Enhancing Homology-Directed Repair for Treatment of Murine Phenylketonuria

By

Michael Martinez

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CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of Michael Martinez has been approved.

Cary O. Harding, MD

Stephen Lloyd, PhD

Amanda McCullough, PhD

Melanie Gillingham, PhD

Joshua Saldivar, PhD

Renee Ryals, PhD

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List of Abbreviations

AAV: Adeno-associated virus

AAV5: AAV serotype 5

AAV8: AAV serotype 8

AAVS1: Adeno-associated viral site 1

ALT: Alanine aminotransferase

APAP: Acetaminophen

APOBEC: Apolipoprotein B mRNA editing enzyme, catalytic polypeptide

ApoE: Apolipoprotein E

AV: Adenovirus

BC: Bile canaliculus

BD: Bile duct

BH4: Tetrahydrobiopterin

Cas: CRISPR-associated sequence

cDNA: Complementary DNA

CMV: Cytomegalovirus

CNS: Central nervous system

CNV: Copy number variation

CRISPR: Clustered regularly interspaced short palindromic repeats

CRISPR RNA: crRNA

DHFR: Dihydrofolate reductase

DHPR: Dihydropteridine reductase

DNA: Deoxyribonucleic acid

DNA-PKCs: DNA protein kinase catalytic subunit

DSB: Double-strand break

EC: Endothelial cell

EF: Human elongation factor 1-α promoter

ERTs: Enzyme replacement therapies

FACS: Fluorescent-activated cell sorting

FAH: Fumarylacetoacetate hydroxylase

GABA: Gamma-aminobutyric acid

HAEs: Hypersensitivity adverse events

HC: Hepatocyte

GMP: Good manufacturing practices

HCR: Hepatic control region

HDR: Homology-directed repair

HIV: Human immunodeficiency virus

HR: Homologous Recombination

HSC: Hepatic stellate cell

HTV: Hydrodynamic tail vein injection

IAP: Isoamylase activity protein

IDUA: Alpha-L-iduronidase

IEM: Inborn error of metabolism

IF: Immunofluorescence

IHC: Immunohistochemistry

IP: Intraperitoneal

ITR: Inverted terminal repeat

KC: Kupffer cell

LNP: Lipid nanoparticles

LSP: Liver-specific promoter

LV: Lentivirus

mGch1: Murine guanosine triphosphate cyclohydrolase I gene

mPah: Murine Pah gene

mRNA: Messenger RNA

MMEJ: Microhomology-mediated end joining

NHEJ: Non-homologous end joining

NGS: Next Generation Sequencing

NGS: National Genetics Administration

NIH: National Institutes of Health

NTBC: Nitisinone

NVB: Novobiocin

PAH: Phenylalanine hydroxylase

PAL: Phenylalanine ammonia lyase

PEG: Polyethylene glycol

Phe: Phenylalanine

PKU: Phenylketonuria

PT: Portal triad

R408W: Specific mutation in the PAH gene (common in PKU patients)

ROS: Reactive oxygen species

RSV-LTR: Rous sarcoma virus long terminal repeat

SA: Succinylacetone

SaCas9: Streptococcus aureus Cas9

scAAV: Self-complementary AAV

sgRNA: Single guide RNA

SpCas9: Streptococcus pyogenes Cas9

SpCas9-PE: Staphylococcus aureus-based Cas9 prime editor

TALENs: Transcription activator-like effector nucleases

TBG: Thyroxine binding globulin

THA: The hepatic artery

TPV: The portal vein

TP53: Tumor protein P53

tracrRNA: Trans-activating CRISPR RNA

UCDs: Urea cycle disorders

vg: Viral genomes

δ-ALA: δ-Aminolevulinic acid

Chapter 1: Introduction

Preface

The goal of this dissertation is to improve our understanding of the factors governing genetic repair to facilitate whole gene insertion into the genome of mature organisms. In this setting, a gene is defined as “a discrete genomic region whose transcription is regulated by one or more promoters and distal regulatory elements and which contains the information for the synthesis of functional proteins or non-coding RNAs”[1]. While this work will mainly focus on developing a sustained cure for phenylketonuria (PKU), the ultimate goal of this research is to invent a framework from which many genetic diseases could be treated, if not cured.

The first chapter explores the fundamentals of the target disease and the current landscape of gene editing technology. This foundational review begins with an overview of the liver, which is the primary organ of interest in this work, followed by an examination of inherited metabolic disorders and a detailed breakdown of PKU. This is complemented by a discussion of the genetic engineer’s toolkit, which is mainly comprised of two parts: programmable gene editing machinery and the delivery vehicles necessary to deliver that machinery to the appropriate cells.

After establishing a solid foundation of the field, Chapter 2 and 3 will examine *in vitro* findings that utilize small molecules and adeno-associated virus (AAV)-delivered proteins for enhancement of gene corrections, offering insights into strategies for improving *in vivo* gene editing. Finally, Chapter 4 will delve into our current work developing transgenes and combinatorial small molecule cocktails to enhance homology-directed gene editing.

Any discussion of a new and powerful technology must also coincide with a discussion of its ramifications and ethical uses. To that end, this work concludes by: describing the differences between somatic and germline editing; exploring the accessibility and ownership of scientific research; and investigating the ethical ramifications posed by two previous case studies in human gene editing. At its core, this is a moral inquiry into the purpose of medicine and the responsibilities scientists bear in shaping the future of medical technology. The dissertation concludes with a summary of progress made, alternative strategies for achieving clinically relevant gene therapies, and with a recommendation to form a new administration to fund and conduct long-term goals in gene editing.

Outline

Chapter 1: Reviews anatomy and metabolic functions of the liver, inherited errors of metabolism, mechanism of PKU, and the tools and vehicles used for *in vivo* gene editing.

Chapter 2: Examines the current state-of-the-art approaches for treating PKU using gene therapy.

Chapter 3: Explores methods for improving *in vitro* gene editing using combinations of small molecules and virally packaged proteins.

Chapter 4: Details strategies to enhance the efficacy of *in vivo* gene editing in murine models of PKU.

Chapter 5: Investigates the ethical and moral dimensions of human gene editing.

Chapter 6: Provides a summary of our contribution to *in vivo* gene editing and discusses alternative approaches for enhancing the efficacy of gene editing.

Inborn Errors of Metabolism

Biology operates in a perpetual cycle of destruction and construction. Across all multicellular life, a shared ability allows organisms to transform air, food, and even sunlight into essential building blocks to maintain life. This remarkable process enables the conversion of carbon dioxide into carbohydrates, proteins into nucleic acids, and one amino acid into another. We call this intricate biochemical symphony metabolism, a system divided into two complementary processes: catabolism, the breakdown of macromolecules, and anabolism, the synthesis of macromolecules. Together, these pathways sustain the hypercomplex machinery of life.

As such, an error in our metabolic pathways does not simply result in some kind of biochemical detour; but a serious disease that traumatically impacts the quality of life for its victims and their families. The following discussion will provide a concise review of metabolic pathways and diseases common in genetic and biochemical research. However, a special focus will be given to errors in nonpolar aromatic amino acid metabolism, as it is germane to the research that will follow in later chapters. For a more comprehensive review of metabolic diseases, you may find valuable references in Eipel et al.[2], Arnold et al.[3], and Saudubray et al.[4].

Inborn errors of metabolism (IEMs) are inherited disorders caused by mutations in genes that produce metabolic enzymes[4]. The term “IEM” was first coined by Sir Archibald Garrod in 1908, who not only predicted that they were the source of four distinct diseases (alkaptonuria, pentosuria, cystinuria, and albinism)[5], but also that protein/enzyme dysfunction was the root cause of these

diseases[6]. In one-way IEMs are all very similar; many can be caused by a frameshift or missense mutation in a single gene, their pathology frequently manifests with neurological defects such as seizures and intellectual disabilities, and they often result in the aggregation of one or more metabolites in the blood or urine. However, despite these shared features, IEMs represent a highly diverse group of diseases with vastly different pathophysiologies, each requiring unique diagnostic and therapeutic approaches. The discussion that follows provides real-world examples of how mutations in a single gene can break a solitary thread in the metabolic web, leading to profound tissue damage and systemic biochemical failure.

Urea Cycle disorders: Anabolic metabolism branches and diverges as the catalytic pathways reach their terminal end points, creating a plethora of molecules from a limited number of precursors. Meanwhile, catabolic metabolism converges from a plethora of precursors to create the same raw byproducts for excretion, such as ammonia[7]. The urea cycle is a process that converts ammonia into urea, which can then be excreted in the form of urine. In this way, the body can safely use nitrogen to create vital biomolecules, such as proteins, nucleic acids, and ATP, without being damaged by its reactivity. However, it wouldn't be completely correct to call the urea cycle a catabolic system, in fact, it produces arginine, fumarate, and ornithine from simpler compounds. As such, it would be truer to say that it is both a catabolic and anabolic process. In a beautiful display of efficiency, the urea cycle creates more value from the dregs of metabolism, while still processing a toxic biproduct. However, when there is a mutation that

inhibits one of the steps in the urea cycle it often results in the aggregation of ammonia in the blood[7]. This aggregation may lead to a disruption of neurotransmitter production[8], leading to disorientation, loss of energy, and seizures[9]. Astrocytes convert this ammonia into glutamine, causing an increase in intracranial pressure[10]; and a disruption of the Kreb's Cycle by depleting α-ketoglutarate and inhibiting pyruvate dehydrogenase[11,12].

Ornithine transcarbamylase (OTC) deficiency, resulting from a mutation in the X-linked OTC gene, is one such error in the urea cycle. OTC is vital for the production of citrulline from ornithine and carbamoyl phosphate. Due to the build-up of ammonia, patients with OTC deficiency experience mild-to-severe developmental and cognitive impairments, seizures, confusion, nausea, migraines, seizures, coma, and death[13]. Prognosis is much improved for those who present in adolescence or young adulthood. OTC deficiency has been successfully treated with liver transplantation from unaffected donors, which, as of this writing, is the only reliable cure for OTC deficiency and remains a standard of care for patients at risk of death[14]. Liver targeted gene addition (**see Chapter 2**) with a wild-type OTC gene has also demonstrated some attenuating results in animal models of the disease[15] and in human clinical trials using Adeno-associated viral vectors[16]. The most common treatment for UCDs is a dietary restriction of protein to decrease the amount of nitrogen that is metabolized. However, it should be noted that this diet aims to reduce, but not eliminate, protein from the diet: since elimination of all protein from the diet would be worse than the disease itself, this is not an option.

Amino acid metabolism disorders: Protein is made up of long strings of amino acids connected along a repeating backbone of nitrogen-carbon-carbon. After these proteins are digested into their constitutive amino acids they are often converted into other amino acids, neurotransmitters, and hormones or broken down and excreted from the body in the form of carbon dioxide, water, and urea. However, when one of the steps involved in this process becomes inactivated, the body lacks an efficient method to safely remove these amino acids from the system. This is especially true for amino acids that contain a phenyl ring such as tyrosine and phenylalanine. However, tryptophan does not typically follow this trend since it has three unique pathways with multiple enzymes that can convert or degrade it: the serotonin pathway (tryptophan dehydrogenase), the indole pathway (aromatic amino acid transferase), and the kynurenin pathway (indoleamine deoxygenase/tryptophan deoxygenase)[17]. Errors in these processes, such as the serotonin pathway, have observable symptoms due the concomitant lack of the neurotransmitters, but symptoms are often milder than in pathways with no alternatives[18,19]. Metabolism of histidine, the fourth aromatic amino acid, is similarly complex[20].

Tyrosinemia, alkaptonuria, and phenylketonuria (PKU) are three diseases that result from errors in tyrosine and phenylalanine metabolism (**Fig. 1-1**). Phenylketonuria will be discussed in greater detail in the section below, but the key feature of this disease is a biallelic mutation of the phenylalanine hydroxylase (PAH) gene, leading to a decrease in tyrosine, accumulation of phenylalanine, and an increase in phenylketones in the blood and urine.

There are three types of tyrosinemia, which are variable in their severity and treatment approach[21]. Tyrosinemia type 1 results from a mutation in the fumarylaceotacetate hydroxylase (FAH) gene. This results in an aggregation of fumarylacetoacetate, which is converted into succinylacetone. Succinylacetone (SA) is highly toxic in the liver and kidney due to its inhibition of glutathione and an aggregation of reactive oxygen species (ROS)[22]. SA also inhibits a vital step in heme synthesis (δ-aminolevulinic acid dehydratase) and porphyrin metabolism causing “intermittent porphyria-like” symptoms in some patients[23]. Over the long term, tyrosinemia is associate with cirrhosis, seizures, acute neurological impairment, liver failure, and hepatocellular carcinoma[21]. To treat Tyrosinemia Type 1 and alkaptonuria the drug nitisinone (NTBC), which inhibits 4-hydroxyphenylpyruvic dioxygenase, is given to patients (**Fig. 1-1**). This prevents the accumulation of fumarylacetoacetate and succinylacetone, thereby reducing much of the damage to the liver, kidney, and brain. In effect, this treatment essentially gives a patient tyrosinemia type 3, but removes tyrosinemia type 1 in the process. Patients who take NTBC will still be unable to properly metabolize tyrosine and must also undergo life-long dietary restriction of protein.

Liver-directed gene therapy has been investigated for treatment of tyrosinemia[24]. However, due to the continual cell death caused by toxic metabolites, the viral episomes, which cannot be repaired or replicated by the cells, are slowly lost. Gene editing would have an advantage since the edited cells would have a selective growth advantage over the unedited cells. However, unless you could correctly edit the vast majority of hepatocytes, the liver would

still face succinylacetone exposure. As such, NTBC and/or dietary restriction of tyrosine and phenylalanine remain the standard of care for those with tyrosinemia and alkaptonuria until a highly efficient method for gene editing can be developed[25,26].

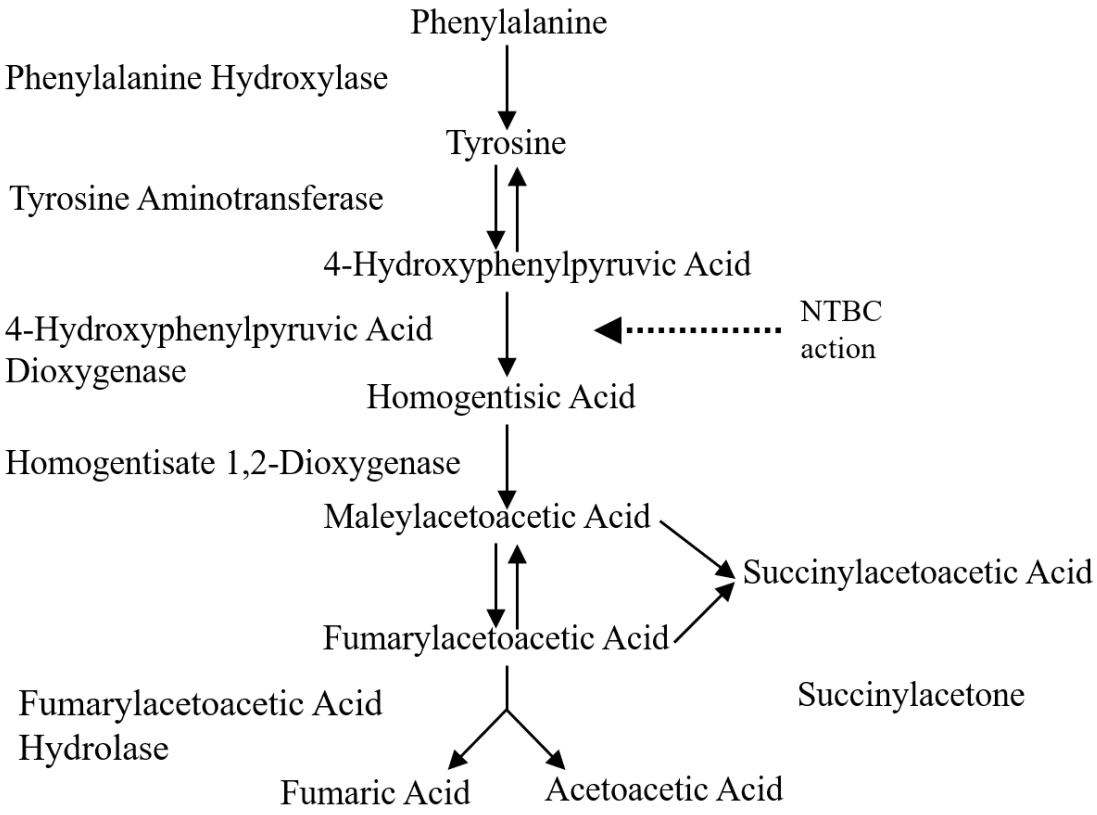

Figure 1-1. **Phenylalanine and Tyrosine Metabolic Pathway.** Published by Taylor et al, *Osteoarthritis and Cartilage*, 2012[27].

IEMs are individually rare, but as a group they make up more than 1 in 2,500 live births or approximately 56,000 cases worldwide each year[28]. Fortunately, many of the above diseases are diagnosed during newborn screening, which has provided timely and life-changing treatment for tens of thousands of families. However, even among first-world nations the ability to screen, diagnose, educate, and continually treat a diverse patient population has proved challenging. For many patients, the cost and availability of treatments like low protein medical food is unrealistic. Even for those who have access to treatments, patient fidelity is far from absolute since the treatments can be isolating, unfulfilling, and unpalatable. As we have seen throughout this review of IEMs, these diseases share many characteristics; they often damage the brain or nervous system, are typically autosomal recessive monogenetic diseases, dietary restriction often offsets some of the more serious ailments, with many IEMs treatable with systemic liver gene therapy. As such, there is a yawning gap in the treatment approach for tens of thousands of patients with IEMs, and, for many of these patients, that gap could be filled using liver-directed gene editing. Of these diseases, phenylketonuria is possibly the best IEM for the refinement of *in vivo* liver-directed gene editing due to its comparatively high incidence rate, easily collected and quantifiable marker metabolite (phenylalanine), and well characterized pathophysiology. Additionally, PKU itself does not cause any liver disease, so any liver dysfunction occurring during a treatment must be an adverse event of therapy and not a disease effect.

Phenylketonuria (PKU)

The story of PKU research is intriguing, tragic, and inspiring. It's filled with individual triumphs, failures, delays, and breakthroughs, which makes PKU not only a prime model for IEM research, but an excellent example of how scientific progress occurs. I will give a very brief overview of this story to highlight the real-world consequences and costs of PKU. For a more comprehensive reporting of PKU history, I suggest the book "Overcoming a Bad Gene" written by one of the pioneers in PKU biochemistry, Dr. Seymour Kaufman[6].

In the late 1920's, a Norwegian mother named Borgny Egeland became deeply suspicious that the cause of intellectual disability in both of her two children was more than a fluke. She observed that each of her children shared a distinct pathology: each emitted a distinct odor, often called "mousy" or comparable to "a horse stable"[6]; neither child learned how to speak, yet they both began their lives as normal, alert, babies before suffering from progressive mental retardation as they aged. Additionally, while having a single disabled child wasn't unheard of, having two affected children "was uncanny, outrageous, like being struck with lightning *twice*"[6]. Much like a scientist, she developed a hypothesis that her children's disability shared a common and distinct cause, and may therefore share a common cure. With great tenacity, Mrs. Egeland searched for a doctor who would take her observations and concerns seriously. After being rebuffed by many doctors, Mrs. Egeland found a physician-scientist who had the wherewithal to take her observations seriously and the skillset to test her hypothesis.

The discovery of phenylketonuria is often credited to Dr. Asbjörn Fölling. Dr. Folling was a dedicated Norwegian physician and talented biochemist who first identified the unique presence of phenylpyruvic acid in the urine of two siblings, named Dag and Liv Egeland[29]. Phenylpyruvic acid is not typically present/detectable in urine, so this discovery led to Dr. Fölling correctly attributing the intellectual disability of these siblings to an error in metabolism, as well as the naming of the disease: phenylketonuria[30].

Both Mrs. Egeland and Dr. Fölling deserve recognition for their unbridled dedication and work-a-day heroism. Unfortunately, Mrs. Egeland's children never benefitted from Dr. Fölling's findings, but countless other families did. Within a mere 20 years of Folling's publication, a dietary treatment would be established for children with PKU, and, within 30-years, newborn screening of the disease would be instituted across most developed nations. Egeland and Fölling's contribution to public health likely changed the lives of tens of thousands of patients and is part of the reason our research into PKU stands where it does today.

Dr. Fölling's seminal 1934 report on PKU suggested that phenylalanine metabolism could be the root cause of the disease due to the structural similarities of phenylalanine and phenylpyruvic acid[30]. However, it wasn't until Dr. George Jervis demonstrated that phenylalanine is converted to tyrosine in the human body that a specific mechanism for the disease could be proposed[31]. Dr. Jervis found that when phenylalanine was injected into patients with PKU their blood tyrosine levels remained static, unlike in healthy patients where tyrosine

increased. He also developed a biochemical assay for determining phenylalanine-to-tyrosine conversion in liver samples, which, when performed on PKU biopsies, showed no tyrosine production[31]. This both established the first steps in phenylalanine metabolism and proved that phenylalanine's conversion into tyrosine was inhibited in PKU patients, leading to accumulation of phenylalanine and its concomitant excretion as phenylpyruvic acid (**Fig. 1-1**).

