received no drug. After 72 hours of growth, the media was changed to ketogenic media and appropriate quantities of each drug were added. This was performed in duplicate. Oil Red O staining was then performed and bright field images acquired at 10x magnification.

siNeg cells are shown as a positive control for effective lipid clearance. Ethanol, a vehicle for several of the drugs, is shown as a negative control to demonstrate the lipid droplet accumulation in knockdown cells that do not receive drug. Carbamazepine is shown to represent positive results following treatment. Verapamil is shown to demonstrate high death caused by cytotoxicity.

Figure 42: Representative images of Oil Red O stained G6pc siRNA knockdown AML-12 cells.

G6pc siRNA knockdown (or random siRNA as a control, as indicated in the top-left image as “siNeg”) AML-12 cells were given three different doses of each drug treatment. siNeg cells received no drug. After 72 hours of growth, the media was changed to ketogenic media and appropriate quantities of each drug were added. This was performed in duplicate. Oil Red O staining was then performed and bright field images acquired at 10x magnification.

siNeg cells are shown as a positive control for effective lipid clearance. Ethanol, a vehicle for several of the drugs, is shown as a negative control to demonstrate the lipid droplet accumulation in knockdown cells that do not receive drug. Carbamazepine is shown to represent positive results following treatment. Verapamil is shown to demonstrate high death caused by cytotoxicity.
**Table 4: Lipid reduction scoring of Oil Red O-stained, drug-treated, G6pc knockdown AML-12 cells.**

<table>
<thead>
<tr>
<th>Drug\Dose</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Lipoic acid</td>
<td>+</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>0 (Spotty +)</td>
<td>- (Spotty ++)</td>
<td>---</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Carbazamazine</td>
<td>+++</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Lithium Chloride</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Metformin</td>
<td>+</td>
<td>+/-+</td>
<td>0</td>
</tr>
<tr>
<td>Methylene Blue</td>
<td>+</td>
<td>--</td>
<td>---</td>
</tr>
<tr>
<td>Mifepristone</td>
<td>+</td>
<td>+ (Spotty ++)</td>
<td>++ (Great survival)</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>--</td>
<td>- (High death)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>-</td>
<td>+ (But high death)</td>
<td>-- (Very high death)</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>--</td>
<td>--</td>
<td>---</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>+</td>
<td>0 (Spotty ++)</td>
<td>---</td>
</tr>
<tr>
<td>DMSO</td>
<td>- (Spotty +)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Ethanol</td>
<td>-</td>
<td>-</td>
<td>--</td>
</tr>
</tbody>
</table>

Drug treatments at three doses each were performed on G6pc siRNA knockdown AML-12 cells. Following oil red O staining the whole plates were evaluated visually for reductions in lipid accumulation. Treatments were performed in duplicate. -/0/++/++/+ indicates the degree of improvement, or lipid reduction, caused by the treatment, with + indicating positive benefits. Subjective comments were also included to note improvements in survival compared with other groups and the consistency or lack thereof regarding the observed benefits.

In addition to Oil Red O staining, treatments were analyzed via Western blots for restored LC3 expression for each drug, under the conditions that had optimal effects in the Oil Red O screen (Figure 43). Since the Western antibody previously used successfully for rapamycin treatment LC3 blots had been
discontinued by the manufacturer, we tested two alternative antibodies with good track records and chose the one produced by Cell Signaling Technologies because it gave the strongest signal-to-noise ratio under our experimental conditions. Western blotting confirmed the autophagy-enhancement effects of many drugs that showed lipid reduction through the Oil Red O stain, and in particular it corroborated the results of bezafibrate, carbamazepine, lithium chloride, and mifepristone application by demonstrating the increase in LC3 autophagic marker predicted based on their lipid-reducing effects (Figure 43).

Figure 43: LC3 Western blot of drug-treated AML-12 cells corroborates the benefits observed in Oil Red O staining.

Western blotting was performed on AML-12 cells incubated for 24 hours with the described drug conditions. LC3 was quantified and normalized against each condition's β-actin protein quantification. Western blot results corroborated the Oil
Red O findings and gave further indication of the most beneficial drugs for increasing autophagy in the G6pc siRNA knockdown AML-12 system, and thus which were most likely to yield benefits in mice.