The hydroxylation of phenylalanine into tyrosine was found to be the function of PAH. Based on the research of Fölling and Jervis, a mutation in the *PAH* gene was suspected to be the root cause of PKU, as suggested by Linus Pauling in 1956[6]. However, this wasn't fully confirmed until 1986 when the lab of Dr. Savio Woo identified a mutation in the *PAH* gene from a patient with PKU. After the *PAH* gene had been identified, isolated and cloned by Dr. Woo, animal models of the disease could be readily produced[32]. Additionally, the advancement and ease of DNA sequencing made the identification of *PAH* mutations in patients far easier to find. It was quickly understood that there wasn't a single mutation that causes PKU, but several hundred, with the exact impact of each mutation dependent on its location within the *PAH* gene.

Mechanism and Genetics of Phenylalanine Hydroxylase (PAH)

PAH operates in a system with phenylalanine, NADPH, a cofactor named tetrahydrobiopterin (BH_4), and two enzymes: dihydropteridine reductase (DHPR) and dihydrofolate reductase (DHFR), which convert BH_4 back into its reduced state[6,33]. PAH itself has three domains: a regulatory domain that provides for the allosteric activation of PAH in the presence of Phe; the catalytic domain that hydroxylates Phe; and a tetramerization domain that links four PAH monomers

into a complex that maximizes their catalytic activity (**Fig. 1-2**)[34]. PAH retains some activity as a dimer, and even as a monomer, as such many mutations in the tetramerization and regulatory domains cause a much milder, and more manageable, version of hyperphenylalaninemia. Meanwhile, missense mutations in the catalytic domain are typically correlated with the near complete ablation of PAH activity leading to PKU.

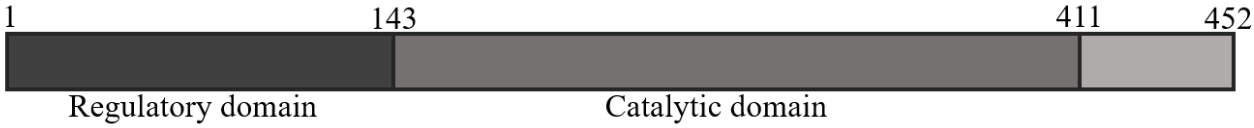

Figure 1-2. Structure of the *PAH* gene. The human *PAH* gene is 452 amino acids in length and consists of three major domains. The regulatory domain allows the allosteric activation of PAH in the presence of phenylalanine. This allows a baseline level of phenylalanine to persist in the blood while preventing a toxic aggregation of the amino acid. The larger catalytic domain performs the hydroxylation that converts phenylalanine into tyrosine. However, this activity is significantly enhanced when a tetramer of the monomer forms, which is facilitated by the tetramerization domain in the C-terminus of the protein. Reprinted with permission from Trunzo et al, *Clin Chim Acta*, 2015[34].

Over 1500 unique mutations in the *PAH* gene have been shown to give rise to the disease, though there are some, such as the R408W mutation, which are far more common and have manifested independently in populations throughout the world, particularly Ireland and Eastern Europe[35,36]. This has led some scientists to suggest that there may in fact be an evolutionary advantage for those who are heterozygous for the PAH mutation, though this avenue of research remains incomplete[1*]. There are some populations that seem nearly devoid of PKU, such as the Japanese population, while others, largely in Africa, remain unknown or unreported[38]. However, as modern populations continue to shift and grow, we can be certain that PKU will be found on every populated continent on the globe: as long as there are those heterozygous for PAH mutations the disease will continue to occur.

One of the notable aspects of heterozygotes is the dominant negative affect where a single intact *PAH* allele merits only 35-43% of normal PAH activity due to the addition of mutant monomers in the PAH tetramer[39,40]. Not only does this result in elevated Phe levels in heterozygotes, sometimes even requiring medical advisement, it also makes PKU especially tricky to cure as we will explore more fully in later chapters.

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[*] LI Woolf reported in 1986 that pregnant mothers who were heterozygous for a PKU mutation had 7.4% more live births than the control population[37]. Woolf hypothesized that societies that stored grains for food, or to feed livestock, could be exposed to species of fungus, such as *Aspergillus ochraceus*. This fungus produces a derivative of phenylalanine called ochratoxin A, which is especially dangerous for expectant mothers. As such, heightened blood Phe may be able to compete with ochratoxin A, thus diluting its toxic effects and providing an evolutionary advantage to those with decreased PAH activity.

Common Mouse Models of PKU

There are several PKU mouse models currently available for study. The first model was called the *Pah*^{enu1} (hereafter: Enu1) strain, and had a mutation in the 104th codon acid of *Pah*[41]*.* However, as shown in Figure 4, this mutation occurred in the regulatory domain and, as such, did not yield a classical PKU phenotype in mice. The most common models for studying PKU are the *Pah*^{enu2} (hereafter: Enu2) model[42], which is likely the most robust murine model of PKU, and the *Pah*^{R408W} (hereafter: R408W) model[43], which replicates the most common *PAH* mutation found in human PKU patients[44,45,46].

While these three genotypes provide serviceable models for studying PKU, there are a few aspects that make them inconvenient in gene therapy experiments. First, the presence of the dominant-negative effect means that the true benefits of providing correct PAH monomers is muted when there is an abundance of mutant monomers waiting to bind with them. Additionally, identifying and isolating cells producing correct/corrected PAH monomers is impossible through immunohistochemistry because there is no antibody that can distinguish between the two. As such, the Dexon1 mouse model was created that produces no PAH monomers[47]. This prevents the dominant negative affect from confounding assessment of the given gene therapy and allows for immunofluorescence (IF) for the identification of treated cells[47].

The research reported in herein will largely use the Dexon1 model due to these benefits. However, it should be noted that this mouse line is not the most accurate for modelling a human response to PKU treatment due to the lack of a dominant negative effect. It should also be noted that there are many other PKU

models, some that have common human PAH mutations inserted into their genome, and each model has its particular benefits that should be taken into account depending upon study design.

Newborn Screening and Dietary Management

With the breakthrough research of Jervis and Fölling, several researchers began investigating the possibility of developing a low-phenylalanine diet for patients with PKU[6]. This diet typically consists of low protein foods with medical food that have all the necessary amino acids with the exception of phenylalanine. The production of this medical food typically consisted of boiling casein (milk protein) in sulfuric acid to break down the protein into its constitutive amino acids and then running the lysate through charcoal, which is able to filter aromatic amino acids, including Phe[6]. The rest of the amino acids could then be added into the mixture and fed to the patient. As you can imagine this medical food was very unpalatable, but was easy enough to provide to infants and small children. The first study that used dietary restriction of phenylalanine on a patient was performed by Horst Bickel and colleagues in 1951 and lasted two years[6]. This led to a recovery of hair pigmentation, an elimination of the hallmark 'mousy odor' in treated patients, a near elimination of seizures, and a reduction of disruptive behaviors. However, significant intellectual changes were only observed when patients were treated as infants: when untreated as infants the brain damage caused by PKU was irreversible[48].

This created a new problem, if dietary treatment was optimally effective in newborns, then we needed a way to diagnosis every baby at the time of birth. Dr. Fölling had used a colorimetric assay of urine with ferric chloride, often used in

diagnosis of diabetes, to confirm the presence of phenylpyruvic acid. However, getting the required urine from newborns was not a viable solution for diagnosis on a nationwide or worldwide scale. Additionally, newborn excretion of phenylpyruvate is quite low and unreliable for early disease detection. It wasn't until 1961 when Dr. Robert Guthrie developed a clever assay that could be used with only a drop of blood[49]. This assay used bacteria grown in a culture of thienylalanine, an 'antimetabolite' of phenylalanine, which inhibits bacterial growth. When phenylalanine is added to the culture, bacterial growth is rescued. As such, filter paper, spotted with a drop of blood, will lead to growth of the culture in proportion to the concentration of phenylalanine in the blood[49]. This assay provided a simple and scalable assay for diagnosing hyperphenylalaninemia in infants. Within just a few short years, hospitals throughout the United States were taking blood through a heel prick, sending the blots for analysis, and quickly providing a diagnosis and treatment plan for affected newborns. In this way, newborn treatment and screening was born. Today, blood Phe is quantitatively measured along with many other amino acids, which also screens for a multitude of IEMs. The general process of newborn screening followed by dietary restriction of Phe remains the standard of care for patients throughout the medically-developed world.

One of the problems with this process is in the dietary restriction itself. For many years, it was thought that this dietary restriction only needed to occur through infancy and after a few years the majority of damage had been avoided. However, patients taken off diet soon began experiencing disruptive behaviors

and intellectual decline: adolescence was not fully protective against phenylalanine causing brain damage[50]. Over the past 25 years, the standard of care for those in the United States has been a life-long dietary restriction of phenylalanine[51,52]. However, considering the rigorous and unpalatable nature of this treatment, it has been estimated that the majority of patients go off-diet for some period of their life[53]. Additionally, Phe-free medical food will cost patients and their insurers millions of dollars over their lifespan. For this reason, as well as a rejection of its benefits in adult patients, many developed countries do not support dietary restriction after patients enter adulthood. As such, there is an unmet treatment need for patients with PKU that cannot be filled through dietary restriction alone.

Sapropterin Dihydrochloride (Kuvan)

The description of PKU as a disease caused by mutations in the *PAH* gene is slightly inaccurate. Since PAH relies upon the cofactor tetrahydrobiopterin to aid in its catalytic activity, mutations that result in a deficiency of tetrahydrobiopterin (BH_4) can also lead to hyperphenylalanemia[33]. Therefore, supplementation of this cofactor has been a long-theorized treatment option for those with BH_4 deficiency or those with mild hyperphenylalaninemia, such as those with mutations in the regulatory or tetramerization domains of *PAH*[33]. The pharmaceutical variant of BH_4, sapropterin dihydrochloride, more commonly known by the brand name 'Kuvan', was approved as the first pharmacologic PKU treatment in 2007[54]. The oral ingestion of Kuvan has been shown to restore healthy blood Phe levels in those with BH_4 deficiency and

significantly lowers the dietary Phe concentration in PKU patients with low Phe levels[55–57].

PEGylated Recombinant Phenylalanine Ammonia Lyase (Pegvaliase/Palynziq®) Treatment

The exogenous supplementation of PAH has been a goal for PKU treatment since the protein was initially identified. However, there were two factors that made development of such a protein as a therapeutic challenging. The first is the same obstacle that all protein supplementation therapies face: injected proteins are rapidly degraded by proteases in the bloodstream. The second is that PAH, as we have discussed, cannot efficiently function without its cofactor: tetrahydrobiopterin. This cofactor's presence in the bloodstream is not sufficient to facilitate PAH activity.

Since the 1960's, scientists have theorized that attaching polyethylene glycol (PEG) to proteins, or PEGylating them, would cloak them from immune detection and increase their lifespan[58]. This has led to the PEGylation of proteins, aptamers, peptides, small molecules, and siRNA's[59]. PEGylation was thought to be a possible solution for PAH degradation as well, but the second problem, regarding cofactor availability remained. As a solution, research into plant and fungi metabolism has characterized phenylalanine ammonia lyase (PAL), which requires no such cofactor in the conversion of phenylalanine into ammonia and trans-cinnamic acid[60]. In 2018, after many years of development, BioMarin Pharmaceutical had their PEGylated PAL protein, dubbed "Pegvaliase" or "Palynziq", approved by the FDA[61].

Daily injections of Pegvaliase allow patients with classical PKU to consume a nearly unrestricted diet. From my personal interactions with patients recently prescribed Pegvaliase, it is the change of dietary behavior that is the most challenging. After spending a lifetime under strict dietary restrictions, the aversion of new foods does not easily subside. In fact, many patients struggle to bring their protein intake up after going on Pegvaliase, which highlights how stressful a protein restricted diet is for patients and suggests that many of the behavioral outcomes that we attempt to measure, such as depression, social isolation, and generalized anxiety, may be a result of an medically induced eating disorder.

While Pegvaliase represents a significant advancement, it is not universally restorative or accessible. Adverse events, including hypersensitivity adverse events (HAEs), are common and necessitate gradual dose escalation under medical supervision, with some patients discontinuing treatment due to intolerability[62–64]. Additionally, BioMarin estimated a gross profit of $192,000 annually for each patient on Pegvaliase, which makes the drugs unaffordable in most of the world[65]. As such, a truly curative treatment option would still provide great benefit to the vast majority of PKU patients in the world.

Liver Anatomy, Function, and Targeting

The liver plays a singular role in human metabolism and is the primary treatment site for all of the diseases discussed thus far. The liver itself is perhaps the most versatile and resilient organ in the body, playing a vital role in bile production, fat and protein metabolism, the urea cycle, and the detoxification of harmful substances. In addition, the liver retains an ability typically reserved for planarians and amphibians: cellular regeneration following chemical or physical damage. This regenerative ability is likely correlated with a predisposition towards polyploidy: murine and human liver cells, are commonly tetraploid or octoploid. As the organ ages, the number of diploid cells decreases while the content of polyploid hepatocytes increases. If this organ is to be the main target for a therapeutic development, it would be useful to know about these kinds of eccentricities as well as its general architecture. The following will begin by exploring the microarchitecture of the liver and how liver cells, called hepatocytes, divide their functions based upon zonation before expanding outward to illustrate the position of the liver in the body.

The liver is largely composed of three different cell types: hepatocytes, Kupffer cells and hepatic endothelial cells. As previously mentioned, hepatocytes are the main cell type of the liver and conduct most of the functions associated with the organ, from metabolism to hormone production. Kupffer cells, also known as stellate macrophages, line the hepatic vasculature and act as the doormen of the liver: allowing nutrients, drugs, and hormones access, while rebuffing and recycling senescent erythrocytes, apoptotic hepatocytes, bacteria, and other enemies of the innate immune system[66–68]. If Kupffer cells are the

“bouncers” of the liver, then endothelial cells are the doorway itself. Hepatic endothelial cells are perhaps the most porous and permeable cells in the human body, with fenestrations pocking their surfaces allowing a physical portal from the circulatory system into direct contact with hepatocytes[69,70]. In short, hepatic endothelial cells are a single layer of cells that comprise the vascular structures of the liver[71].

Just as the pattern of wallpaper is comprised of a repeating series of basic units stacked between, above, and below one another, the liver is composed of a repeating base unit called the hepatic lobule (**Fig. 1-3A**)[72]. The hepatic lobule is essentially a hexagon. At the center of this hexagon is the central vein, which acts as the exit route for blood in the liver. Through the central vein, deoxygenated blood is transported back to the heart via the inferior vena cava. At each of the six corners of the hepatic lobule lies a cluster of three critical vessels: the bile duct, hepatic artery, and portal vein (**Fig. 1-3B**)[72]. Together, these vessels, known as “the portal triad”, supply the liver with a variety of products, such as oxygenated blood, nutrients, and ammonia. Despite its dense vascular network, the liver is not a homogenous organ. Hepatocytes located near the portal triad receive more oxygen compared to those closer to the central vein, creating an oxygen gradient across the lobule[73]. This gradient defines three functional zones of the liver, each with distinct metabolic and physiological roles[72].

The hepatocytes in zone 1, or the periportal hepatocytes, are often the primary therapeutic target for patients with an inherited error of metabolism.

These cells perform much of the oxidative metabolism, gluconeogenesis, and ureagenesis that is vital for the body[74]. Perivenous hepatocytes, in zone three, are largely in charge of glycolysis, ketogenesis, and drug metabolism[74]. Proteins associated with the function of cytochrome P450s are expressed in these cells[75]. Meanwhile, hepatocytes in zone 2 of the liver share some functionality with its neighbors in zone 1 and in zone 3. However, zone 2 hepatocytes perform a majority of the regenerative function after liver damage[76].

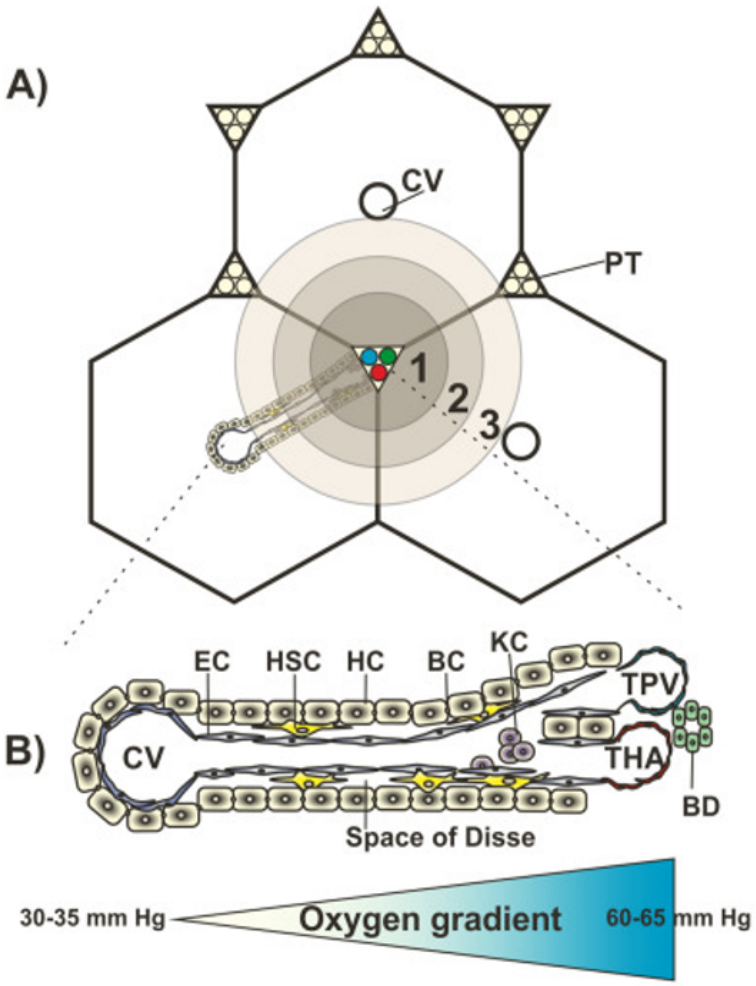

Figure 1-3. **Schema of the Hepatic Lobule**. (A) Layout of three hepatic lobules with depictions of the central vein (CV), portal triad (PT), and hepatic zones 1-3. (B) Illustration of hepatic sinusoid and the cells that compose it, such as endothelial cells (EC), hepatic stellate cell (HSC), hepatocytes (HC), and Kupffer cells (KC). Additionally, bile canaliculi (BC) transport bile from the liver to the bile duct (BD), which composes the portal triad along with the hepatic artery (THA) and the portal vein (TPV). As blood flows from the portal triad to the central vein the oxygen concentration decreases from zone 1 to zone 3. Reprinted with permission from Thomas Kietzmann, *Redox Biology*, 2017[72].

Now that we have a general idea of the composition, structure, and function of the hepatic sinusoid, let us zoom out to discuss the liver at large. In many mammals, such as humans, mice, and whales, the liver is located on the right side of the abdomen, adjacent to the stomach and sitting above the gallbladder (if they have a gallbladder). In humans and mice, the liver is composed of four lobes[77]: the caudate lobe, the right lateral lobe, the median lobe, and the left lateral lobe. As we have discussed, portal triad supplies these lobes with blood and nutrients, though the route and exact contents of each vessel varies dramatically.

Oxygenated blood flows directly from the heart into the liver via the hepatic artery. This artery branches and enters the liver at two sites: the left and right hepatic artery. However, only ~25% of the liver's blood supply is fulfilled through this route[78]. The majority of hepatic blood is supplied via the portal vein, which is formed from the veins of the intestine, spleen, and stomach[78]. As such, the portal vein supplies the nutrients and waste that will quickly be processed and metabolized by the liver. Additionally, the gallbladder, located just beneath the liver, stores bile produced by hepatocytes. Bile is secreted into the intestine through bile ducts connected to the liver, playing a crucial role in the emulsification and absorption of dietary fats during digestion. Finally, as the blood flows through the liver sinusoids, it collects into the central vein that leads into the inferior vena cava.

The CRISPR System

In 1987, a paper was published reporting the partial sequencing of the *iap* gene in bacteria. It briefly noted the presence of short, repeating, palindromic, DNA sequences separated by approximately 20 base pairs[79]. Unknown to the authors at the time, this small observation would end up sparking a revolution in genetic research and invigorating gene editing research to an unprecedented scale. However, until sequencing technologies progressed, the nature of this idiosyncratic bacteria element was not fully elucidated. It was hypothesized that the organization of this fragment suggested some kind of library, for why else would you need **C**lustered-**R**egularly-**I**nterspaced-**S**hort-**P**alindromic-**R**epeats (CRISPR), as Francisco Mojica and Ruud Jansen called them in 2000, if not to organize [80].

I assume these were heady days for genetics and molecular biology research, during a time when sequencing data was as intermittent and vague as a telegram from a far-off corner of the globe, scientists postulated that CRISPR sequences were part of some vestigial gene regulation system, that they were simple 'junk DNA' with no function, and some correctly guessed that they were a part of a bacterial immune system. After all, even bacteria need an ability to defend against viruses, the archfiends of cellular life. However, it wasn't the palindromic repeats that provided the pivotal clue for unraveling this mystery, but the ~20bp sequences, called 'protospacers', between the repeats. These sequences were found to be identical to DNA sequences found in viral genomes, which partially confirmed early suspicions that the CRISPR sequences are involved in a bacterial immune system[81,82]. As it would turn out, these sequences

between the clustered repeats encoded guide RNAs for a programmable nuclease.

There were a number of bacterial elements near these repeated sequences that seemed the be associated with the CRISPR system. Emmanuelle Charpentier and Jennifer Doudna discovered that one of these CRISPR-associated sequences (Cas) was a nuclease that aided or connected with CRISPR RNA (crRNA) encoded by the "protospacers"[83]. In bacteria, the crRNA will form a duplex with trans-activating RNA (tracrRNA), which forms a complex with Cas9. This crRNA/tracrRNA hybrid could be compared to a sword; on one end is the blade (crRNA) that makes contact with the target and on the other end is handle (tracrRNA) that mediates the interaction between the wielder and the target. In modern molecular biology the crRNA and tracrRNA are linked as a single guiding RNA (gRNA)[83]. Once Cas9 has taken up this guiding RNA (gRNA), it will attempt to find a DNA sequence complementary to the gRNA, bind to it, and then cleave both strands of the target strand at that site.

The CRISPR/Cas9 system was initially characterized in *Streptococcus pyogenes* bacteria. One of the limitations of CRISPR nucleases, such as *S. pyogenes* Cas9 or SpCas9, was that the target sequence had to be upstream of a specific sequence called a protospacer adjacent motif (PAM)[84]. For SpCas9 the PAM sequence was N-G-G, but for others, such as *Streptococcus aureus* Cas9 (SaCas9), the PAM sequence is different. Some have attempted to create "PAM-less" Cas9 variants, though the PAM sequence greatly narrows possible binding

sites for Cas9, which likely leads to increased nuclease activity and decreases off-target binding.

An easily programmable nuclease had long been desired by geneticists who had previously relied on TALENs[85] and zinc-fingers[86] to create targeted double-strand breaks. These tools could take the better part of a year for a small lab to design and validate, making their development costs untenable for all but the most dedicated investigations. As such, the characterization of Cas9 marked a breakthrough, offering a new, accessible way to manipulate DNA. By simply altering the sequence of a short RNA, scientists could precisely target any desired location among the 3 billion nucleotides in the human genome. This innovation, combined with Mathew Porteus's discovery of homology-directed repair (HDR)[87,88]—which demonstrated that double-strand breaks (DSBs) could be repaired, or new sequences inserted, using a DNA template flanked by homologous sequences—has revolutionized gene editing and advanced the field of gene-targeting technologies. This work defines HDR as the repair of nuclease-induced DSBs using a synthetically produced repair template, while homologous recombination (described in the section below) refers specifically to the endogenous repair pathway itself

It should be noted that homology-based editing is simply one method for inserting or editing the genome and other editing tools and strategies have been utilized for this end. Such as base editing[89], prime editing, homology-independent targeted insertion (HITI)[90], PASTE editing[91], and DNA/RNA writers being developed by Tessera Pharmaceuticals.

Homologous Recombination (HR)

All living creatures experience DNA damage over the course of their existence. Whether the source is a targeted nuclease or ultraviolet light[92], sooner or later a cell must repair damage to its DNA. Allowing DNA damage to endure in the genome may lead to a loss of transcriptionally active genes or regulatory elements, impaired DNA replication, and chromosomal instability. As such, cells have evolved an arsenal of repair mechanisms to resolve a wide variety of DNA damage. Homologous recombination (HR) is just one of these pathways, which is employed to faithfully repair DSBs using the sister chromatid as a repair template. This pathway is essential for HDR, making it of special interest to genetic editing research. As such, this brief review of HR has been included to describe the notable milestones and biochemical features of the HR pathway.

HR is typically active in S and G_2 phases of the cell cycle, when sister chromatids are in proximity and chromatin is relatively open[93]. One of the early events of HR, which likely directs DSB repair towards HR and away from competitor pathways, is the 5'-to-3' resection along a single strand of the blunt-ended DNA strands, creating two single-stranded 3’ overhangs on either side of the DSB. This resection is first mediated by binding of MRE11, RAD50, and NBS1 (MRN complex)[94]. MRE11, together with CtIP, create a single-strand nick upstream of the DSB and degrade DNA from the nick to the breakpoint, 3'-to-5', to initially produce short 3' overhangs[95]. These overhangs are elongated by the nucleases EXO1 or DNA2 after the Bloom (BLM) helicase binds to the MRN complex[94–96]. While the MRN complex, along with the BLM helicase and either EXO1 or DNA2, extends the resected DNA ends, it should be noted that this

process is complex and is supported by a variety of critical factors. Among these are Ataxia-Telangiectasia Mutated (ATM) kinase, which phosphorylates p53, Chk2, and histone H2AX, and BRCA1, which can itself bind with CtIP to promote end resection[97,98].

After resection is complete, the 3' ssDNA overhangs are rapidly bound and protected by replication protein A (RPA), a complex of three different proteins (RPA70, RPA32, and RPA14). The BRCA2-DSS1 complex then mediates the replacement of RPA with RAD51, which forms helical filaments that act as a scaffold to mediate the search for homologous sequences further downstream in the pathway[99–101]. The search for a homologous donor molecule occurs through a poorly understood process called base-flipping, where double stranded DNA is denatured to sample for homology with the single stranded overhangs[101–103]. For a review of proposed proteins involved in bridging RAD51 filaments with a repair template, such as BRCA1-BARD1 and RAD54, please see the following references[98,101,104,105]. After an acceptable repair template is found, the 3' overhang of the DSB invades the donor molecule, forming a D-loop that is resolved into Holliday Junctions. Here, the DNA heteroduplexes provide a "primer-template junction competent for DNA synthesis"[101]. This DNA synthesis is accomplished using polymerase δ, which is loaded and stabilized by RFC1-5 and PCNA[106,107]. Finally, the Holliday Junctions are cleaved using the Sgs1-Top3-Rmi1 complex, which resolves the DSB and the HR pathway[108].

In conclusion, the canonical HR pathway is a highly coordinated and complex system used to faithfully repair DNA. This process could be divided into

three general stages: end-resection, filament formation and homology search, and DNA synthesis. Much of the work that attempts to increase the frequency of HDR is ultimately attempting to influence the cell's dependence on the HR pathway. Research in Chapters 3 and 4 will explore targeted inhibition of competitive pathways to increase this frequency *in vitro* and *in vivo*. However, the first challenge of this process is developing delivery systems to transport these tools of genetic editing to their targets, which is discussed below.

Delivery Systems

It is extremely difficult to consistently deliver any molecule to billions, or trillions, of cells, especially if it is too large to diffuse through the cell membrane, *a la* small molecules. While there are a handful of truly manmade transduction methods—such as "gene guns" that physically shoot microscopic DNA-coated gold particles into cells—the most efficacious and elegant tools are, of course, those already developed by evolution. In fact, it's our great enemy The Virus that reigns supreme in the world of gene delivery, and, with some new and notable exceptions, it is through the manipulation of viral capsids that many gene therapies are delivered.

Viral Vectors: AV, AAV, and Lentivirus

Since Adenovirus (AV) genomes are large (36 kb), meaning scientists aren't as constrained by vector size limitations, AV was initially explored as gene delivery vehicles due this large packaging limit, as well as their ability to transduce a broad range of cell types. However, their application in gene therapy suffered a major setback after the tragic death of Jesse Gelsinger in a clinical

trial[109]. This incident highlighted the severe immune responses associated with adenoviral delivery and led to the near complete abandonment of AVs as a therapeutic delivery system.

Lentivirus is what HIV uses as a delivery system. It can carry a relatively large genetic payload (10 kb), and it randomly inserts throughout the genome and has proved carcinogenic in clinical trials[110]. Initially, this was thought to be perfect for gene editing: the only materials required are a transgene cassette packaged in a lentivirus and we could provide patients with plenty of copies of their missing gene. However, further research has suggested that lentivirus has a tendency to insert at oncogenes, thus providing a severe danger to patients treated with the virus. This concern is amplified by the results of several clinical trials in which patients treated with lentiviral vectors contracted leukemia and a variety of immune disorders[111–113]. Another major limitation is that lentiviral transduction is greatly facilitated by infecting dividing cells, so it is most useful in cell cultures and *ex vivo* approaches, and not post-mitotic cells *in vivo*. Additionally, high doses of lentivirus can cause clinically significant immune responses in patients making lentiviral gene delivery treacherous[111].

Adeno-associated virus (AAV) was initially identified as an impurity in adenovirus titers. As previously mentioned in the discussion of fenestrated endothelial cells, AAVs are a fraction of the size of adenovirus, which is likely a double-edged sword. On the one hand their small size aids in their evasion of the immune system and helps them pass through cellular fenestrations; on the other hand, it limits their genetic payload to ~4.8 kb, which significantly limits their

utility. However, unlike lentiviral and adenoviral vectors, AAV triggers a minimal immune response and does not directly affect the host DNA[114]. As such, AAV gene editing is the most commonly used viral vector for gene editing clinical trials[115]. Researchers have also developed a bouquet of serotypes from one of the first discovered, each with its own immunotoxicity and preferential cell type[116]. For liver-directed gene editing, AAV8 has been the standard, with doses of $1X10^{12}$ vg/kg capable of transducing nearly >90% of hepatocytes in mice[117].

Despite its low immunogenicity, AAV has proven lethal at high doses (above $5x10^{14}$ vg/kg)[118]. As such, there has been some interest in delivery of smaller doses directly into the portal vein of the liver instead of intravenous injections that will be transported across the body[119]. Another strategy of interest, especially in neonates, is the cannulation of the umbilical cord for delivery of viral vectors directly into the liver, which reduces the required viral dosage, while reducing the invasiveness of the treatment.

All viral vectors share the immune system as their major weakness. Even viral vectors with low immunogenicity, such as AAV, a single large dose of a serotype will prime the immune system and marshal an immune response against repeated doses. As such, gene therapy treatments that utilize these vectors can only be applied to a patient a single time. This makes them less like a therapy, which implies a continual application, and more like a moonshot, where a single application will dictate the treatment's success or failure.

For genetic engineers targeting the liver, delivering therapeutic agents to the majority of hepatocytes may be achievable with a single dose. If not for the

heart's central role in the circulatory system, the liver might well be considered the vascular Rome—where all roads lead. This is advantageous for those of us who would like to deliver drugs or transfection vehicles to the liver, as any substance entering the circulatory system inevitably passes through it. As such, many small molecules can be delivered via intraperitoneal (IP) injection, while more valuable vehicles, such as viral vectors, can be injected into the tail vein or retro-orbital vein. These routes ensure the payload reaches the liver via the hepatic artery (through the heart) or the portal vein (via the digestive organs).

One of the only confounding elements of hepatic targeting is the Kupffer cells, which have been known to absorb as much as 90% of intravenously injected adenovirus (AD)[120]. Unsurprisingly, some research has shown that depletion of Kupffer cells with chlodronate liposomes can significantly increase parenchymal hepatocyte expression prior to gene therapy[121]. Naturally, the low size and low immunogenicity assists AAVs in their role as a genetic vehicle, though a significant fraction of AAV dosing is likely still lost to Kupffer cells. As such, it isn't unusual for gene therapy treatments to deliver 1,000-1,000,000 viral genomes per hepatocyte.

Liposomes/Lipid Nanoparticles

Liposomes and lipid nanoparticles (LNPs), are essentially lipid-based micelles that incorporate additional components enabling them to function analogously to extracellular vesicles. Upon endocytosis into target cells, liposomes deliver their encapsulated cargo, commonly RNA, into the cytoplasm[122]. They are typically composed of four distinct components: ionizable cationic lipids, zwitterionic phospholipids, cholesterol, and poly(ethylene glycol)

(PEG) lipids[123]. A significant advantage of LNPs is their ability to avoid eliciting a pronounced immune response when correctly formulated, even with repeated administration. However, the inclusion of permanently cationic lipids, used in products such as Lipofectamine, have been shown to be limited *in vivo* due to "rapid plasma clearance, immune activation, and adverse toxicity"[124,125]. As such, it is largely the inclusion and modification of ionizable cationic lipids that allows for safe *in vivo* delivery and cell type targeting of LNPs. However, this cell targeting is largely dependent upon LNP's ability to bind to serum proteins, such as apolipoprotein E (ApoE), upon injection; facilitating further opsonization and cell type specificity[126,127].

Liposomes have two primary limitations. First, despite the modulation of ionizable cationic lipids, they lack the intrinsic cell-type specificity of many viral vectors. While the specific composition of a liposome can influence its preferential interaction with certain cell types, this specificity remains limited compared to that of viral systems and is entirely dependent upon serum proteins. Second, liposomes are generally ineffective at delivering their payload into the nucleus, a critical drawback for applications requiring nuclear delivery, such as the delivery of a DNA repair template into the nucleus. This limitation is less consequential when delivering short-lived RNA, as RNA can access the ribosomes in the cytoplasm for translation without necessitating nuclear entry. Nevertheless, the inefficiency of DNA delivery via liposomes constrains their utility in applications demanding precise genomic integration or modification. As

such, most of the following research utilizes viral delivery vectors, particularly AAV, instead of LNPs.

Chapter 2: Review of State-of-the-art Gene Therapy for Phenylketonuria

State-of-the-Art 2023 on Gene Therapy for Phenylketonuria

Michael Martinez,[1] Cary O. Harding,[1] Gerald Schwank,[2] and Beat Thöny[3]

[1]Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR, USA, [2]Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland, [3]Division of Metabolism, University Children's Hospital Zurich and Children's Research Centre, Zurich, Switzerland

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Abstract

Phenylketonuria (PKU) or hyperphenylalaninemia is considered a paradigm for an inherited (metabolic) liver defect and is, based on murine models that replicate all human pathology, an exemplar model for experimental studies on liver gene therapy. Newborn screening of hyperphenylalaninemia has been available for two generations, providing robust characterization of pathogenic *PAH* variations and biochemistry. Life-long dietary protein restriction provides relief from many classical PKU symptoms though significant shortcomings of contemporary dietary treatment of PKU remain. A long list of various gene therapeutic experimental approaches using the classical model for human PKU, the homozygous *enu2/2* mouse, witnesses the value of this model to develop treatment for a genetic liver defect. The list of experiments for proof of principle gene therapy treatments includes recombinant viral (AV, AAV, and LV) and non-viral (naked DNA or LNP-mRNA) vector delivery methods, combined with gene addition, genome, gene or base editing, and gene insertion or replacement. In addition, a list of current and planned clinical trials for PKU gene therapy is included. This review summarizes, compares, and evaluates the various approaches for the sake of scientific understanding and efficacy testing that may eventually pave the way for safe and efficient human application.

Introduction

Phenylketonuria (PKU) or hyperphenylalaninemia due to phenylalanine hydroxylase (PAH) deficiency (OMIM #261600) is among the most common inborn errors of metabolism (IEM) and is caused by recessively inherited biallelic variants in the *PAH* gene[128]. PAH (EC 1.14.16.1) catalyzes the irreversible hydroxylation of L-phenylalanine (Phe) to L-tyrosine (**Fig. 2-1**). While the global prevalence of PKU is estimated to be 1:23,930 live births with 0.45 million individuals that have PKU, in Europe and in the USA, the mean prevalence is approximately 1:10,000 newborns[129]. PAH is predominantly expressed in liver, and PAH activity requires molecular oxygen and the reduced pterin cofactor tetrahydrobiopterin (BH_4) for activity. PAH deficiency leads to hyperphenylalaninemia and relative tyrosine deficiency. Liver PAH deficiency has no pathologic effect on the liver, but the accumulation of Phe, through several pathogenic mechanisms, is toxic for both the developing and also for adult brain[128]. If left untreated, hyperphenylalaninemia leads to postnatally acquired microcephaly, global developmental disability, and seizures. In many medically advanced countries, newborns are screened for hyperphenylalaninemia shortly after birth, and infants with confirmed PAH deficiency are treated with dietary Phe restriction. Strict adherence to this complicated and unpalatable diet prevents the major manifestations of PKU, but the incidence of attention deficit and specific learning disabilities remains greater in treated PKU patients than in the general population[130]. The effectiveness of dietary therapy wanes dramatically during adolescence and adulthood, with only a small fraction of

treated individuals maintaining desired blood Phe concentrations with dietary therapy alone[131–133]. Elevated Phe concentrations are associated with the emergence of significant cognitive impairment including problems with attention, memory, and executive function, and a high incidence of psychiatric symptoms including anxiety and depression[134–136]. Chronically elevated blood Phe can lead to permanent neurologic damage manifesting as balance issues or seizures[137,138]. In addition, elevated maternal blood Phe during pregnancy is an extremely potent teratogen causing microcephaly, global developmental disability, and a high incidence of congenital heart disease in the (unaffected) infants of women with uncontrolled PKU[139]. Because the ultimate outcomes of contemporary dietary therapy are imperfect, novel therapies, and ultimately a cure, that do not rely upon dietary Phe restriction are highly desired[131].

The mainstay of therapy for PAH deficiency is a dietary restriction of Phe intake[52,140] through restriction of intact protein and supplementation with synthetic medical food products that provide adequate Phe-free nutrition. Dietary Phe tolerance improves modestly in a subset of individuals with PAH deficiency in response to treatment with oral sapropterin dihydrochloride, a synthetic form of the natural BH_4 cofactor[141]. These individuals carry *PAH* genotypes that yield some residual PAH enzyme activity[142], and sapropterin is likely performing a chaperone function to stabilize and activate the mutant PAH protein[143]. Only 20%–50% of PAH-deficient patients respond to sapropterin therapy[144,145]; and in most, partial dietary Phe restriction is still

necessary. For adults with PAH deficiency, enzyme substitution therapy is now available. Pegvaliase, a recombinantly produced PEGylated phenylalanine ammonia lyase (PAL) from the cyanobacterium *Anabaena variabilis*, is administered daily by subcutaneous injection, converts circulating Phe to trans-cinnamic acid and ammonia, and lowers blood Phe concentration to even allow some individuals to have typical dietary intact protein intake.[146,147] This treatment approach successfully reduces blood Phe but still demands daily injections, is associated with immune-mediated hypersensitivity reactions in some individuals, and is not a cure.

The pathophysiology of PKU is caused by the effects of Phe upon the brain, as described above. Therefore, any novel therapeutic approach that lowers blood and brain Phe while allowing unrestricted consumption of dietary intact protein will successfully treat PKU. Several innovative new treatments are in various preclinical stages of development, but here we will concentrate only on those genetic therapies that utilize nucleic acids as pharmaceuticals and ultimately aim to restore liver PAH expression. All preclinical testing depends on a valid animal model, and most gene therapeutic studies for PKU have been carried out in *Pah*$^{enu2/enu2}$ mice which are a model for human PKU[148,149]. In addition to this classical PKU mouse strain, other valuable models have been generated recently including animals harboring other *Pah* missense variants[43,149] and deletions[47,150]. The state-of-the-art of current gene therapy approaches for PKU are illustrated in **Figure 2-1**. The four primary approaches can be categorized into messenger RNA

(mRNA) therapy, gene addition (also called gene transfer), and two forms of gene editing, either gene correction of the malfunctioning *PAH* allele variant or independent insertion of a fully functional *PAH* expression cassette into the genome. We have previously described in detail the physiological requirements necessary for successful liver-directed gene therapy for PKU[151]. Here, we provide an update on recent progress toward the clinical implementation of these potentially curative therapies.