Carbamazepine and lithium have potent psychoactive effects. Lithium compounds were among the first mood-stabilizing drugs used to treat bipolar disorder and schizophrenia circa 1949, and anticonvulsants, particularly carbamazepine, have been combined with lithium in the treatment of bipolar disorder and schizophrenia for over three decades.\textsuperscript{164-167} The fact that lithium is still prescribed as a mood stabilizer in the treatment of bipolar disorder after 75 years stands as a testament to the potency of its psychoactive effects. These beneficial effects for patients suffering from psychiatric illnesses would instead become serious side effects in patients prescribed lithium and/or carbamazepine to ameliorate GSD Ia symptoms, so lithium chloride and carbamazepine have not been pursued in mice as potential GSD Ia treatments at this time.

Mifepristone is used to induce chemical abortions up to day 70 of pregnancy.\textsuperscript{168} It also has effects on endometriosis.\textsuperscript{169} These on- and off-label purposes could produce significant side-effects in GSD Ia patients using mifepristone to improve their autophagic activity. Furthermore, the side effects of the drug itself as described on the FDA label include nausea, vomiting, and diarrhea, which could make it difficult for patients to intake sufficient calories for combating hypoglycemia, potentially negating the benefits.\textsuperscript{168} Therefore, administration of mifepristone was not pursued in GSD Ia mice.

Thus, Bezafibrate emerged as the best candidate from the screen.
Bezafibrate is a PPARα agonist used to lower cholesterol levels and prevent hyperlipidemia to reduce the risk of heart disease. Since hyperlipidemia is a symptom of GSD Ia, this pre-established on-label effect could have benefits in addition to autophagic enhancement in patients. In terms of side effects, bezafibrate does induce loss of appetite and elevation in circulating liver enzymes. While these could complicate therapeutics taking advantage of the medication for GSD Ia, the overall effects on autophagy and hyperlipidemia would likely outweigh the downsides, so we chose bezafibrate to move forward as our best drug candidate in GSD Ia mice.

G6pc-/- knockout mice were initially given intraperitoneal injections of 100mg/kg/day bezafibrate suspended in 10% DMSO/90% PBS IP for 7 days, but none survived to the endpoint for tissue collection, out of the three that were dosed as such. Consequently, we halved the dosage to 50mg/kg/day, and reduced the treatment duration to 4 days to increase the chances of mice surviving to the endpoint. Three G6pc-/- mice received this treatment, but two died prior to tissue collection. We therefore further reduced the dose to 25mg/kg/day for 3 days. We were confident that even this short treatment would yield results based on our positive autophagy and lipid clearance results in AML-12 cells after just 24 hours of drug incubation. We obtained tissues from 4 G6pc-/- mice treated with bezafibrate, 3 WT mice treated with bezafibrate, and 3 affected mice treated with 10% DMSO/90% PBS as vehicle-injected negative controls.
At the time of tissue collection, blood glucose was analyzed and affected mice were found to have no increase when treated with bezafibrate, their levels consistently below the threshold of detection (data not shown; it would be a blank graph). However, liver and kidney weights were recorded at the time of sacrifice, and these data revealed a slight difference in kidney size as a percentage of body weight. Bezafibrate caused a very small but quantitatively significant increase in kidney weight expressed as a percentage of total body weight (p<0.046) (Figure 44). This runs contrary to the expectation that bezafibrate would reduce kidney size in G6pc/- mice.

![Figure 44: Bezafibrate-injected GSD Ia mice liver and kidney weights did not show improvement (reduction) following bezafibrate administration.](image)

Livers and kidneys from mice undergoing bezafibrate injections were weighed at the time of collection, weights expressed here as percentage of body weight. No significant difference emerged in liver weights between knockout mice treated with or without bezafibrate, but bezafibrate did produce a small but significant increase in kidney weight as a percent of body weight. N=3 for all groups, and * indicates p<0.05. Error bars: mean ± SD.
The collected livers were analyzed by Western blot for LC3 to determine whether bezafibrate enhanced autophagy. The single surviving 4-day-treated mouse was pooled into the treatment group for purposes of the Western blot since the effects should have peaked well before 3-4 days, whereas organ size would be a much slower change and the 4-day mice were therefore excluded from that experiment. We found a trend toward increased LC3 autophagic marker when mice were treated with low-dose bezafibrate, but the difference between groups was not statistically significant (Figure 45).