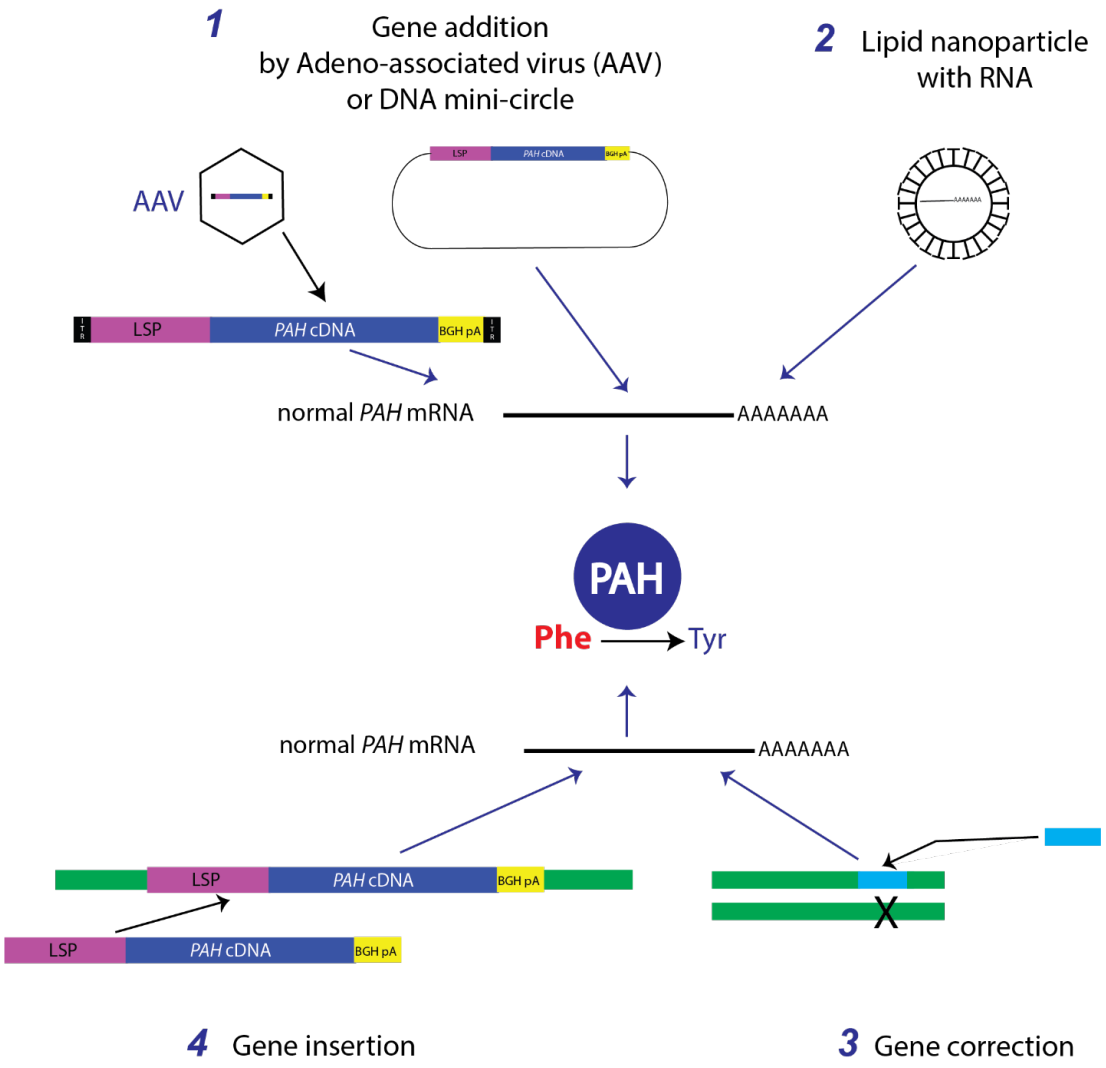

Figure 2-1. Gene Therapy for PKU. Phenylketonuria (PKU) is caused by recessively inherited variants in the phenylalanine hydroxylase (*PAH*) gene (Panel A). Phenylalanine hydroxylase (PAH) is a homotetramer that catalyzes the irreversible conversion of phenylalanine (Phe) to tyrosine (Tyr). The reaction requires reduced tetrahydrobiopterin (BH_4), iron, and molecular oxygen as cofactors (not shown). In the absence of PAH activity, phenylalanine accumulates in tissues and is non-enzymatically deaminated to phenylpyruvate and further oxidized to other phenylketones, leading to the

eponymic name phenylketonuria (PKU). Biallelic *PAH* variants encode variant *PAH* messenger RNA (mRNA) which then leads to either unstable, poorly active, or inactive PAH protein and impaired ability to hydroxylate Phe to Tyr in the liver. Genetic therapies (Panel B) aim to restore liver PAH expression by gene addition, or CRISPR/Cas-based gene or base editing; i.e. several different treatment approaches to accomplish this goal are under preclinical investigation with mice, including (1) gene addition, (2) delivery of therapeutic mRNA via lipid nanoparticles (LNP), (3) gene editing/correction, or (4) gene insertion. Gene addition is currently most frequently attempted through the delivery of a *PAH* expression cassette to hepatocytes using either recombinant adeno-associated virus (rAAV) vectors or nonviral (minicircle) vectors. rAAV genomes penetrate into the hepatocyte nucleus and predominantly remain episomal, not interacting with the host genome, but expressing the therapeutic transgene. Several different gene or base editing technologies are available to accomplish site-specific correction of the pathologic variant back to the wild-type sequence in gene correction. Some of these editing methods suffer from low correction frequency; all must be redesigned for every specific pathologic variant to be targeted. Gene insertion yields a combination of gene addition and gene correction by permanently inserting an entire *PAH* expression cassette somewhere into the hepatocyte genome (see text for more details).

Gene Addition

Various viral and nonviral delivery methods have been explored to deliver the PAH expression cassette to animal models (**Table 2-1**). The first demonstration of a successful gene addition approach utilized a recombinant first-generation adenoviral vector to express human PAH under the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter in the liver of the *Pah*$^{enu2/enu2}$ mouse model[152]. This resulted in complete normalization of blood Phe levels for approximately 2 weeks until the adenoviral infected hepatocytes were recognized and destroyed by the treated animals' immune system. Readministration of the adenoviral vector yielded no change in blood Phe due to the inhibitory effect of antiviral neutralizing antibody over cell transduction. The use of a similar adenoviral vector to deliver an ornithine transcarbamylase (OTC) expression cassette in a human clinical trial yielded only low levels of gene transfer, insignificant metabolic correction, and revealed a severe adverse inflammatory response that resulted in the death of a trial subject due to the treatment emergent effects[153].

Vector system	Transgene(s)[a]	Promoter[b]	Target Organ	Injection Method[c]	Duration of Treatment[d]	References
Gene addition						
Adenovirus	*hPAH*	RSV-LTR	Liver	i.pv.	1 week	Fang et al.[152]
Adenovirus	*hPAH*	CAG	Liver	i.pv. or i.v.	11 days	Nagasaki et al.[154]
rAAV2/2	*hPAH*	EF	Liver	i.p.	up to 25 weeks	Oh et al.[155]
rAAV2/5	*mPah*	CBA	Liver	i.p.	up to 40 weeks	Mochizuki et al.[156]
rAAV2/8	*mPah*	CBA	Liver	i.v. or i.p.	up to 42 weeks	Ding et al.[157]
rAAV2/8	*mPah*	LSP	Liver	i.p.	up to 17 weeks	Harding et al.[158]
rAAV2/1	*mPah, mGch1, mPts*	CMV	Muscle	i.m.	up to 70 weeks	Ding et al.[159]
rAAV2/1, rAAV2/2, rAAV2/8	*mPah*	CBA	Liver	i.m.	up to 53 weeks	Rebuffat et al.[160]
scAAV2/8	*mPah*	LP1	Liver	i.p.	up to 52 weeks	Yagi et al.[161]
rAAVHSC15	*hcoPAH*	CBA	Liver	i.v.	up to 48 weeks	Ahmed et al.[162]
rAAV2/8	*rAvPAL*	APOE-hAAT	Liver	i.v.	up to 24 weeks	Tao et al.[163]
rAAV2/Anc80	*hPAH*	Alb-A1AT	Liver	i.v.	up to 85 days	Kaiser et al.[164]
rAAV2/proprietary capsid	*mPAH, rAvPAL*	A1MB2-mTTR482	Liver	i.v.	up to 41 days	Manek et al.[165]
Non-viral (naked DNA)	*mPah*	P3	Liver	HTV	up to 52 weeks	Viecelli et al.[166]
Non-viral (naked DNA)	*mcoPah*	P3, endogenous	Liver	HTV	up to 60 weeks	Grisch-Chan et al.[167]
LNP-mRNA	*mPah*	not required (mRNA)	Liver	i.v.	repeated injections required	Perez-Garcia et al.,[168] Cacicedo et al.[169]
Lentiviral (LV)	*hPah*	mTTR	Liver	facial vein	up to 42 weeks (males only)	Vonada et al.[170]
Gene, base, and prime editing						
Dual rAAV2/8 intein	APOBEC-nSaCas9, sgRNA	P3, U6	Liver	i.v.	persistent (up to 26 weeks)	Villiger et al.[171]
Dual rAAV2/8	repair-template, SpCas9, sgRNA	LSP, U6	Liver	facial vein	serum Phe lowering only	Richards et al.[172]
LNP-mRNA	*SaKKH-BE3*-mRNA, sgRNA	not required (mRNA)	Liver	i.v.	persistent	Villiger et al.[173]
AdV	*SpCas9*-PE (PE2$^{\Delta RnH}$),	P3, U6	Liver	i.v.	persistent	Böck et al.[174]

Table 2-1. Overview of experimental gene therapy for PKU using PAH-deficient mice. *Note*: This table is based on a previously published overview article with updates[151].

[a]APOBEC, apolipoprotein B mRNA editing enzyme, catalytic polypeptide; *hPAH*, human *Pah* gene; *mcoPah*, murine, codon-optimized *Pah* gene; *mGch1*, murine guanosine triphosphate cyclohydrolase I gene; *mPah*, murine *Pah* gene; *mPts*, murine 6-pyruvoyltetraphydrobiopterin synthase gene; nSA*Cas9*, is a adenine base editor; pegRNA, prime editing guide RNA; SaKKH-BE3, variant of a *Cas9* ortholog from *Staphylococcus aureus*-base editor; sgRNA, single guide RNA; Sp*Cas9*-PE, a *Staphylococcus aureus*-based *Cas9* prime editor.

[b]A1MB2-mTTR482, two copies of alpha1-microglobulin enhancer with a modified mouse transthyretin promoter; Alb-A1AT, human albumin promoter and alpha-1-antitrypsin promoter; APOE-hAAT, apolipoprotein E enhancer and the human alpha-1-antitrypsin promoter; CAG, CMV early enhancer/chicken β-actin; CBA, CMV enhancer chicken β-actin promoter; CMV, cytomegalovirus; EF, human elongation factor 1-α promoter; LNP, lipid nanoparticle; LP1 promoter consists of the human apolipoprotein E/C-I hepatic control region (HCR) and the human α1-antitrypsin promoter (hAAT)[161]; LSP; liver-specific promoter is a combination of two copies of a human a1-microglobulin/bikunin enhancer and the promoter from the human thyroid hormone-binding globulin gene[158]; mTTR, mouse transthyretin promoter (liver specific); P3, synthetic liver-specific promoter[175]; rAAV,

recombinant adeno-associated virus vector; RSV-LTR, Rous sarcoma virus long-terminal repeat; scAAV, self-complementary AAV; U6, RNA polymerase III promoter.

[c]HTV, hydrodynamic tail vein injection; i.pv., intraportal vein infusion; i.m., intramuscular infusion (*M. gastrocnemius*); i.p., intraperitoneal injection; i.v., intravenous tail vein infusion.

[d]Blood Phe level below defined threshold for treatment of 360 µM. In the LV approach, *in vivo* transfected hepatocytes needed APAP selection to treat males while females were resistant to treatment.

All *Pah*$^{enu2/enu2}$ mice were treated as adults except for the gene editing study by Richards et al[172]. and Böck et al.[174] that treated newborn *Pah*$^{enu2/enu2}$ mice.

After many years of preclinical experimentation, as well as success with the human application, liver-directed recombinant adeno-associated viruses (rAAV) are currently the most advanced vectors for in vivo gene addition therapies[163]. DNA encoding a *PAH*-cDNA driven by the powerful liver-specific promoter and enhancer sequences is delivered to hepatocyte nuclei where wild-type *PAH*-mRNA is then produced. *PAH*-mRNA then traffics to the cytoplasm for translation to active PAH protein. The delivered PAH expression cassette typically does not directly interact with the endogenous *PAH* gene or other elements of the hepatocyte genome and predominantly remains as an independent episome. AAV is generally viewed as a non-pathogenic parvovirus with a single-stranded DNA genome. Wild-type AAV integrates its genome into a specific genomic site on human chromosome 19 and awaits adenovirus co-infection to become replication competent and to generate fresh infectious AAV virions. In rAAV vectors engineered for therapeutic purposes, nearly all AAV sequences except the inverted terminal repeats (ITRs) at the ends of the viral genome are removed and replaced with the therapeutic expression cassette. These vectors only rarely integrate into the host genome and are not replication competent, even if the host cell were to be infected with adenovirus. Nevertheless, the development of liver tumors upon insertional mutagenesis of rAAV vectors has been observed in mice and initiated an ongoing debate on potential risks for human application[176]. Following receptor-mediated endocytosis into hepatocytes, rAAV traffic to the nucleus and uncoat to deliver their single-stranded

genome. Either through recruitment and annealing of (+) and (-) genomes or through second-strand synthesis, single-stranded genomes become double-stranded duplexes; duplexes frequently form multimeric circular concatemers in which multiple genomes are arranged head-to-tail. These episomes are the predominant driver for the expression of the therapeutic protein. A comprehensive review of AAV vector biology has recently been published[177].

The choice of the particular AAV serotype, which refers to the type of capsid protein residing on the exterior of the AAV virion, dramatically influences the infectivity and the tissue distribution of the intravenously administered vector. AAV serotype 8 (AAV8), originally isolated from Rhesus macaques[178], has proven repeatedly to yield robust transduction in rodent liver and has been used by several laboratories to successfully treat murine PKU[157,158,161]. AAV8, however, transduces human hepatocytes farmed in a mouse xenograft model less efficiently than other serotypes[151]. AAV8 has been successfully used clinically to treat human hemophilia B due to Factor IX deficiency[179] and more recently, in data only released in abstract form but not yet published, in trials for glycogen storage disease type 1A and partial OTC deficiency. It will be difficult to objectively discern which AAV serotype will be best for human liver transduction as the majority of clinical trials are not performing liver biopsies and cannot, therefore, directly assess the frequency of cell transduction. To date, *only* adults with PAH deficiency have been treated in two separate industry-sponsored clinical trials of liver-directed

AAV-mediated gene addition. The trials differ in the details of the liver-specific promoter used in the PAH expression cassette and in the choice of AAV capsid. *Homology Medicines* is utilizing a proprietary AAV capsid labeled AAVHSC15, which has been shown to correct hyperphenylalaninemia in *Pah*$^{enu2/enu2}$ mice[162]. *BioMarin Pharmaceutical Corp* has designed an AAV5 vector very similar to that employed in their successful Hemophilia A trial[180]. No published data have yet been released from either clinical trial (**Table 2-2**).

Gene addition therapy using rAAV vectors, however, does present several disadvantages. First, although initial clinical trial experience with AAV-mediated liver-directed gene addition has been positive and has recently led to the first approval of an AAV5 vector for the treatment of Hemophilia A disease due to Factor VIII deficiency by the European Medicines Agency[181], ultimately the transduction efficiency in humans remains disappointing. Clinical efficacy in most trials to date has required rAAV doses of over 10^{13} vector genomes per kg body weight. For an adult, this equates to an absolute rAAV dose of at least one trillion (10^{15}) virions to attempt to transduce approximately 2 billion hepatocytes, corresponding to at least 500,000 virions per hepatocyte. Only a handful of clinical trials have performed liver biopsies to actually assess the frequency of successful hepatocyte transduction. For the recently approved Factor VIII-rAAV5 vector, *BioMarin* has published hepatocyte transduction data on five trial participants who consented to liver biopsy. In the two participants (numbers 3 and 4)

who received the highest vector dose of 6 x 10^{13} vector genomes/kg body weight injected intravenously[180], both enjoyed sustained Factor VIII expression with dramatically reduced bleeding episodes and need for prophylactic factor infusions for at least 3 years postinfusion[182]. Based upon their published body weights[180], these participants each received total AAV5 vector doses of 5.3 x 10^{15} and 3.6 x 10^{15} vg, respectively, which equate to approximately 2 x 10^{6} virions per hepatocyte. At liver biopsy 4 years after rAAV5 infusion[183], 45% and 52% of hepatocyte nuclei were positive for rAAV genomes by in situ hybridization, and 3.11 and 4.24 vector copies/diploid liver genome were measured in isolated liver DNA. We calculate that these measurements account for only about 0.0002% of the vector genomes originally injected. Liver biopsy was not performed earlier in the trial or repeatedly in the same study participant, so it is not known whether these data reflect stable transduction efficiency achieved immediately after initial rAAV treatment or are the remains of a steadily decreasing episomal vector genome population 4 years after the initial injection. Circulating Factor VIII concentrations have decreased over time in some trial participants, suggesting that the number of episomal vector genomes and consequently the amount of therapeutic Factor VIII expression in the liver is gradually decreasing and/or silenced over time, likely because of slow but typical hepatocyte turnover.

These results illustrate a major potential drawback for liver-directed rAAV-mediated gene addition therapy: the gradual loss of episomal vector

genomes from the liver due to hepatocyte turnover. In the course of cell regeneration, when the nuclear membrane dissolves for mitosis and cell division, no mechanism for replication and retention of the rAAV episomes exists. Any environmental insult or disease process that increases hepatocyte turnover could accelerate the rate of rAAV episome loss. At some point, the number of hepatocytes expressing the therapeutic gene could fall below a therapeutic threshold and allow reemergence of the disease phenotype. The threshold for the development of phenotypic symptoms will differ by disease; diseases such as hemophilia that may be ameliorated through low level expression of a secreted protein may be satisfied with transduction in only a few % hepatocytes, but a disorder of intermediate metabolism such as PKU (or OTC) deficiency may require significantly greater numbers of stably transduced hepatocytes to effectively clear the offending metabolite from circulation and prevent symptoms. Moreover, the therapeutic effects of rAAV-mediated gene addition are only temporary in juvenile animals as the vector genomes are quickly diluted in the rapidly proliferating hepatocytes of the juvenile liver[184–186]; the minimal age at which liver rAAV expression might remain relatively stable in human adolescents is unknown. Another bottleneck of rAAV therapies is the development of anti-AAV immunity and neutralizing antibodies following the initial infusion, which prevents vector readministration. Preexisting anti-AAV immunity in the population is also a significant limitation as many adults with metabolic disease who would otherwise be candidates for gene addition therapy have

already been exposed to naturally occurring AAV in the wild and harbor antibodies against various AAV serotypes. Efforts to circumvent both preexisting anti-AAV immunity and to prevent the development of antibodies against the vector to allow readministration in case of waning therapeutic efficacy are however under investigation (for more details see[88,187,188]).

Another aspect of rAAV vectors one should emphasize for treating human diseases is the “manufacturing challenges” and, as a consequence the associated costs to generate pharmaceutical products, when it comes to up- scaling for large scale good manufacturing practices (GMP) production of rAAV vectors (for an overview see[189]).

Most liver-directed trials to date have been relatively free of significant adverse events. Hepatocytes harboring AAV capsid proteins appear to trigger an asymptomatic cell-mediated immune response several days to weeks after infusion in many study participants. This is signaled by an asymptomatic rise in the serum ALT concentration and can be associated with a loss of therapeutic efficacy, first noted in hemophilia trials[190,191]. Treatment with oral corticosteroids, either prophylactically beginning at the time of rAAV vector treatment or initiated reactively at the first sign of ALT increase, appears to arrest this response and preserve gene expression. For some therapeutic indications, namely in those disorders in which skeletal muscle or the CNS is the primary target organ, rAAV doses of 10^{14} vg/kg or larger are administered intravenously to achieve penetration into the target tissue. Although the liver is not the primary target in these situations,

substantial liver transduction frequently occurs and at these doses is often associated with acute inflammatory reactions which have been lethal in some cases. Both acute cytokine storm occurring within a few hours of rAAV infusion and hypocomplementemic thrombocytopenic microangiopathy resembling hemolytic uremic syndrome, typically occurring a few days after rAAV infusion, have been described[192]. Extremely high doses of rAAV8 administered to boys with X-linked myotubular myopathy, a disease that causes intrahepatic cholestasis and liver dysfunction in addition to myopathy, have been associated with fatal subacute liver failure. Finally, rAAV-mediated gene addition is associated with a theoretical genotoxicity risk. Although the majority of rAAV genomes remain episomal, a substantial minority will integrate into the host cell genome[193], typically into sites of random double-strand breaks, and these integrations carry a theoretical risk for liver tumor development. Several studies have reported insertional mutagenesis in neonatal mice using rAAV vectors, although at low frequency but with consequences of hepatocellular carcinoma; a list of recommendations has been established to minimize the risk of adverse long-term effects upon using high doses of rAAV vectors for liver gene therapy, especially in young or very young patients[176].

Nonviral mediated gene addition using naked DNA, so-called "minicircle" or "mini-plasmid" vectors, lead to sustained, life-long transgene expression in experimental mouse studies without any evidence for integration[166,167]. Naked DNA vectors offer several benefits, including

straightforward and inexpensive manufacturing processes, including high-quality GMP material for human application, the potential for reapplication (upon loss of vector DNA due to hepatocyte turnover), and the (theoretically) unlimited DNA-vector size. Naked DNA delivery was employed to treat the *Pah*$^{enu2/enu2}$ mouse model. *Pah* was expressed on a minicircle DNA using either a liver-specific promoter ("P3") or the native (murine) *Pah* promoter, the latter in combination with a truncated 5^0 intron. Administration of this vector through hydrodynamic intravenous injection resulted in therapeutic PAH activity in the liver, which led to the sustained correction of blood Phe levels[167]. Naked DNA delivery into hepatocytes in large animals and humans remains challenging requiring methods involving open surgery for intraportal[194] or retrograde intrabiliary injection[195]. However, delivery by ultrasound-guided percutaneous transhepatic portal vein injection with small pigs is a minimally invasive method that has been well tolerated in piglets and has the potential for human (neonatal) application[196].

Company	Clinical trial identifier	Vector used (and other details, if know)
Homology Medicines	NCT03952156	AAVHSC15 (gene addition, phase 1, 18 - 55 years, not yet recruiting)
Homology Medicines	NCT05222178	AAVHSC15 (gene editing, phase 1, 18 years to 55 years, recruiting)
BioMarin Pharmaceutical	NCT04480567	AAV5-*PAH* (phase 1, 15 years or older, not recruiting)

Table 2-2. Clinical trials for PKU gene therapy. See also text for more details and/or on www.clinicaltrials.gov (as of May 2023).

mRNA Therapy

Delivery of a synthesized PAH-encoding wild-type mRNA to hepatocyte cytoplasm will restore liver PAH activity and correct blood Phe concentrations. The concept and the history of using mRNA as a therapeutic agent have been reviewed previously[197]. The key limiting issues for this approach are the method of delivering the mRNA to the liver and the short half-life of mRNA. Most commonly, delivery is accomplished through intravenous administration of mRNA complexed in a lipid nanoparticle (LNP)[198]. Liver-targeted LNP-mRNA complexes with ApoE in circulation and then is taken up via endocytosis into the hepatocyte. During endosomal maturation, acidification of the endosomal interior causes a critical cationic lipid in the LNP to become positively charged. Interaction between the positively charged cationic lipid in the LNP with negatively charged endogenous lipids causes disruption of the endosomal membrane and release of the mRNA cargo into the cytoplasm for translation. The design and preadministration processing of the mRNA, including codon optimization of the coding sequence and 5^0 capping with O-methylated guanosine, are critical to mRNA survival and expression. Replacement of uridine with pseudouridine in the primary mRNA sequence has been found to be necessary to avoid triggering innate immunity via Toll-like receptors in antigen-presenting cells[199,200]. Successful preclinical demonstration of restored liver PAH activity and correction of blood Phe concentrations in *Pah*$^{enu2/enu2}$ mice has been reported by several groups using different proprietary lipid nanoparticles[168,169]. Normalization of serum Phe levels over prolonged

periods, however, required repeated dosing every few days. This would be a limitation for clinical application, and to our knowledge, no clinical trials of mRNA therapy in PKU are currently active; nevertheless, trials in other IEM including ornithine transcarbamylase (OTC) deficiency, propionic and methylmalonic acidemias, and glycogen storage disease type 1a (GSD1a) are currently enrolling.

Genome Editing

Genome editing methods hold the promise of permanently altering the genome of the host hepatocytes to express intact PAH. Thus, after editing, PAH expression will be sustained even if the edited hepatocyte divides and its genome is replicated. Gene editing may include gene correction in which the patient-specific pathogenic variants in the *PAH* gene are corrected back to the wild-type sequence, or gene insertion, in which a PAH expression cassette that includes a *PAH*-cDNA driven by a strong promoter is permanently inserted somewhere into the host cell genome. Gene correction has the advantage that PAH expression is regulated (for liver-restricted expression) by the endogenous *PAH* promoter. In addition, it could also be used to treat patients with mutations that encode for (partially) dominant negative alleles, which mute or inactivate the wild-type PAH monomers provided in gene addition or gene insertion therapies. Circumstantial evidence for such effects was found by comparing a novel PAH-deficient murine model harboring a recessively inherited deletion of the *Pah* gene with the dominant negative *enu2* allele in the classical $Pah^{enu2/enu2}$ PKU mouse[191].

Furthermore, one of the most common mutant alleles in PKU patients, PAH-R261Q, was found to be a misfolding variant with a strong tendency to form toxic amyloid-like aggregates in the liver[149]. The gene insertion approach, nevertheless, may yield greater PAH protein expression per cell with a strong liver-specific promoter. The challenge for both approaches is to achieve PAH expression in a physiologically relevant number of hepatocytes and restore Phe clearance.

Gene correction may be accomplished through three different approaches: (i) HDR from template DNA following nuclease-mediated DNA double-strand cleavage, (ii) base editing using clustered regularly interspaced short palindromic repeats (CRISPR)-Cas guided deaminases, or (iii) prime editing using a CRISPR-Cas guided reverse transcriptase. A variety of DNA endonucleases have been developed to generate DNA double-strand breaks (DSB) at specific genomic sites including zinc finger nucleases[201], transcription activator-like effector nucleases (TALENs)[202], the homing meganuclease I-*Cre*I[203], and the extremely versatile CRISPR-*Cas* system[204,205]. Following the introduction of a DSB near a pathogenic variant in the *PAH* gene, repair of the DSB by HDR from an exogenously supplied DNA template can restore the variant allele to the wild- type sequence. Regardless of which nuclease is chosen to introduce the DSB, the success of gene correction is determined by the efficiency of HDR. Nonetheless, the default DSB repair pathways are non-homologous end joining (NHEJ) or alternate end joining (alt-EJ), which are not favorable for gene correction as they either

fix the DSB back to the original sequence, leaving the pathogenic variant intact, or generate insertion/deletion (indel) mutations[206]. However, HDR is more commonly utilized in dividing cells, and reports of successful in vivo gene correction using targeted nucleases have been documented in neonatal or juvenile animals.

Richards and collaborators have employed CRISPR-Cas9 technology with HDR-mediated repair in neonatal mice to correct the pathogenic variant that causes PAH deficiency in *Pah*$^{enu2/enu2}$ mice[172]. Here, two rAAV8 vectors were used for the treatment: one expressing the Cas9 nuclease, and the other expressing the guide RNA which directed Cas9 to cut the *Pah* gene at exon 7 (the site of the pathogenic c.835T>C variant) and a DNA repair template homologous to exon 7 with thymine at position c.835. The HDR frequency was only about 1%, which was insufficient to correct hyperphenylalaninemia in the mice. This low repair frequency is similar to that achieved in mouse models of OTC deficiency[207], hereditary tyrosinemia[208], and hemophilia B[209]. However, if the PKU mice were treated with vanillin, a potent NHEJ inhibitor[210], after rAAV vector delivery, the frequency of HDR increased to 13%. In this cohort, the mean liver PAH activity was 9.5% of wild-type liver activity, and mean serum Phe was reduced to 685 μM (range = 252–1168 μM). Thus, the treatment approach showed a notable improvement but was not sufficient to fully correct hyperphenylalaninemia, and further enhancements in editing efficiency will be necessary to achieve complete correction of blood Phe.

Base editors are novel genome editing tools that work independent of HDR and therefore also enable precise single base pair conversion in non-dividing cells. They are chimeric proteins that fuse a nuclease-impaired Cas9 domain with a DNA deaminase. The RNA-guided Cas9 recruits the DNA deaminase, either a cytosine deaminase or an adenosine deaminase to the targeted locus, converting C to T via uracil intermediates[211] or A to G via inosine intermediates[212]. A cytidine base editor delivered via an AAV8 vector has been successfully used to achieve correction of the *Pah*^{enu2} c.835T>C variant back to the wild-type thymine in more than 20% of hepatocytes, fully correcting hyperphenylalaninemia in adult *Pah*$^{enu2/enu2}$ mice[171]. The same base editor has also been delivered as messenger RNA via lipid nanoparticles, allowing the correction of hyperphenylalaninemia using a nonviral approach with transient expression of the genome editor[173]. Prime editing is a more recently developed Cas9-derived genome editing tool. Like base editing, it works independent of HDR and DNA double-strand break formation. A nuclease-impaired Cas9 molecule, which only nicks the DNA strand that does not hybridize with the guide RNA, is fused to an engineered reverse transcriptase. Using a template sequence that is fused to the 3' end of the guide RNA, the prime editor replaces the sequence at the site of the nick. This makes prime editing an extremely versatile tool, capable of repairing nearly all small sized mutations. Recently, prime editing has also been employed to treat neonatal *Pah*$^{enu2/enu2}$ mice[174], achieving average correction efficiencies of 11%. The drawback of this latter system is the lower

activity compared with Cas9 nucleases or base editors, and their large molecular size. A high dose of recombinant adenovirus, which has sufficient packaging capacity to contain the molecular size of the prime editor, was therefore required to reach editing rates sufficient for the therapeutic reduction of serum Phe levels.

Gene correction approaches described above are designed to target only a single specific disease-causing mutation. More than 1500 *PAH* variants are known in humans with PAH deficiency. Thus, the application of targeted nucleases, base or prime editing to the entire PKU population would require the development of potentially hundreds of personalized variant-specific reagents. Alternatively, along with gene addition, permanent insertion of an entire PAH expression cassette into the hepatocyte genome would be agnostic to the patient's disease-causing variant and allow the development of a single set of broadly applicable gene therapeutic reagents. *Homology Medicines* has reported achieving insertion of a promoterless human PAH cDNA into 6% of *PAH* alleles in human hepatocytes farmed in FRG mice, a humanized mouse model[213]. In this approach, the human *PAH*-cDNA is flanked by sequences homologous to *PAH* exon 1 and delivered as a rAAV vector by intravenous injection. *Homology* uses its proprietary capsid AAVHSC15 to deliver this *PAH* editing vector. Following the delivery of the rAAV vector genome to the hepatocyte nucleus, the human *PAH*-cDNA is permanently inserted into *PAH* exon 1 via homologous recombination, and *PAH* expression from the cDNA is driven from the endogenous *PAH*

promoter. Based upon preliminary results treating hyperphenylalaninemia in adult *Pah*$^{enu2/enu2}$ mice, *Homology Medicines* has initiated a Phase 1/2 clinical trial of this gene insertion approach.

The frequency of targeted gene insertion may be increased if a site-specific nuclease is employed to create a DSB at the desired insertion site. Again, this strategy relies upon endogenous DNA repair to insert the expression cassette via HDR into the site of the nuclease-induced DSB. Preclinically, OTC deficiency has been successfully treated using this mutation-agnostic approach in a murine model of the disease[214]. 25%–35% of hepatocytes harbored human *OTC*-cDNA insertions 8 weeks after neonatal administration of rAAV8 vectors expressing Cas9 and the repair template; OTC activity in liver homogenate measured 79% of wild-type activity. This level of successful correction was associated with a significant reduction in blood ammonia concentrations and improved survival during a dietary protein challenge. This nuclease-assisted treatment approach promises to yield a much greater initial integration frequency than without a site-specific DSBs and thereby produce a greater physiologic benefit. The risk of the procedure is the chance of off-target activity of the nuclease creating DSBs at sites other than intended. Insertions of a full expression cassette elsewhere in the genome should still produce therapeutic protein; the major concern is the potential for genotoxicity of DSBs and their imperfect repair at off-target sites, as well as chromosomal rearrangements occurring as a result of simultaneously generated DSBs at the on-target and off-target

sites. Still, this mutation-agnostic approach holds great promise as a permanent treatment for genetic diseases like PKU which exhibit a broad mutation spectrum.

Competitive Growth Selection of Genetically Modified Hepatocytes

The frequency of successful in vivo gene correction or gene insertion in the liver using currently available technologies is relatively low and struggles to reach physiologic significance in some diseases, such as PKU. In certain disorders that are associated with hepatocellular dysfunction, a genetically modified hepatocyte may have a survival and growth advantage over its unedited neighbors. For instance, genetically modified fumarylacetoacetate hydrolase (FAH) positive hepatocytes enjoy a tremendous selective advantage in animal models of human tyrosinemia type 1 and will almost completely repopulate the liver of *Fah*$^{-/-}$ animals[215–219]. A similar selective advantage exists for hepatocytes expressing methylmalonyl-CoA mutase in murine methylmalonic acidemia[220]. No such selective mechanism exists for PAH-expressing hepatocytes in PAH-deficient mice, so methods that might provide a selective advantage to genetically modified hepatocytes regardless of target disease or therapeutic transgene have been avidly sought. Recently, a selection method based on protection for the genetically modified hepatocyte against acetaminophen toxicity has been developed[170]. In this work, neonatal mice were treated with a lentiviral vector expressing both a therapeutic transgene and an inhibitory short hairpin RNA (shRNA) against NADPH-cytochrome P450 reductase (CYPOR). CYPOR is an

essential cofactor in the cytochrome P450 system that converts acetaminophen to its toxic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). Hepatocytes lacking CYPOR activity cannot convert acetaminophen to NAPQI and are protected from acetaminophen toxicity. When therapeutic lentiviral vectors are integrated into hepatocytes, they lose their CYPOR activity, which shields them from NAPQI toxicity. As a result, these protected hepatocytes will proliferate and repopulate the treated liver while their CYPOR-positive counterparts succumb to NAPQI toxicity. This selection method has been used to expand the relatively small population of hepatocytes with initial lentiviral integrations into a physiologically relevant population to (partially) treat murine hemophilia B or PAH deficiency[170].

Conclusions

Dietary Phe restriction remains the mainstay of treatment for PAH deficiency, but shortcomings in this therapy drive the continued search for novel treatments that do not depend so heavily upon dietary restrictions. Sapropterin supplementation improves Phe tolerance but does not eliminate dependence upon the Phe-restricted diet. Subcutaneous pegvaliase injection is the first available treatment that can in many patients significantly or fully increase the intake of dietary intact protein but also requires daily injection and is associated with immune-mediated hypersensitivity reactions. Significant unmet treatment need, particularly in adolescents and adults with PAH deficiency, remains; genetic therapies that aim to directly restore liver PAH activity are a promising and active research field. *PAH*-mRNA delivered

by LNPs has shown very promising results in preclinical models but has not yet been transferred to clinical trials; this technology is being studied clinically in other liver-based IEMs. Clinical trials of rAAV-mediated gene addition therapy in adults with PAH deficiency are underway; the release of definitive trial data is eagerly awaited. Gene addition approaches will not yield stable therapeutic gene expression in juveniles and ultimately the long-term stability of PAH expression in adult liver following AAV vector administration is also unknown, so permanent gene correction or gene insertion methods are under investigation. The low initial frequency of genetic modification leading to a subtherapeutic population of PAH-expressing hepatocytes as well as potential off-target editing remains the central challenge for these technologies. Methods to increase initial rates of gene modification or to yield a selective growth advantage for corrected hepatocytes will be necessary to bring most gene editing strategies up to therapeutic relevancy.

Acknowledgements
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Chapter 3: Protein & Small Molecule Enhancement of Cas9-Mediated Gene Correction *in vitro*

Abstract

The success of gene correction following a nuclease-induced targeted double-strand break (DSB) is dependent upon conditions favoring HDR involving a provided repair template rather than non-template directed repair of the DSB via non-homologous end joining (NHEJ). Our lab has previously demonstrated that vanillin, an inhibitor of the NHEJ repair pathway, significantly increases *in vivo* gene correction, suggesting that successful gene correction relies upon modulation of gene repair in favor of homologous recombination (HR). Based upon these results, we hypothesized that more potent inhibition of competitory DNA repair pathways would further facilitate HR and thereby increase gene correction frequency. To perform a high-throughput screen of potential small molecules and protein DNA repair modulations we utilized a modified HEK-293T EGFP cell line. Through a the Cas9-mediated correction of a premature stop codon in the EGFP sequence, we were able to ascertain the efficiency of HDR by measuring the percentage of EGFP positive cells in culture. Our results indicated that Olaparib and PIP6, inhibitors of Poly (ADP-ribose) polymerase (PARP) 1 and 2, are capable of increasing the baseline gene editing frequency nearly four-fold relative to controls. However, the mechanism of this effect remains poorly understood. Additionally, combined use of Novobiocin (NVB) and vanillin inhibit both NHEJ and microhomology-mediated end joining (MMEJ), providing increased editing by 54% *in vitro*.

Introduction

PKU is the most common inborn error of metabolism, affecting approximately 16,500 live births in the United States alone[221,222]. The classical treatment for this disease is lifelong restriction of phenylalanine through strict ingestion of Phe-free medical foods. However, due to the unpalatable, expensive, and isolating nature of this diet the vast majority of patients will struggle with treatment adherence despite knowing that it will likely cause irreparable harm to their cognitive abilities[223]. Alternatively, a new drug has recently been approved by the FDA called pegvaliase, which is a pegylated derivative of phenylalanine ammonia-lyase. This protein, commonly found in plants and fungi, converts phenylalanine to trans-cinnamic acid, and has been shown to lower Phe blood levels without the need of Phe-free medical foods[146]. However, a significant portion of PKU patients do not respond to this treatment or experience an immune response to daily pegvaliase injections, which prevents its universal adoption[146]. As such, our lab has endeavored to correct the genetic mutations that cause PKU, thereby providing permanent and potent relief from this debilitating disease.

The utilization of the CRISPR/Cas9 system has had an immense effect on the gene editing field over the past decade. This has led to the development of reengineered CRISPR products such as Cas9-HR[224] and AceCEcas9[225], which have broadened our gene editing arsenal. Despite these novel advances in the field there has been limited success in repairing pathogenic mutations *in vivo*. This is largely due to (1) difficulty sufficiently transfecting the target cell population of a developed organism and (2) the poor editing frequency observed

even among cells that receive all the necessary tools for editing. This second consideration suggests that the mode and method of inducing a double-strand break (DSB) is of little value if the endogenous systems that mediate editing are not harnessed. To this end, there has been a large effort in both industry and academia to identify factors that are capable of inducing the homologous recombination (HR) gene repair pathway over the more common non-homologous end joining pathway (NHEJ) (**Fig. 3-1A**). From this research a plethora of small molecules and proteins have been observed to increase gene editing, though most of these factors have not been validated in an *in vivo* context[226,227]. Additionally, the efficacy of these editing enhancers varies drastically depending upon the model, cell type, and loci of interest, which underlies the current gap in knowledge regarding what is necessary and sufficient for one gene repair pathway to be utilized over another. Here, we report a series of experiments to systematically measure the ability of a select group of these DNA repair modifiers to enhance HDR for the potential *in vivo* correction of murine Phenylketonuria.

Results

Addition of i53 and E18 Increase HDR in vitro

The addition of HR enhancing proteins with conventional CRISPR-Cas9 machinery has been shown to increase gene correction and integration. Recently, Rad18, and E3 ubiquitin ligase, has also been identified as a uniquely potent HR enhancer[228]. leading to the development of E18, an engineered Rad18 variant with a greater potential for facilitating HR[226]. Alternatively, a modified ubiquitin protein with affinity for 53BP1, dubbed i53, has been shown to increase gene integration through stimulation of DNA end-resection (**Fig. 3-1A**)[229].

To evaluate correction efficiency, a HEK-293T cell line harboring an EGFP sequence with a premature stop codon integrated into the AAVS1 locus was used. Confluent cultures were co-transfected with two plasmids: the first expressing SpCas9 and a validated guide RNA that directs targeted nuclease activity to a site near AAVS1. The second plasmid carried an EGFP repair template with an intact open reading frame. Thus, the cells expressed functional EGFP only after HR-mediated gene correction. In addition to plasmid transfection, the cells were treated with the selected NHEJ inhibitors. Following a 72-hour treatment, cells were harvested and sorted by EGFP fluorescence using flow cytometry. In this way, expression of EGFP acts as a direct indicator for successful gene editing in a facile and high-throughput platform without the need for sequencing (**Fig. 3-1B**).

FACS analysis demonstrated successful editing in control samples and in those treated with HDR protein enhancers (**Fig. 3-2A**). Addition of i53 caused a 2-fold increase in EGFP positive cells (**P = 0.0016), while the addition of E18

was correlated with a 54% increase but was statistically insignificant (ns = 0.4625) (**Fig. 3-2B**). Meanwhile, CLOCK, SPARTAN, and Rad51, which are respectively involved in cell cycle timing[230], DNA stabilization[231], and homologous strand pairing[232], displayed no such increase.

Our lab has previously demonstrated a ~50% drop in blood phenylalanine levels in *Pah*$^{enu2/enu2}$ mice following neonatal AAV8 facial vein injections with Cas9-Exon7 sgRNA and a repair template encoding the correction of the enu2 mutation[192]. However, this strategy only proved efficacious with daily injections of vanillin (100 mg/kg) immediately after injection. To test the *in vivo* efficacy of E18 and i53 for HDR enhancement, we devised a triple vector treatment strategy. E18 and i52 sequences were cloned into a plasmid downstream of a liver specific promoter (TBG promoter with a bikunin enhancer) for packaging in an AAV8 vector. In addition to our previously validated Cas9 and repair template vector, either E18-AAV8 or i52-AAV8 were then injected into *Pah*^{enu2} mice at P3. However, after weaning all mice displayed blood phenylalanine levels greater than 2,000 μM (**Fig. 3-2C**), indicating that the promotion of HR from our enhancing proteins was practically nonexistent for *in vivo* gene editing.

PARP-1 Inhibition Enhances Successful Gene Correction in vitro

In order to identify small molecule compounds that promote HR, several small molecule NHEJ inhibitors were evaluated *in vitro* for their ability to facilitate gene correction. Among those tested, Olaparib, an inhibitor of Poly (ADP-ribose) polymerase 1 (PARP-1) and 2, was capable of increasing the baseline gene editing frequency nearly four-fold relative to our control (**Fig. 3-3A**). For the results of the full *in vitro* small molecule screen see **Fig. S3-1**.

Prior published work has indicated that inhibiting PARP-1 can lead to hyper-resection of free DNA strands and increased HR frequency[233–235]. Resection of DSBs is required for HR, therefore the connection between these two features is plausible and a promising premise for enhancing HR (**Fig. 3-3B**). However, other studies conversely suggest that PARP-1 activity is required for HR[236], suggesting the possibility that inhibition of PARP-1 is incidental to our observed results. Finally, the mechanism of PARP inhibition may also play a role in this observed increase of HDR. Type 1 PARP inhibitors are defined by their ability to allosterically lock PARP-1 to DNA[237]. Olaparib, being a type 2 PARP inhibitor, binds to the NAD+ binding site of PARP1 and does not share this activity. However, previous research has shown that even PARP-1 can mediate the continuous dissociation and rebinding of PARP to DNA in a process known as "PARP trapping"[238]. Therefore, it's possible that our *in vitro* results in **Fig. S3-3A** may have been due to the attachment of a protein adduct at the DSB, which may be PARP-1 or PARP-2 based on Olaparib's reported affinity.

To help determine the exact cause of Olaparib's success, we used our *in vitro* assay to screen other PARP inhibitors with different specificities to PARP-1 and varying tendencies to 'trap' PARP on the sites of DSBs. Olaparib replicated its previous performance, but NMS-P118, one of the most discerning PARP-1 inhibitors with a 150-fold selectivity for PARP-1 over PARP-2[239], showed no enhancement of HDR (**Fig. 3-3C**). Simultaneously, Velaparib, a PARP-1 and PARP-2 inhibitor, demonstrated only a marginal increase in HDR efficiency (**Fig.**

3-3C). Interestingly, a type 1 PARP inhibitor called PIP6, produced in the lab of Michael Cohen, demonstrated a 4-fold increase of HDR[240] (**Fig. 3-3C**).

Small Molecule Toxicity in Murine Neonates

The benefits of PIP6 as an *in vivo* enhancer of HR are blunted by its high cytotoxicity, which is noted in our tissue cultures as well as a report of it having 90-fold greater cytotoxicity than AZ0108, a similar type 1 PARP inhibitor[241]. Three other small molecules, Novobiocin, Olaparib, and Fludarabine, which have been reported to increase HDR, were administered to neonates at therapeutically relevant doses. However, all three compounds resulted in significant mortality. Only Novobiocin, at one-ninth of its reported subcutaneous LD50, allowed a survival rate exceeding 75% (**Fig. 3-3D**).