**Figure 45: Bezafibrate increases LC3 autophagic marker.**

Livers from mice undergoing bezafibrate treatment were analyzed for autophagic activity by LC3 Western blot. No significant differences were observed comparing groups of the same genotype that received or did not receive bezafibrate, though there is an emergent trend showing that bezafibrate may increase autophagy in knockout mice. WT n=1; WT+Bez n=3; KO n=2; KO+Bez n=3.
4.4 Autophagy manipulation discussion

The current approach to GSD Ia therapy focuses on preventing lethal hypoglycemia by providing constant calories throughout the day. This fails to prevent many of the chronic symptoms, including hepatomegaly, hyperlipidemia, and glycogen accumulation. While gene therapy approaches appear very promising for long-term treatments and are likely to be extremely beneficial down the line, gene therapy as a treatment field overall is still immature. It takes many years to develop gene therapeutics, and the manufacturing process is still slow and difficult to scale efficiently. As such, stopgap and combinatorial treatments for GSD Ia will be extremely valuable, in that they can provide benefit to patients living with the disease in a much shorter timeframe than can gene therapies.

Autophagy manipulation has only recently been explored as a therapeutic approach to many diseases in which toxic accumulation of endogenous products causes health problems, including prion diseases, Alzheimer’s disease, and NAFLD. The theory behind these treatments is that enhanced autophagy may be able to break down the excess products trapped in cells that causes clinical defects. Since much of GSD Ia’s symptom set derives from excess lipids, glycogen, and even amino acids, it stands to reason that autophagy could be useful for treating aspects of GSD Ia to reduce the symptoms and improve the quality of life for patients living with it long term.

In exploring this route, we first found that autophagy is reduced in GSD Ia mice livers and kidneys as well as in the G6pc knockdown AML-12 mouse hepatocyte cell line model (Figures 25, 26, and 28). What this meant to us was
that increasing autophagy in GSD Ia would not actually mean raising its levels above normal, but rather *restoring* its levels closer to normal. The distinction means that pro-autophagic treatments are likely to have fewer and less intense side effects in patients, further indicating that this course of treatment investigation is a strong contender for future GSD Ia therapeutics. We believe the autophagic reduction occurs because excess G6P that accumulates as a result of insufficient G6Pase to hydrolyze it signals the cell that the cell is under fed conditions—conditions under which cells try to store excess energy by activating lipogenesis and inhibiting autophagy and fatty acid oxidation. Reversing this state could potentially be done by inhibiting mTORC1 or through other pathways that could out-compete the mTORC1 inhibitory effect.

Using rapamycin, the prototypical mTOR inhibitor to induce autophagy, we confirmed that inhibiting mTORC1 can enhance autophagy in GSD Ia model cells and mice, and that doing so reduces lipid and glycogen accumulation characteristic of GSD Ia (*Figures 31-38*). The effects were further examined in GSD Ia canines by analyzing the reduction in hepatomegaly and liver damage (as indicated by circulating GGT and ALT levels) induced by rapamycin treatment (*Figures 39-41*).

While rapamycin administration showed great effects, its known toxicity, off-target effects, and side effects in humans due to its general inhibition of mTORC1, which controls a wide variety of cellular pathways, makes it a relatively poor option for long-term human treatment. Therefore, we decided the next
step was to look for alternative drugs with similar pro-autophagic effects to rapamycin that may produce fewer side effects through long-term administration. We began by using our newly-developed $G_6pc$ knockdown AML-12 cell model as a screening system for several drugs with known autophagy-enhancing effects, and analyzed these drugs using Oil Red O staining and LC3 western blots to determine their ability to reduce lipid accumulation in GSD Ia-like cells and confirm their ability to enhance autophagy in the face of GSD Ia (Figure 42, Table 4, and Figure 43). We found several drugs with pro-autophagic effects in the face of GSD Ia symptoms, and ultimately chose the one with the least toxicity and potential for deleterious side-effects to proceed with in $G_6pc^{-/-}$ mice, bezafibrate. This drug has the added benefit of not yet being FDA approved for any kind of therapy in the U.S., but is a well-documented drug approved for use in Europe. This makes it enticing for future research for commercialization because it could be picked up and its research funded by the pharmaceutical industry while already having many toxicity studies completed.

We found that bezafibrate shows trends in enhancing autophagy in GSD Ia mice, but the effect is not significant. However, the study is ongoing and as additional mice are added to treatment groups to increase statistical power, we anticipate the improvements becoming significant. Furthermore, hepatic lipid and glycogen accumulation assays have yet to be performed, and they are planned for the future.