Partial Hepatectomy

Considering the HR pathway is almost exclusively active in the S/G2 phases of the cell cycle, we were forced to consider the contribution of cytotoxicity in increasing HDR. We hypothesized that the *in vitro* enhancement we observed in our small molecule screen could be explained by cell death, leading to increased cell cycle and HDR in surviving cells as they expanded to fill the vacant space. Indeed, our previously reported HDR enhancement with vanillin was only successful in neonates, most likely due to the high turnover rate of the growing neonatal liver. To mimic this effect in adult mice we performed partial hepatectomies prior to injection of AAV8/Cas9- mediated gene editing tools and vanillin (100 mg/kg) in a small cohort of *Pah*^{enu2} mice. However, we found no significant biological response in these animals despite full regeneration of the liver, though only ~30% of the liver was removed (**Fig. 3-4**).

Figures

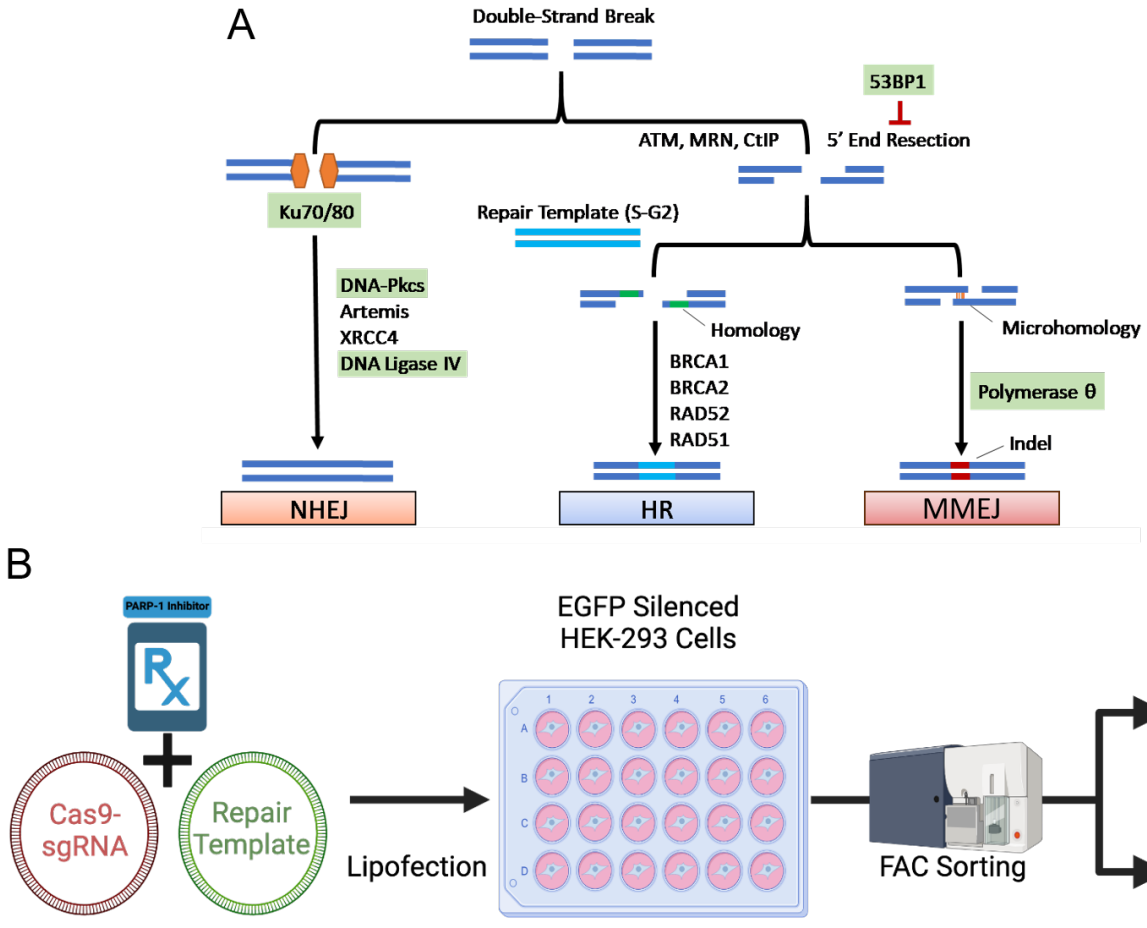
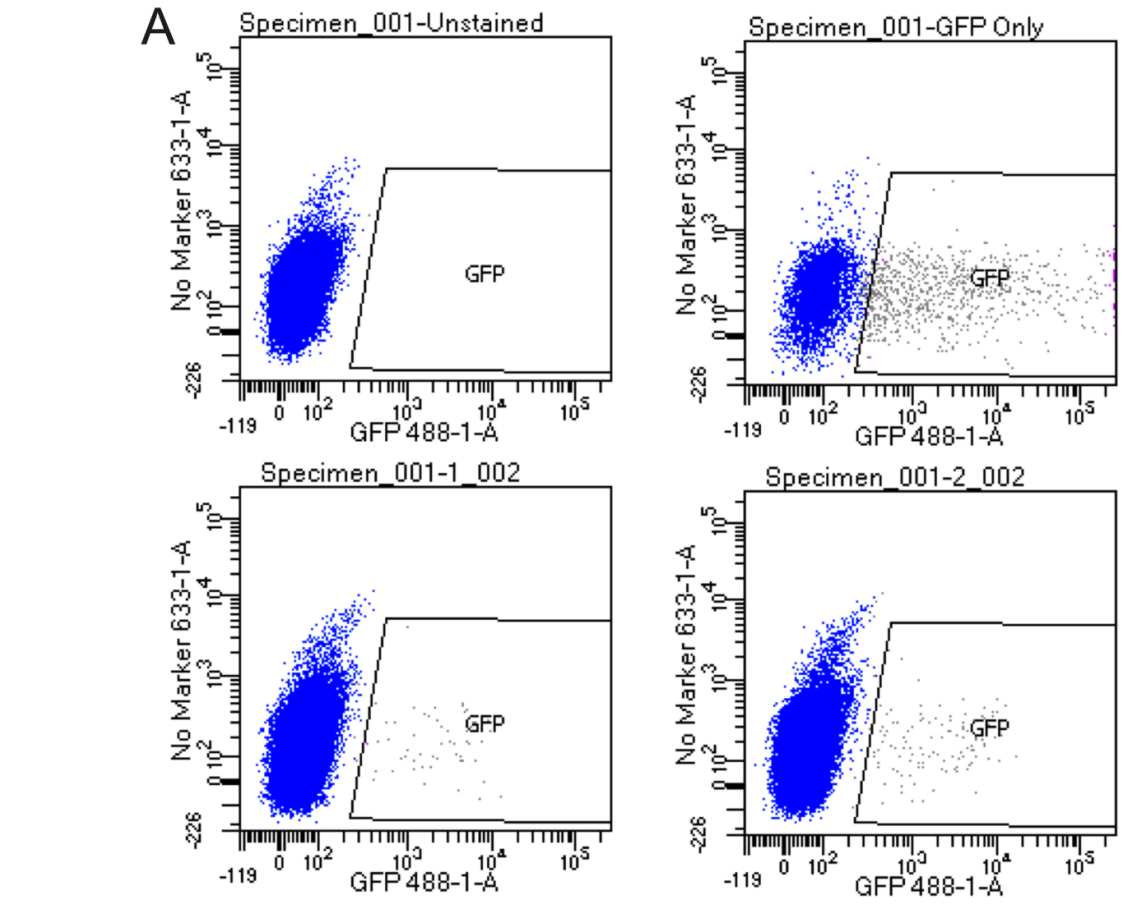
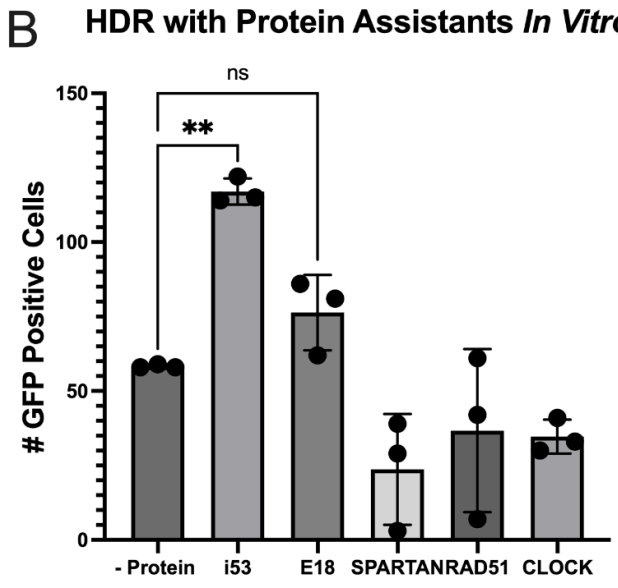

A
Double-Strand Break
53BP1
ATM, MRN, CtIP
5' End Resection
Ku70/80
Repair Template (S-G2)
DNA-Pkcs
Artemis
XRCC4
DNA Ligase IV
Homology
Microhomology
BRCA1
BRCA2
RAD52
RAD51
Polymerase θ
Indel
NHEJ
HR
MMEJ
B
PARP-1 Inhibitor
Rx
Cas9-sgRNA
Repair Template
Lipofection
EGFP Silenced HEK-293 Cells
FAC Sorting
%EGFP Cells = HDR Frequency

Figure 3-1. High-Throughput Workflow for Evaluating *in vitro* HDR Protein Enhancers. (**A**) Simplified DSB repair tree of non-homologous end joining (NHEJ), homologous recombination (HR), and microhomology mediated end joining (MMEJ). Targeted factors are highlighted. (**B**) HEK-293 cells were edited with an intact EGFP sequence, but with a premature stop codon between the promoter and cDNA. These cells were seeded on a 24-well plate and transfected with plasmids encoding Cas9, sgRNA targeting the stop codon, and a repair template to convert the stop codon to the first codon of EGFP. Simultaneously, small molecules or plasmids expressing HDR protein enhancers are added to the cell medium. The percent of fluorescent cells are analyzed by fluorescent-activation cell sorting (FACS) to measure HDR efficiency under these diverse set of conditions.


A
Specimen_001-Unstained
Specimen_001-GFP Only
Specimen_001-1_002
Specimen_001-2_002
No Marker 633-1-A
GFP 488-1-A
GFP
-226
-119
0
10^2
10^3
10^4
10^5


B
HDR with Protein Assistants *In Vitro*
GFP Positive Cells
ns
**
150
100
50
0
- Protein
i53
E18
SPARTAN
RAD51
CLOCK

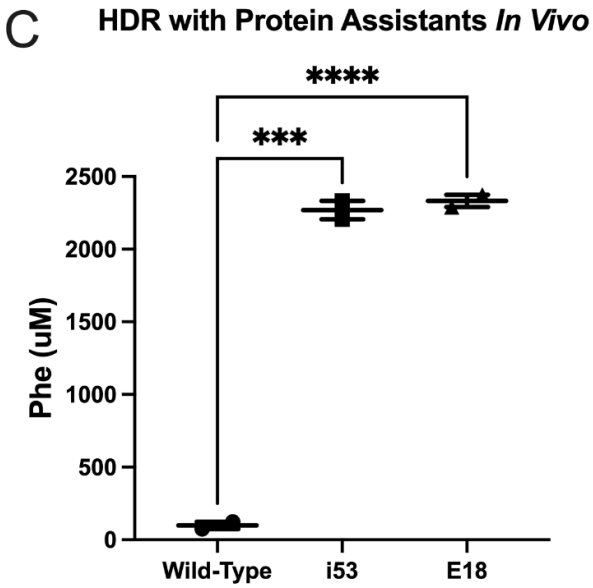

C
HDR with Protein Assistants *In Vivo*
Phe (uM)
****
***
2500
2000
1500
1000
500
0
Wild-Type
i53
E18

Figure 3-2. *In Vitro and In Vivo* Screen of HDR Protein Enhancers. (**A**) FACS analysis of untreated cell culture (Top Left), cell culture mixed with GFP positive cells (Top Right), treated culture with E18 expression plasmid (Bottom Left), and treated culture with i53 expression plasmid (Bottom Right). (**B**) Number of GFP positive cells in 10,000 cell culture with co-expression of i53, E18, SPARTAN, RAD51, CLOCK, or null vector (- Protein). Significance calculated using one-way ANOVA (***P* = 0.0016, ns = 0.4625). (**C**) *Pah*$^{enu2/enu2}$ neonates received facial vein injection of three AAV8 vectors at a dose of 1X10^{10} vg/kg: AAV8-LSP-Exon7-RepairTemplate, AAV8-LSP-Cas9, and either AAV8-LSP-i53 or AAV8-LSP-E18. Two mice were treated for each condition. All blood phenylalanine levels were above 2,000 μM indicating severe hyperphenylalaninemia indistinguishable from untreated *Pah*$^{enu2/enu2}$ animals.

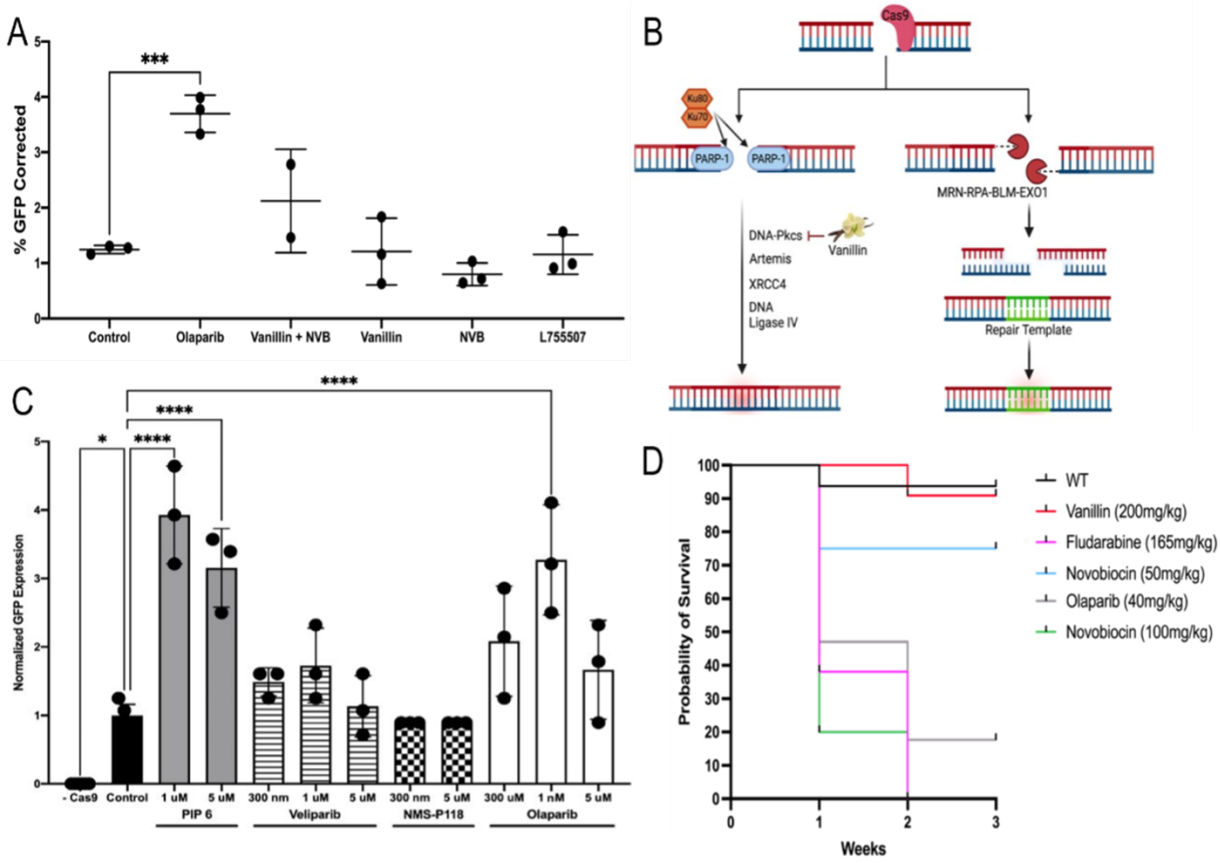

A
% GFP Corrected
***
Control
Olaparib
Vanillin + NVB
Vanillin
NVB
L755507
B
Cas9
Ku80
Ku70
PARP-1
PARP-1
MRN-RPA-BLM-EX01
DNA-Pkcs
Artemis
XRCC4
DNA
Ligase IV
Vanillin
Repair Template
C
Normalized GFP Expression
****
****
*
****
- Cas9
Control
1 uM
5 uM
300 nm
1 uM
5 uM
300 nm
5 uM
300 uM
1 nM
5 uM
PIP 6
Veliparib
NMS-P118
Olaparib
D
Probability of Survival
Weeks
WT
Vanillin (200mg/kg)
Fludarabine (165mg/kg)
Novobiocin (50mg/kg)
Olaparib (40mg/kg)
Novobiocin (100mg/kg)

Figure 3-3. PARP-1 and MMEJ Inhibitors Enhance HDR *in vitro*. (**A**) The percentage of CRISPR/Cas9-mediated correction of EGFP is displayed without the presence of a small molecule (Control) or with an inhibitor of PARP-1 (Olaparib), DNA-PKCS (Vanillin), DNA polymerase theta (NVB, Novobiocin), or 3-adrenergic receptor (L755507). Olaparib (1 μM) was the sole condition with a significantly increased EGFP correction compared to the control. All conditions were performed in triplicate sampling; 100,000 cells were screened per sample. (**B**) Simplified model of non-homologous end-joining and homology directed repair. PARP-1 inhibits end-resection, thereby inhibiting homologous recombination (Right), mediating DNA repair through the non-homologous end joining pathway (Left). (**C**) Effect of PARP-1 inhibitors on gene correction. Cells were treated with four PARP-1/2 inhibitors with varying mechanisms of action at three doses (300 nm, 1 uM, and 5 uM). Veliparib and NMS-P118 are PARP-1 specific inhibitors and did not display a significant increase on gene correction in comparison with Olaparib and PIP6, which are potentially more likely to 'trap' PARP-1 on DNA. (**D**) Survival of *Pah*$^{Dexon1/Dexon1}$ neonatal mice after injection of small molecules at P3 until weaning. Mouse cohorts between 15 and 23 individuals were used for each condition.

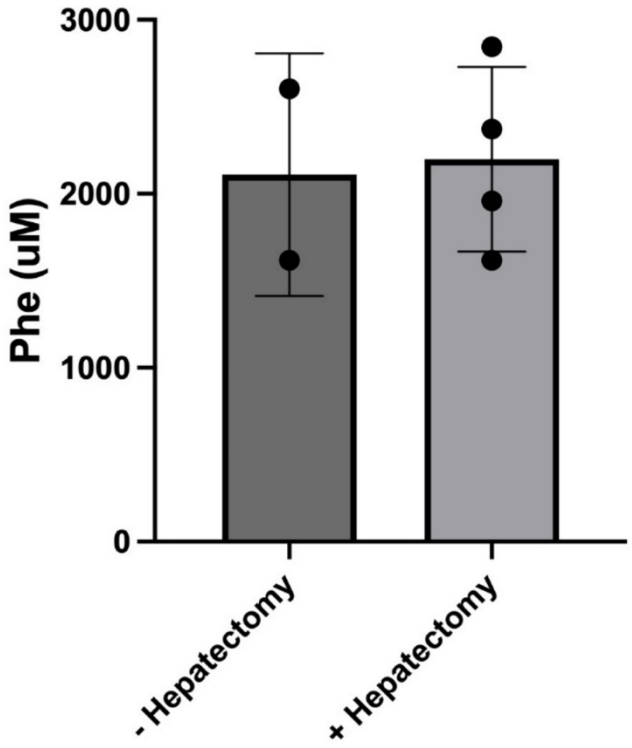
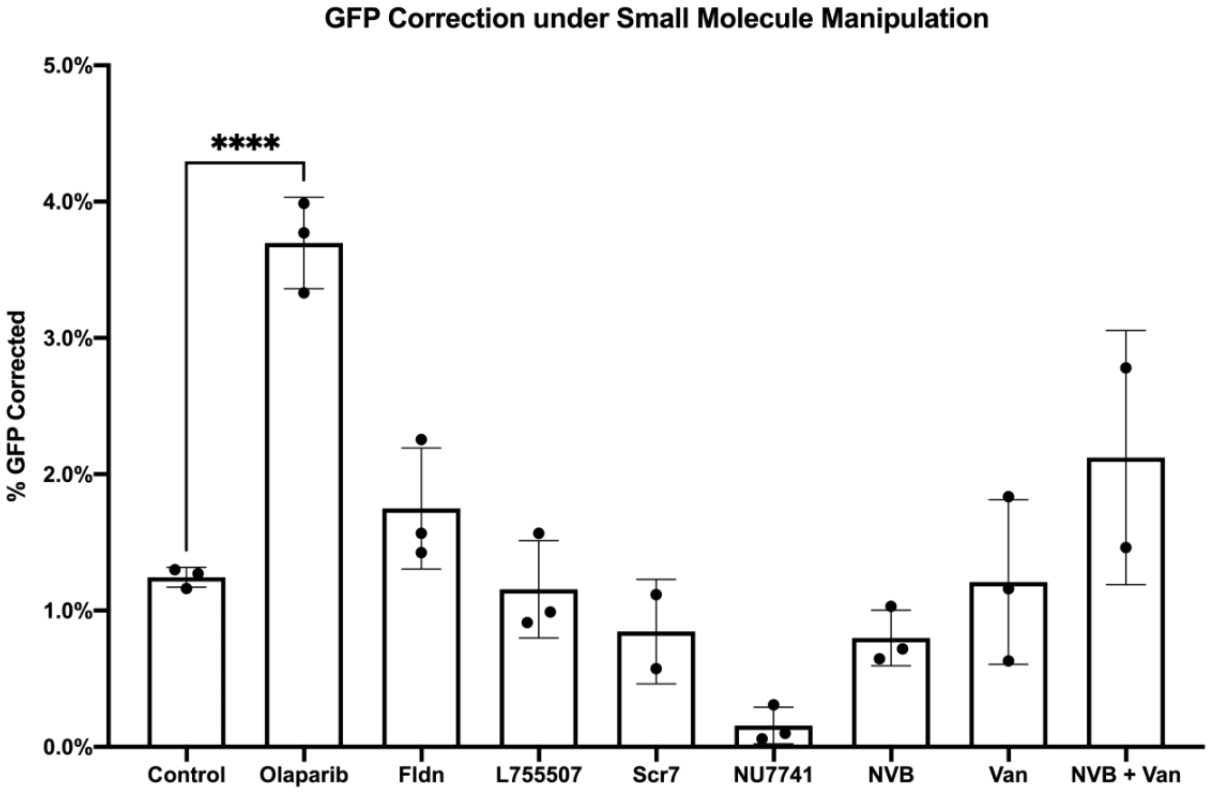


Figure 3-4. Partial Hepatectomy during dAAV-Vanillin Treatment of Adult *Pah*$^{enu2/enu2}$ Mice. Adult mice were injected with AAV8 vectors expressing Cas9 with a repair template for the Enu2 mutation, as previously reported. However, injections occurred one day after removal of 30% of the liver. Mean blood phenylalanine level of mice that received hepatectomy was 2,122 μM.