Overall this study has shown that autophagy manipulation has great
potential to provide therapeutic benefits for GSD Ia. Rapamycin may not be the best drug for these purposes, but it has opened the door on this new approach. Our small screen has turned up several drugs, and additional screens may reveal yet more as the field of autophagy enhancement grows and more pro-autophagic drugs become known. Ongoing work with bezafibrate is so far promising, and our other top drug candidates, carbamazepine, lithium chloride, and mifepristone, are excellent options to further pursue this course in mouse treatments. We expect that autophagy induction will prove to be an approach rich with new GSD Ia therapies that will be brought to bear in the years to come, dramatically improving the quality of life and clinical outcomes for GSD Ia patients in the near future.
5. Conclusions and the Future of GSD Ia Therapies

Despite the use of dietary regimens to fight hypoglycemia as means of enhancing patient survival, many GSD Ia symptoms remain untreatable, and the risk of death due to hypoglycemia is never eliminated, only controlled. Current treatment entails frequent meals of uncooked cornstarch to maintain normoglycemia, and the use of a nasogastric feeding tube overnight for this purpose overnight in infants. Nasogastric feeding tubes may fail, however, leading to sudden hypoglycemia and death, while the alternative, waking up periodically throughout the night to consume cornstarch, seriously affects the patient's quality of life.

Even with the arduous treatment course, once a patient survives into adulthood, they are at extreme risk of developing hepatic adenomas that may progress to hepatocellular carcinoma, and also of progressive renal disease, on top of less life-threatening but not insignificant chronic hyperlipidemia and hepatomegaly. The quality of life for these patients is bleak despite the ability of medical treatment to keep them alive, yet due to the disease's rarity, it has been an unpopular field with far to go to catch up with modern medicine. This is why we continue to seek more advanced and superior means of treating GSD Ia, including gene therapy and autophagy manipulating small molecule therapies.

Our lab has worked on gene therapies for GSD Ia for many years, and has made great strides in the field. We have found in both mouse and dog models that AAV vectors encoding either canine or human \textit{G6PC} can enable many
months or even years of survival in animals that, without treatment, survive only
days or weeks. However, our past research has determined that the
replicative nature of hepatocytes, especially those in GSD Ia livers, causes AAV
gene therapies to provide only temporary effects, since DNA delivered by AAV
exists as non-replicative episomes, providing a finite number of therapeutic
transgenes for an essentially infinite number of cells that need to be treated as
they constantly replicate and die off.

ZFNs have been a promising approach to genome editing for some time,
though they have recently fallen out of favor with advances in other nucleases.
They have been successfully used for gene correction in and out of living
models, and for gene insertion in vitro. We have performed the first
successful in vivo gene knock-in at a safe harbor locus using a small ZFN
encoded by an AAV genome. This has the advantage over gene correction
approaches in being applicable to multiple autosomal recessive disorders,
whereas ZFN approaches that edit dysfunctional genes must always be specific
to a single mutation. By combining our safe harbor site-cutting ZFN with a
different donor gene, the system can be adapted across different mutations and a
variety of genes.

Our system uses two AAV vectors: one encoding the ZFN that specifically
binds and cleaves the ROSA26 mouse safe harbor locus; and one encoding the
human G6pase catalytic domain, with the active gene flanked by ROSA26
homology arms. Once the mouse genomic ROSA26 locus is cut by the ZFN, the
other vector genome’s homology arms allow the active gene to be taken into the
mouse genome as part of the normal homology-directed repair pathway. Once
integrated into the genome of targeted mouse hepatocytes, the gene replicates
with each cell division to prevent the previously-observed dose-dependent loss of
effect over time.\textsuperscript{113} We indeed found that mice given both vectors had
dramatically improved survival compared with mice that received the \textit{G6PC}
vector alone, demonstrating that the ZFN has the desired effect of improving the
longevity of therapeutic effects in GSD Ia mice (\textbf{Figures 13-14}). We furthermore
found that administration of the combined vector therapy improved the
biochemical readout and visually-observable cell correction from mice compared
with mice that received AAV-G6P alone (\textbf{Figures 19-21, 24-25}). Overall we
conclude that this novel approach to gene therapy holds potential as a new
means of providing long-term treatment for the wide array of symptoms
associated with GSD Ia in older patients. This is, of course, a goal far down the
line, as this is only the first proof-of-principle experiment showing that gene
integration is possible at a safe harbor locus using ZFNs to drive the genome
modification.