Supplemental Figure



Supplemental Figure 3-1. Small Molecule Perturbation of Cas9-mediated HDR rescue of EGFP *in vitro*.

Discussion

Decades of research have elucidated the sequential steps in HR, NHEJ, and MMEJ, but the mechanisms governing pathway determination are not fully understood. If targetable regulators of this process are identified, our ability to upregulate HR to increase the efficacy of gene editing would be far easier. In this study, we have targeted some of the earliest factors associated with NHEJ, MMEJ, and HR. However, DNA repair is a dynamic and flexible system. Maintaining genomic integrity is of vital importance to cellular life, and, when challenged, the cell will expend massive resources to repair damage or will undergo apoptosis to avoid endangering the body. As such, simplifying DNA repair into a binary system, when it is in fact manifold, may provide more dead ends than answers.

Both the literature and our own data suggest PARP proteins play an influential role in pathway determination. PARPs have been shown to bind to DNA at the site of DSB and recruit DNA repair factors, including the Ku70/80 complex, in one of the initial steps of NHEJ[233]. Additionally, a previous study that inhibited PARP-1 *in vitro* observed a significant increase in blunt end resection, which is a vital initial step in HR and MMEJ, and is typically mediated by attachment to the MRN-BLM-EXO1 complex [233]. This same study demonstrated that PARP-1, and not PARP-2 or PARP-3, occupies blunt-ended DNA, preventing accumulation of EXO1 at that site while simultaneously facilitating loading of Ku proteins to the break, thereby directing repair towards NHEJ[233].

In contradiction with this report, PARP-1 has been shown to facilitate binding of the MRN complex (specifically MRE11) to DSBs[242]. Another study observed a

reduced frequency of HR in PARP-1/2 chicken DT40 cells in the presence of Ku proteins, suggesting PARP-1 protects HR from interference from NHEJ associated proteins[243]. Additionally, PARP-1 has been implicated in the recruitment of BRCA1 and RAD51 onto DNA, which are both vital for HR[244]. Therefore, the premise of PARP-1 as an inhibitor of HR and a promoter of NHEJ is not clearly supported by the literature. Our data demonstrate that PARP inhibitors, such as Olaparib, can enhance HDR in an *in vitro* system (**Fig. 3-3**). However, our results may suggest a more nuanced role for PARP-1, where, due to PARP-1's interactions with EXO1, BRCA1, 53BP1, and Ku70/80[233,245,246], low levels of PARP inhibition can bias DNA repair towards HR, but a near total inhibition prevents DSB repair altogether, leading to cell death post-nuclease activity.

This hypothesis is further complicated by the important role PARP-1 has in other types of DNA repair, including nucleotide excision repair (NER), single-strand break repair, and base excision repair (BER)[247–249]. As such, we cannot ignore the possibility that inhibition of these pathways, which are typically not associated with DSB repair, may provide benefit for increasing the frequency of HDR. Since PARP-1 can bind to DNA during inhibition, specifically when utilizing a type I like PIP6, it is possible that our treatment created a DNA adduct in addition to a DSB. Such adducts may be eliminated through BER, in which PARP-1 recruits XRCC1 to act as a scaffold for DNA ligase 3 and polymerase β[250]. Given the potential inhibition of BER, these pathways were likely downregulated in our experimental context. This inhibition could explain the

cytotoxicity in treated PIP6 cells as well as mortality in treated mice. However, it is unclear how such a situation would potentiate HR and repair template integration. Future studies should examine the effects of BER inhibition on HR, possibly through the silencing of XRCC1. Additionally, shRNA-mediated silencing of PARP-1 may be used to prevent adduct formation and pharmacological toxicity.

Interestingly, our previously published gene correction experiment using vanillin demonstrated promising HDR results but a greater number (21%) of *Pah* alleles with indels[192]. Considering MMEJ also relies on MRN-mediated end resection, and inherently creates indels, we hypothesize this increase in homologous recombination causes a concomitant increase in MMEJ as well. The final step of MMEJ is catalyzed by polymerase Theta[251]. Novobiocin (NVB) is an antibiotic that has recently been shown to inhibit polymerase theta activity by binding to its ATPase pocket[252]. Furthermore, NVB has been shown to inhibit growth of HDR-defective tumors and acts synergistically with PARP inhibitors in both *in vitro* and *in vivo* tumor models[252]. Our *in vitro* data suggests that simultaneous administration of NVB, along with vanillin, inhibits both MMEJ and NHEJ to achieve a greater frequency of HR than could otherwise be achieved. Ultimately, further research in more representative hepatocyte cell lines or *in vivo* models will be required to understand the full biological implications of this work.

Methods

Animal husbandry

Animal care and experimentation were performed in accordance with the guidelines of the Department of Comparative Medicine, Oregon Health & Science University, and the NIH Guide for the Care and Use of Laboratory Animals. All mice were fed tap water and standard mouse chow (LabDiet Picolab Rodent Diet 5LOD, St. Louis, MO, USA) *ad libitum*. Given that adult mice consume approximately 5 g chow per day, daily L-Phe intake was estimated to be approximately 50 mg/day. The animals were housed under a standard 12-h:12-h on-off light cycle. Mice that exceeded 20% weight loss were given 2 days on a high fat diet to regain weight. Animals were euthanized through CO_2 or isoflurane inhalation followed by cardiac puncture.

Genotyping of Enu2 mice

To assess whether mice were homozygous for the *Enu2* mutation, PCR of the *Pah* exon 7 was performed as previously reported[192].

Vector design and viral production

Plasmids were designed in Snapgene (Version 7.0.1). Protein vectors were assembled using Gibson assembly cloning procedures using fragments synthesized by GenScript.

The OHSU Molecular Virology Support Core produced large-scale preparations of recombinant AAV2/8 virus, using standard triple plasmid transfection procedure (a plasmid with our transgene of interest, an AAV8 helper

plasmid, and a plasmid encoding the AAV Repl and Cap genes) into cultured HEK293 cells and purification by iodixanol gradient ultracentrifugation. AAV titers were determined using ITR-based qPCR analysis using ITR specific primers.

In vitro culture of HEK-293T cells

24-well plates were seeded with 10,000 HEK-293T cells and grown to 75% confluency. DMEM with 10% FBS and 0.1% penicillin-streptomycin. Cells were incubated at 37 °C with 5% CO_2 with saturating humidity. Each well was transfected with 0.2 µg of Cas9 and repair template plasmids at a 1:1 vector ratio with the RT template using lipofectamine 3000. After transduction and small molecule addition, cultures were incubated for 3 days before FACS analysis. Between 10,000 to 50,000 cells per well were sorted.

Delivery of small molecules and viral vectors

Vanillin was dissolved in saline (0.9% w/v) and heated in 50°C water bath with frequent vortexing. Novobiocin sodium salt (Thermo Fisher Scientific, Waltham, MA), Fludarabine, Olaparib, and other PARP-1 inhibitors were dissolved in 400 µL of DMSO and heated in a 50 °C water bath before slowly adding 600 µL of saline preheated to 50 °C. Viral vectors, diluted in saline, were injected into the facial vein of neonatal mice at either P3 or P5. An IP injection of the desired small molecule cocktail was performed immediately after facial vein injection and would continue for 3-5 days.

Serum phenylalanine

Serum phenylalanine was measured using an established fluorometric protocol[253]. This process requires the incubation of 10 µl of serum with an equal volume of 0.6 M tricarboxylic acid (TCA) for ten minutes prior to centrifugation at 10 K rpm for 5 minutes at room temperature. 10 µl of serum was then decanted and mixed with 93.75 µl of 600 mM succinate buffer, 37.50 µl of 30 mM ninhydrin, and 18.75 µl of L-leucyl-l-alanine. This solution is incubated for 2 hours at 60˚C. The resulting mixture is immediately combined with 0.6 ml of 25 mM Rochelle salt and 0.4 mL of 0.6 mM copper sulfate.

Graphical illustrations

Graphical illustrations of DNA repair and experimental workflow shown in **Figure 3-1** and **Figure 3-2** were created in powerpoint.

Statistical analysis

Dunnett's multiple comparison test was used to establish significance in all ANOVAs. All statistical analyses were performed using Graphpad Prism version 10.2.2 for Mac (GraphPad Software, La, Jolla, California, www.graphpad.com).

Acknowledgements
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Chapter 4: Enhancement of Therapeutic Transgene Insertion for Treatment of Murine Phenylketonuria

Enhancement of Therapeutic Transgene Insertion for Murine Phenylketonuria

Michael A Martinez[1], Daelyn Y Richards[1,2, α,] Shelley R Winn[1], Adrian M Baris[1], Anne Vonada[3], Sandra Dudley[1], Laura Harper[1], Cary O Harding[1,3*]

Affiliations: [1]Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR, USA. [2]Washington State University Elson S. Floyd College of Medicine, Spokane, WA, USA. [3]Department of Pediatrics, Oregon Health & Science University, Portland, OR, USA

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Abstract

Low *in vivo* transgene integration frequency limits the therapeutic efficacy of homology-directed repair (HDR)-mediated gene insertion as a treatment for Mendelian disorders. This study demonstrates improved efficacy of HDR-mediated gene insertion for the treatment of murine phenylalanine hydroxylase (PAH) deficiency, a model of human phenylketonuria (PKU), through pharmacologic inhibition of competing DNA repair pathways. Targeted integration of a *Pah*-expressing transgene into the hepatocytes of neonatal mice was enhanced with vanillin, a potent inhibitor of non-homologous end joining (NHEJ). This was further improved following combination of vanillin and novobiocin, an inhibitor of microhomology-mediated end joining (MMEJ). Combined NHEJ and MMEJ inhibition yielded PAH-expressing transgene insertions in approximately 10% of targeted alleles and was associated with a 70.6% decrease in serum phenylalanine. Demonstrating that pharmacologic inhibition of DNA repair pathways that compete with HDR can significantly enhance HDR-mediated transgene insertion *in vivo*.

Introduction

Significant progress has been made in developing gene addition and gene correction methods for the treatment of inherited disorders. However, current gene addition techniques, which most frequently rely upon transient expression from viral episomes, offer only temporary relief due to their limited stability in dividing cells[254]. Meanwhile, gene correction methods that target single-nucleotide variants, such as base editors, remain impractical for treating genetic diseases associated with hundreds or thousands of pathogenic variants.

Phenylketonuria (PKU) is one such genetic disorder that exemplifies these challenges. PKU results from variations in the phenylalanine hydroxylase (*PAH*) gene that cause liver PAH enzyme deficiency, leading to neurotoxic accumulation of phenylalanine in the bloodstream. To date, over 1,500 unique variants have been associated with PAH deficiency in humans, with many individuals being compound heterozygous for two different *PAH* variants, challenging the clinical utility of gene correction tools targeting a single variant. Therefore, targeted insertion of a complete functional *PAH*-expressing transgene into the liver genome is an attractive alternative, offering the potential to treat individuals harboring any *PAH* variants using a single therapeutic approach. To meet this need, researchers have made clever alterations to existing CRISPR/Cas9 machinery to create tools, such as PASTE editors, that are capable of large insertions[201,255]. However, while these inventions represent an exciting development for creating targeted insertions, they have not demonstrated robust *in vivo* editing success.

Alternatively, gene insertion through homology-directed repair (HDR) is a method that largely relies upon the endogenous repair machinery of the cell to

incorporate a desired edit. A full expressing transgene is integrated into the genome by creating a targeted double-strand break (DSB) while simultaneously providing a repair template with the desired transgene flanked by regions of homology to the cut site. Repair of the DSB using the homologous recombination (HR) DNA repair pathway permanently incorporates the repair template into the genome. We previously demonstrated that pharmacological inhibition of a competing repair pathway, non-homologous end joining (NHEJ), significantly enhances the efficacy of HDR-mediated gene editing when correcting the *Pah*^{enu2} missense variant[192]. In the present study, we demonstrate that this strategy can facilitate the integration of a large (>2,300 bp) murine *Pah* expression cassette resulting in a significant reduction of serum phenylalanine (Phe) concentration in a mouse model of PAH deficiency (**Fig. 4-1**). Subsequently, we observed that the simultaneous pharmacologic inhibition of two separate DSB repair pathways, NHEJ and microhomology-mediated end joining (MMEJ), resulted in a 70.6% reduction in serum Phe concentration when combined with our whole gene integration strategy. To our knowledge, this represents the most complete phenotypic correction of murine PAH deficiency achieved through HDR-mediated transgene insertion to date.

Results

Pharmacologic Inhibition of NHEJ Promotes Pah Transgene Insertion into the PAH-Deficient Mouse Liver Genome

Previous work in our laboratory demonstrated significant reduction in serum Phe concentrations in the *Pah*$^{enu2/enu2}$ mouse model. This was achieved through the co-administration of a recombinant adeno-associated virus (AAV) serotype 8 vector (AAV2/8) expressing *Streptococcus pyogenes* Cas9 (SpCas9) and a second AAV expressing a single guide RNA (sgRNA) and carrying a repair template designed specifically for the correction of the *Pah*^{enu2} missense variant in *Pah* exon 7[192]. However, this approach proved effective only in neonates that received injections of vanillin, an inhibitor of the NHEJ pathway[256], to promote DNA repair through HDR (**Fig. 4-1A**)[192,196]. We hypothesized that vanillin treatment would similarly promote whole transgene insertion *in vivo*.

To test this hypothesis, three-day old *Pah*$^{\Delta exon1/\Delta exon1}$ mice (hereafter Dexon1 mice)[257] received two separate AAVs (hereafter dual-AAV) via facial vein injection at a 1:1 ratio: one expressing SpCas9 and another carrying the repair template (4.2 x 10^{13} vg/kg total dose). Intraperitoneal (IP) injections of 100 mg/kg vanillin accompanied dual-AAV injection and were administered daily for five days following AAV delivery (**Fig. 4-1B**). The repair template vector delivered a 2,379 bp murine *Pah* expression cassette that was flanked by two 864 bp sequences with homology to *Pah* exon 1 (**Fig. 4-2A**).

Serum Phe concentrations were reduced in the treated cohort to 916 ± 342 μM (mean ± SD) in comparison to 2,122 ± 163 μM in untreated animals (P <0.0001) (**Fig. 4-2B**). At euthanasia, mean liver PAH activity in treated mice was 7.67 ±

3.17% that of wild-type mice, significantly increased from 0.72 ± 0.45% measured in the untreated group (P <0.0001) (**Fig. 4-2C**).

Unlike other PAH-deficient models that produce a mutant PAH protein product, the PAH-null Dexon1 model allows visualization of successful repair events by PAH immunostaining (**Fig. 4-2D**). Quantification of PAH-positive hepatocytes revealed that 3.76 ± 2.07% of hepatocytes expressed PAH in the treated cohort (**Fig. 4-2E**). As murine hepatocytes are often polyploid it can't be assumed that each PAH positive hepatocyte represents a single edited allele. Therefore, we employed qPCR on bulk liver genomic DNA using junction gap primers between *Pah* exons 7 and 8 to estimate the percentage of genomes with *Pah* cDNA insertions, yielding a mean insertion rate of 3.91% per haploid genome (**Fig. 4-2F**). However, this reaction detects the presence of the *Pah* cDNA anywhere in the liver genome and is therefore agnostic to the genomic integration site.

To assess specific integration into the target site DNA sequences flanking the targeted cut site, along with sequences from the transgene, were enriched from bulk liver genomic DNA using custom probes and then sequenced on an Illumina next-generation sequencing (NGS) platform. This analysis confirmed integration of the *Pah* cDNA transgene into *Pah* exon 1 in treated mice (**Fig. 4-S1A**). Interestingly, we also detected a significant amount of reads that included the U6 promoter and sgRNA from the AAV8 repair template, sequences that would not have integrated into the liver genome with the expected HDR-directed event. Two possible alternate molecular events would explain this result. First, integration of

the entire AAV genome into the Cas9-induced DSB through NHEJ-mediated repair. Second, AAV concatemers, composed of serial repeats of a viral genome, may have been used as the substrate for the HDR-mediated repair, leading to integration of multiple copies of the whole viral construct. Previous studies have also observed frequent insertion of concatemers when delivering a repair template via AAVs[258]. Additionally, we observed a small number (<120 reads) of partial Cas9 sequences within the integrated expression cassette, suggesting that a hybrid concatemer of the repair template and Cas9 episome was integrated into the target site in rare events (**Fig. 4-S1B**).

HDR-mediated Transgene Insertion into Pah Exon 7

One of the major advantages of whole transgene insertion is the ability to design a single sgRNA and repair template that can be efficiently advanced through clinical trials and produced at scale for use in most afflicted individuals. However, the homology arms of the repair template used in Figure 2 were tailored specifically to the unique deletion in the *Dexon1 mouse* model, which lacks most of the first exon of wildtype *Pah*. To enhance the rigor of this approach, and to create a universal repair template applicable to all known murine models of PKU, we designed a new repair template. This construct utilizes the *Pah* exon 7-targeting sgRNA, previously used to correct the *Pah*^{enu2} mutation[192], and includes 800 base pair homology arms (**Fig. 4-3A**). Treatment of neonatal Dexon1 mice with the exon 7 repair template and Cas9 nuclease yielded a reduction in serum Phe concentrations, measured at 7 weeks of age, to 896 ± 325 μM (**Fig. 4-3B**). This is significantly reduced in comparison to mice that received the repair template without Cas9 (2026 ± 232 μM, P <0.0001). Liver PAH activity increased

to 6.57 ± 2.11% in contrast to 0.67 ± 0.12% measured in RT-only treated animals (*P* <0.0001) (**Fig. 4-3C**). PAH-positive hepatocytes were observed in liver sections (**Fig. 4-3D),** with 7.44 ± 2.49% of bulk liver genomes possessing the *Pah* transgene (*P* <0.0001) (**Fig. 4-3E**).

AAV vector genomes form episomal concatemers that are capable of PAH expression without integrating into the genome[259,260], but these episomes are unstable in dividing hepatocytes[261]. The persistent hyperphenylalaninemia in neonatally treated mice, given only the transgene-containing repair template, demonstrates three key points: (1) AAV episomes were largely lost during juvenile liver development, and physiologically-significant episomal PAH expression was not sustained into adulthood; (2) presence of homology arms in the repair template alone, without expression of a targeted nuclease, is insufficient for generating a clinically relevant number of genomic insertions, as has been previously shown[88,262]; and (3) although NHEJ-mediated insertions of fully intact AAV vector genomes into DSBs have been reported[263], the frequency of these events was insufficient to correct serum Phe concentrations in Dexon1 mice treated with the repair template alone.

Acetaminophen Selection of Dexon1 Hepatocytes Harboring Transgene Insertions

To achieve a full phenotypic correction of Dexon1 mice, we sought to provide edited hepatocytes with a selective growth advantage. This would allow expansion of the edited hepatocyte population and increase their physiologic impact. Previous research has shown that the inclusion of a cytochrome P450 reductase (*Cypor*)-targeting short hairpin RNA (shRNA) can provide a selective

advantage to edited hepatocytes in the presence of acetaminophen (APAP)-induced toxicity[211]. We hypothesized that addition of a *Cypor* shRNA to our transgene would promote the expansion of the PAH-expressing hepatocyte population during APAP-induced cell selection, resulting in further blood Phe reduction in PAH-deficient mice.