The safe harbor locus used in this experiment was ROSA26, which does not
have an analog in humans or canines. This means that the experiment cannot
simply be moved into large animal models and eventually clinical trials like small
molecule drug therapy experiments can. There are known safe harbor loci in
humans, including AAVS1 and the albumin locus (as long as 100\% gene
modification is not achieved), but in dogs there are no pre-established safe harbor loci. Likely, the albumin locus could be used, but it is not yet established as a therapeutically targetable gene in canines. Still, should we wish to continue pursuing the route of whole-transgene integration at safe harbor loci, the canine albumin locus would be the most likely candidate. And rather than going through the arduous task of designing and characterizing a zinc finger nuclease, we would instead use the simpler and faster, but less well-developed, CRISPR/Cas9 system.

We have begun working with CRISPR/Cas9 technology as the successor to the ZFN-based genome editing project. Through a collaboration with the Cullen lab, we have designed a CRISPR/Cas9 guide RNA that can be packaged with a small Cas9 gene in AAV to direct cleavage, though instead of targeting a safe harbor locus in canines, we chose to target the defective canine genomic G6pc gene. This work is still unique in that no genome editing has been reported in a large animal model for genetic disease. The approach is generalizable in that rather than targeting the canine point mutation for genome editing, we have targeted a site just prior to the first intron. This allows the transgene vector to use the first exon and part of the canine promoter as the 5' homology arm (Figure 46). This allows us to increase the amount of homology available since the first homology arm doubles as part of the functional gene, which should improve the likelihood of HDR occurring.\textsuperscript{172} By targeting integration at the genomic canine G6pc locus, integration (which is not necessary for activity) would place the
functional transgene in its natural context, allowing full control via enhancers as if it were the endogenous gene, all without disrupting functional parts of the canine genome.

Figure 46: The CRISPR/Cas9 canine G6pc-targeting process is designed to integrate active G6PC at the endogenous canine G6pc locus.

The guide RNA used for CRISPR/Cas9 targeting to canine G6pc was designed to target the junction between exon 1 and intron 1. Cleavage at that site allows for homology directed repair using homology with the end of the full promoter and all of exon 1 at one end, and the beginning of intron 1 at the other. HDR results in an integrated G6pc in the canine genome without inclusion of any ITRs. NHEJ is also possible at the cleavage site without relying on homology, in which case the ITRs at each end are included in the canine genome.

Another future direction of the ZFN project could seek to examine the long-term therapeutic effects beyond 3 months of age, using a model whose survival
would not depend on how well the animals respond to gene therapy. A mouse model is available in which \textit{G6pc} is selectively knocked out in the liver, and these mice survive even without gene therapy, unlike our constitutive knockouts, possibly due to kidney G6Pase activity being enough to sustain the mice. These mice, while able to survive, still exhibit enlarged livers and go on to develop hepatic adenomas and hepatocellular carcinoma. We would like to see enhanced biochemical effects beyond just 3 months, since these types of therapies would be expected to work much longer than that if they are to be used in humans. However, so far we have not seen a difference between the double- and single-vector treatment groups in these mice. It is possible that further experiments could yield results, especially if we examine additional biochemical markers such as hepatic triglycerides or perform staining to look at the G6Pase expression patterns in these mouse livers, the latter of which revealed key improvements in the 3-month-old vector-injected mice that were not apparent in normal G6Pase activity assays.

One last route of exploration for the ZFN project could be to adapt it to kidney correction. As performed herein, the ZFN was under control of a liver-specific promoter, but both the livers and kidneys of patients, mice, and canines with GSD Ia show symptoms. Therefore, it could be of benefit to treat the kidneys alongside the liver with the pro-integration therapy.

In addition to gene therapy approaches, we have also begun investigating the possibility of small molecule therapies for enhancing autophagy in GSD Ia.
This arose due to the observed similarities between GSD Ia and NAFLD symptoms such as hepatomegaly and hyperlipidemia, and the beneficial effects in NAFLD observed from pro-autophagic treatments. We first confirmed that autophagy is downregulated in GSD Ia, and that treatments to enhance autophagy could restore the phenotype toward normality. We then determined that rapamycin, the archetypal mTORC1 inhibitor, is able to increase autophagy and reduce lipid and glycogen accumulation in GSD Ia-like AML-12 cells. We further found corresponding effects in mice and canines with GSD Ia, showing that autophagy manipulation has potential for therapeutic benefits in the disease.

By screening a series of potentially pro-autophagic drugs in cells, we were able to find several that increase autophagy in G6pc knockdown cells, and took the least toxic of these, bezafibrate, into mouse experiments. We found that bezafibrate produces a trend of increased LC3 autophagic marker, but the difference was not statistically significant.

Future experiments on autophagy manipulation will focus on testing additional drugs in mice to determine whether their in vitro pro-autophagic effects apply in vivo to GSD Ia mice. The screen revealed that the next candidates are carbamazepine, lithium chloride, and mifepristone. Bezafibrate experiments are also ongoing in mice to increase the size of treated groups and better determine its effects. Additional assays on the bezafibrate experiment would also be of benefit, particularly ATG5, AMPK, and Beclin 1 Western blots to examine the state of autophagy through multiple outputs rather than only LC3.
promising results come about following early additional assays, more complex ones would be of benefit for a publication, including using chloroquine to halt autophagosome degradation and confirm that bezafibrate stimulates autophagosome formation as the means of enhancing autophagic flux rather than decreasing autophagosome breakdown to do so. Seahorse assays to examine beta oxidation rates as an indication of lipid breakdown would also be of interest. Electron microscopy on mouse liver sections to examine lipid vacuoles and autophagosomes would be valuable as a means of directly observing bezafibrate's effects, as was done with rapamycin in our publication.

Finally, performing drug treatments on GSD Ia canines would be the next step following drug tests on the various candidates in mice. Ultrasounds can reveal changes in hepatomegaly, and blood work can show potential reductions in hypoglycemia and hyperlipidemia, as well as reductions in ALT and GGT as signs of liver health. Liver biopsies on the canines would allow us to analyze hepatic glycogen, lipid, and autophagosome content, as well as to examine lipid vacuoles and autophagic vesicles via electron microscopy. These would all be valuable for establishing downstream treatment regimens for future human clinical trials with pro-autophagic drugs in GSD Ia, which is the ultimate goal of this work.

The two approaches to GSD Ia therapy presented herein consider the problem from two highly different angles, the problem itself being that present treatments for GSD Ia focus exclusively on preventing hypoglycemia without
regard to the many other health problems the disease entails, which result from undesirable product accumulation when G6Pase is unavailable. G6P gets converted to amino acids, glycogen, or lipids that build up in hepatocytes, becoming part of the cause of hepatomegaly. The chronic stress state the cells are then put under make them more likely to malfunction and become cancerous later in a patient's life.

Since the long-term complications arise as a result of these toxic accumulations, we sought multiple ways to reduce this buildup. Through gene therapy, we can reduce them by adding back G6pase activity so the normal functionality is restored and G6P stops feeding into those pathways. Using the novel ZFN-based genomic integration approach increases the duration of G6PC AAV vector treatment, ensuring that we see long-term benefits. Through pro-autophagic drug therapy, we reduce these products by breaking them down through an alternative pathway from G6Pase, obviating the need for G6Pase to be present at all as long as the treatment is combined with one to maintain normoglycemia. In combination with current treatment regimens, these two approaches could provide much-needed help to patients presently living with GSD Ia, and dramatically improve their quality of life and long-term prognosis. Both routes of investigation are just emerging, but we hope that the potential this research holds will garner interest and lead to breakthroughs in the field of GSD Ia therapy.
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Biography

Dustin James Landau was born on January 20, 1987, in Torrington, CT. He weighed ten pounds and ten ounces, a fact of which he is inordinately proud. He graduated from Litchfield High School in 2005 after taking many elective courses in a broad array of biological sciences.

He attended the University of Michigan Ann Arbor starting in September 2005 and graduated in May 2009 with a Bachelor of Science, majoring in Cellular and Molecular Biology and minoring in Epistemology and Philosophy of Science, the latter of which honed his skills in critical thinking and communication, which helpful in continuing his work in the biological sciences.

He began PhD work in August of 2009 at Duke University, in the Program in Cell and Molecular Biology, eventually joining the Department of Molecular Genetics and Microbiology. He has published three papers: *Long-term efficacy following readministration of an adeno-associated virus vector in dogs with glycogen storage disease type Ia* in 2012; *Induction of autophagy improves hepatic lipid metabolism in glucose-6-phosphatase deficiency* in 2016, and *In Vivo Zinc Finger Nuclease-mediated Targeted Integration of a Glucose-6-phosphatase Transgene Promotes Survival in Mice With Glycogen Storage Disease Type IA* in 2016.

Dustin is a member of the American Society of Gene & Cell Therapy. He has received Meritorious Abstract Awards and associated travel grant to attend the annual ASGCT meetings in both May 2014 and May 2016, and likewise for the annual meeting of the Society for Inherited Metabolic Disorders in 2015.