To test this hypothesis, we created a third repair template, still targeting *Pah* exon 7, with the addition of *Cypor* shRNA under transcriptional control of a ubiquitous U6 promoter (**Fig. 4-4A**). Neonatal Dexon1 mice were treated with this novel repair template, Cas9-expressing AAV, and vanillin as per prior experiments. At weaning, blood Phe concentrations remained extremely elevated (2128 ± 496 μM), suggesting low initial repair template integration frequency and liver PAH activity. Addition of 1.9% APAP (w/w) to the mouse chow was associated with a gradual decline in serum Phe (**Fig. 4-4B**), particularly in male mice. As a negative control, we treated another cohort of mice identically but did not provide APAP after weaning. Unlike our previous transgene insertion experiments, we observed a significant sex difference in this treatment response, with a mean genome insertion rate of 9.55 ± 1.16% in APAP treated male mice and 3.45 ± 2.48% in females (**Fig. 4-4C**). APAP-treated males achieved an average blood Phe concentration of 425 ± 205 μM (P <0.0001) while APAP-treated females achieved 1309 ± 603 μM (**Fig. 4-4D**).

PAH enzyme activity of APAP-treated males measured 6.23 ± 1.64% of wild-type activity (P < 0.0001), while females exhibited mean activity of 2.36 ± 1.93% (**Fig. 4-4E**). Anti-CYPOR and anti-PAH immunohistology showed clear expansion

of CYPOR deficient hepatocytes (**Fig. 4-4F)** as well as concomitant colonies of PAH positive cells in APAP-treated mice (**Fig. 4-4G**). Females with high blood Phe concentration demonstrated limited expansion of CYPOR deficient colonies (**Fig. 4-S2**), suggesting that, while these mice did receive some low level of transgene integration, the founder population of edited cells failed to significantly expand under APAP selection.

Inclusion of Cypor shRNA Sequences Alters AAV Vector Genome Replication

Loss of AAV genome homogeneity and lower effective titers has been reported in vectors containing shRNAs[264]. DNA sequencing of the '*Pah* Exon7-shCypor' (Fig. 4A) AAV vector stocks revealed that only 7.8% of *Pah* Exon7-shCypor vector genomes contained the desired construct while 61.8% of vectors consisted of so-called 'snapback genomes', which consisted of truncated AAV genomes without PAH expression elements[265] (**Fig. 4-S3**). The impaired production of fully functional AAV genomes in the presence of shRNA sequence is likely responsible for the low initial transgene integration frequency present prior to APAP selection.

Severe Motor Disability Observed in Cypor shRNA Treated Animals

Unexpectedly, animals treated with high dose AAV2/8 vectors carrying *Cypor* shRNA developed severe motor impairment, characterized by dystonic movements of all limbs, with a pronounced impact on the hindlimbs (**Supplemental Movies 1 and 2**). Out of 98 mice injected with *Cypor* shRNA-expressing AAV2/8 vectors, 53 exhibited motor dysfunctions within 1-2 weeks post-treatment. Development of this adverse effect did not require co-administration of Cas9 vector. No animals receiving AAV8 repair templates lacking *Cypor* shRNA

ever developed neurologic impairment, suggesting that *Cypor* shRNA expression is the underlying cause of this pathology. However, previous studies utilizing *Cypor* shRNA delivered to neonates via lentivirus vectors did not report such pathology[211].

Administration of high AAV vector doses has been associated with abnormal histopathology of dorsal root ganglia, but minimal clinical symptoms[266–268]. Also, injection of shRNA-expressing AAV vectors directly into brain has resulted in severe motor dysfunction, death, and neurodegeneration in several reported animal models[269–271]. However, gross anatomic examination of the brain, spinal cord, and DRG of our *Cypor* shRNA-treated mice revealed no clear pathology. Histologic examination (Nissl staining) of fixed brain sections from these mice likewise did not reveal any microscopic pathology (data not shown). Although AAV2/8 does not readily cross the blood-brain barrier (BBB) in adult mice, we considered the possibility that the BBB might be compromised in neonates. However, no AAV genomes were detected in brain tissue by qPCR in a limited sample of affected mice (n = 4). Therefore, the precise mechanistic cause of motor deficits in PAH-deficient mice treated with *Cypor* shRNA-expressing AAV8 remains undetermined.

Simultaneous Pharmacologic Inhibition of NHEJ and Microhomology-mediated End Joining (MMEJ) Synergistically Enhances HDR-Mediated Gene Insertion in vivo

Our previous research demonstrated that pharmacologic inhibition of NHEJ with vanillin produced a seven-fold increase of *in vivo* gene correction as well as a corresponding increase in the number of small insertions and deletions (indels) at the targeted DSB[192]. We suspected that inhibition of NHEJ had increased the

frequency of not only HDR, but also of microhomology-mediated end joining (MMEJ), which is inherently mutagenic[272,273]. High-throughput small molecule screens have identified the antibiotic Novobiocin (NVB) as a potent inhibitor of DNA polymerase theta, a key enzyme in MMEJ. These screens also demonstrated that the coupling of NVB treatment with inhibitors of poly (ADP-ribose) polymerase 1 (PARP1), an essential NHEJ intermediate, leads to selective death of HDR-deficient tumors . This result indicates that inhibition of both MMEJ and NHEJ forces the cell to rely upon HDR for DSB repair, a phenomenon that could be leveraged for increasing the efficacy of HDR (**Fig. 4-1A**). This hypothesis is supported by a recent report that used a potent inhibitor of DNA-PK catalytic activity (M3814) in the NHEJ pathway along with NVB to dramatically increase the efficiency of HDR-mediated editing for introduction of small mutations *in vitro*[274].

To test this strategy, we evaluated simultaneous pharmacologic inhibition of NHEJ with vanillin, and of MMEJ with NVB, in a treatment strategy we have dubbed Vanbiocin. Five-day old Dexon 1 mice received vanillin, 100 mg/kg and NVB, 50 mg/kg by IP injection followed by facial vein injections of the AAV2/8 Cas9 and AAV2/8 Pah Exon 7 repair template. Vanbiocin treatment was repeated daily for two additional days (three days total). An initial treatment attempt in 3-day old mice with five-day total Vanbiocin treatment duration had been associated with lethargy, poor feeding, and death of all pups by day 10. Delaying treatment onset and shortening the duration of Vanbiocin therapy improved tolerability and survival (8 out of 11 treated pups). At six weeks of age, serum Phe concentrations in mice that had received both AAV2/8 vectors and Vanbiocin treatment measured 631 ± 211

μM (n = 8) in comparison to 2,134 ± 197 μM in untreated mice (**Fig. 4-5A**). Mean insertion frequency of the repair template at euthanasia in Vanbiocin treated mice was 9.71 ± 3.62% with mean liver PAH activity of 6.14 ± 1.15% of wild-type activity (**Fig. 4-5B-C**). Anti-PAH staining of liver sections showed colonies of PAH positive hepatocytes primarily near the central hepatic veins (**Fig. 4-5D**). Coat pigmentation, which is light brown in C57BL/6 models of PKU due to phenylalanine-mediated inhibition of melanin production, was rescued in mice treated with Vanbiocin (**Fig. 4-5E**).

Female PAH deficient mice, if untreated and hyperphenylalaninemic, are unable to produce viable offspring due a severe maternal PKU effect. Here, gene insertion/Vanbiocin-treated Dexon1 dams successfully gestated and reared pups through weaning while consuming normal chow and without additional Phe-lowering treatment. All progeny from matings between Vanbiocin-treated Dexon1 dams and untreated homozygous Dexon1 sires exhibited severe hyperphenylalaninemia at weaning, indicating that all offspring lacked liver PAH activity. All pups from this pairing were homozygous for the Dexon1 mutation indicating that genomic insertions in the dams were liver specific and had not been transmitted to germ cells (**Fig. 4-5G**).

Hyperphenylalaninemia in Vanbiocin-treated Dexon1 Mice is Responsive to Sapropterin Supplementation

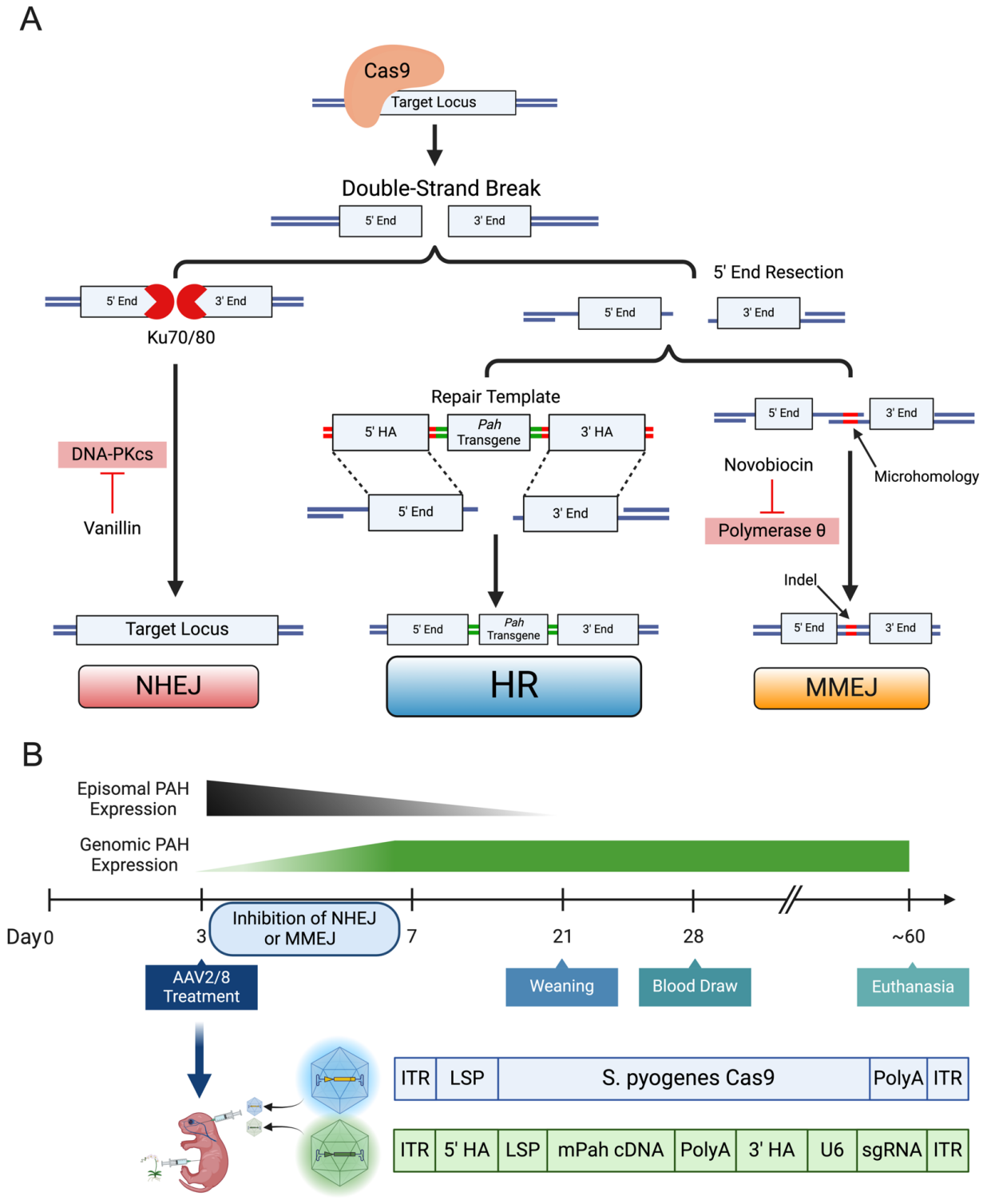
PAH deficiency in humans is represented by a broad spectrum of disease severity that is determined in an individual by the specific *PAH* variants inherited and the resultant amount of residual liver PAH activity. Individuals with 2-5% residual liver PAH activity may exhibit mild hyperphenylalaninemia (blood Phe ≤

600 μM) while consuming an unrestricted diet. In many of these individuals, treatment with pharmacologic doses of tetrahydrobiopterin[275], the natural pterin cofactor for PAH[33], or its pharmaceutical analog, sapropterin dihydrochloride[141], augments PAH activity and lowers blood Phe. We hypothesized that since Vanbiocin-treated Dexon1 mice exhibited partial correction of serum Phe concentrations, sapropterin treatment may improve Phe metabolism in the mice and lead to further reduction in serum Phe concentrations. We provided gene-edited, Vanbiocin-treated animals with 100 mg/kg sapropterin via oral gavage, daily, over the course of 5 days, and observed a static reduction in serum Phe at 396 ± 142 μM (P = 0.0089), near the therapeutic threshold for PKU (**Fig. 4-5F**).

Non-invasive Assessment of in vivo Phe Oxidation Capacity in Vanbiocin-Treated Gene-Edited Dexon1 Mice Using Stable Isotope Loading

Following oral loading with 1-^{13}C-L-phenylalanine and timed collection of breath CO_2, we non-invasively documented restored total body Phe metabolism in Vanbiocin-treated gene-edited Dexon1 mice through measurement of $^{13}CO_2$ excreted in breath (**Fig. 4-S5A**). Plotting total $^{13}CO_2$ production vs. final serum Phe concentration in Vanbiocin treated Dexon1 mice revealed an exponential curve (R^2=0.9655) (**Fig. 4-S5B**), in a relationship consistent with the known Michaelis-Menten enzyme kinetics for PAH with allosteric regulation by Phe[276]. No significant difference in Phe oxidation was detected in Vanbiocin treated animals with or without sapropterin supplementation. These results suggest potential clinical utility for stable isotope breath testing as a method for non-invasively assessing total body Phe oxidation in PKU clinical gene therapy trials, without the need for more invasive liver biopsy.

Figures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Cas9
Target Locus
Double-Strand Break
5' End
3' End
5' End Resection
Ku70/80
DNA-PKcs
Vanillin
Repair Template
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Pah Transgene
3' HA
Novobiocin
Polymerase θ
Microhomology
Indel
Target Locus
NHEJ
HR
MMEJ
B
Episomal PAH Expression
Genomic PAH Expression
Day 0
3
Inhibition of NHEJ or MMEJ
7
21
28
~60
AAV2/8 Treatment
Weaning
Blood Draw
Euthanasia
ITR
LSP
S. pyogenes Cas9
PolyA
U6
sgRNA
mPah cDNA

Figure 4-1. Experimental Design for HR-mediated Gene Editing Treatment of Murine PAH Deficiency. (**A**) Canonical model of DSB repair by non-homologous end joining (NHEJ), homologous recombination (HR), and microhomology-mediated end joining (MMEJ). Inhibition of DNA-PKcs and Polymerase theta, vital factors for NHEJ and MMEJ, is hypothesized to increase frequency of repair through HR. (**B**) Neonatal mice at neonatal day 3 (P3) receive dual AAV2/8 vectors through facial vein injection and daily IP injections of NHEJ or MMEJ inhibiting small molecules. Dual AAV2/8 treatment is composed of a streptococcus pyogenes Cas9 (spCas9) vector expressed from a liver specific promoter (LSP), and a repair template with 3' and 5' homology arms (HA) for integration into target genomic locus. Single-guide RNA (sgRNA) is delivered on the repair template, therefore requiring transduction with both vectors to induce a double-strand break (DSB). Episomal expression of murine PAH (mPAH) dissipates during hepatocyte turnover, while expression of repair template after integration into the genome remains.

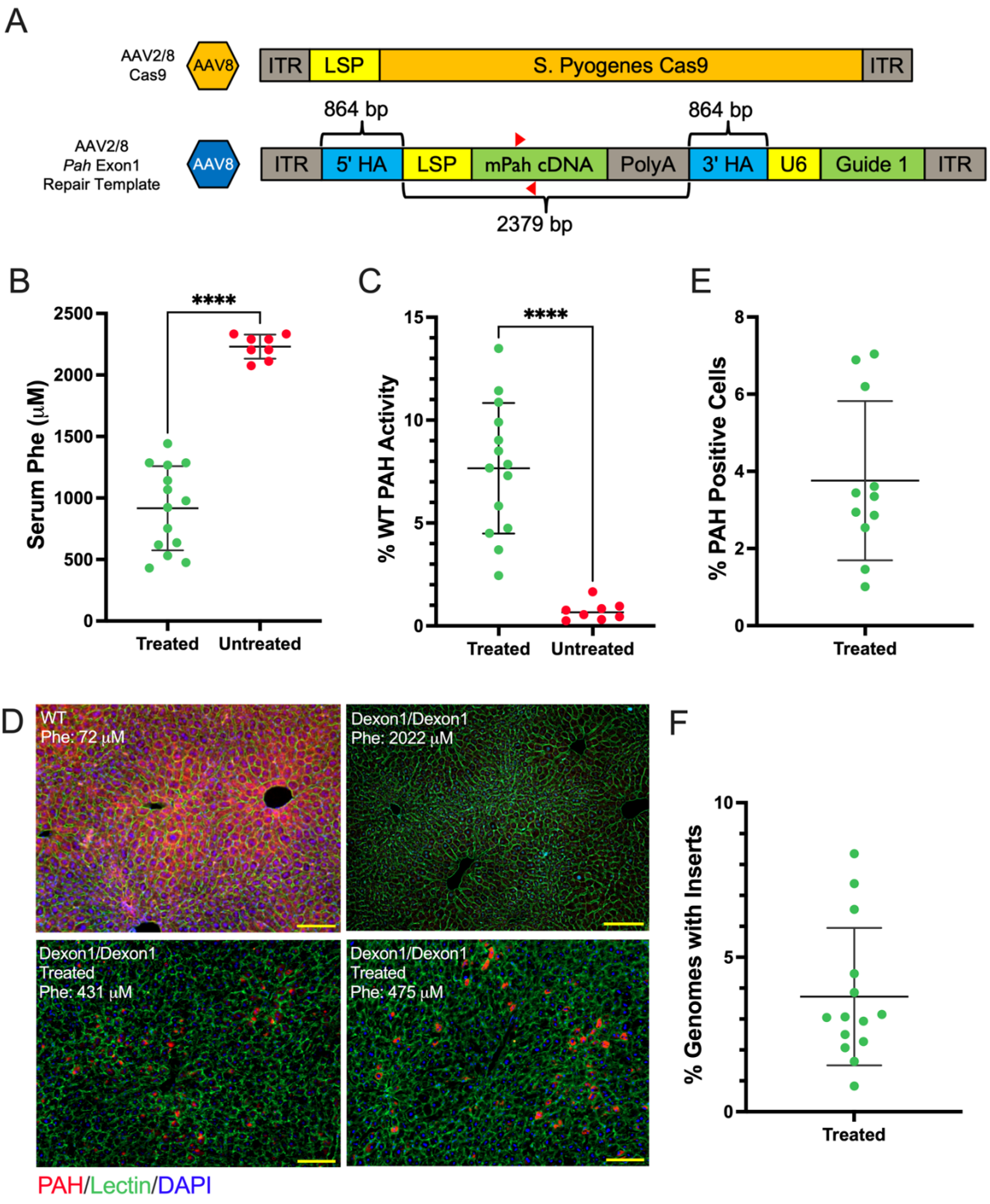

A
AAV2/8
Cas9
AAV8
ITR
LSP
S. Pyogenes Cas9
ITR
AAV2/8
Pah Exon1
Repair Template
AAV8
864 bp
864 bp
ITR
5' HA
LSP
mPah cDNA
PolyA
3' HA
U6
Guide 1
ITR
2379 bp
B
****
Serum Phe (μM)
2500
2000
1500
1000
500
0
Treated
Untreated
C
****
% WT PAH Activity
15
10
5
0
Treated
Untreated
E
% PAH Positive Cells
8
6
4
2
0
Treated
D
WT
Phe: 72 μM
Dexon1/Dexon1
Phe: 2022 μM
Dexon1/Dexon1
Treated
Phe: 431 μM
Dexon1/Dexon1
Treated
Phe: 475 μM
PAH/Lectin/DAPI
F
% Genomes with Inserts
10
5
0
Treated

Figure 4-2. Vanillin Assisted Insertion of *Pah* Transgene in Murine Model of PKU. (**A**) Schematic of dual-AAV vectors including 5' and 3' homology arms (HA), liver specific promoter (LSP) including a bikunin enhancer and human thyroxine binding globulin (TBG) promoter, murine Pah cDNA, HGH poly(A) signal (PolyA), U6 promoter (U6), and sgRNA guide for targeted Cas9 cleavage (Guide 1). Red arrows indicate forward and reverse primers for qPCR. (**B**) Blood Phe concentrations in $Pah^{\Delta exon1/\Delta exon1}$ mice (n = 14) treated with dual AAV2/8 Cas9 and Pah Repair Template (mean = 917 ± 342 μM) versus control mice treated exclusively with AAV2/8 Pah Repair Template (mean = 2,122 ± 163 μM). All animals received facial vein injections of viral vectors (8.2×10^{13} vg/kg, 1:1 ratio) at day P3 with subsequent intraperitoneal injections of 100 mg/kg vanillin for 5 days following viral transduction. (**C**) PAH enzyme activity of treated (mean = 7.66 ± 3.17%) versus control (mean = 0.72 ± 0.45%) animals measured as percent of wild-type PAH activity. (**D**) Anti-PAH (Red, cytoplasmic), anti-lectin (Green, cell membrane), and DAPI (Blue, nuclear) of livers of treated (Bottom) and untreated (Top-left) $Pah^{\Delta exon1/\Delta exon1}$ animals as well as untreated C57BL/6 mice (Top-right). Scale bars, 100 μm. (**E**) Percent of liver genomes containing the mPah cDNA delivered in our repair template, as determined by qPCR, was 3.91 ± 2.5%. (**F**) Percent of hepatocytes with Pah transgene (mean = 3.76 ± 2.07%). $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ by unpaired T-test. Data are means ± SD.

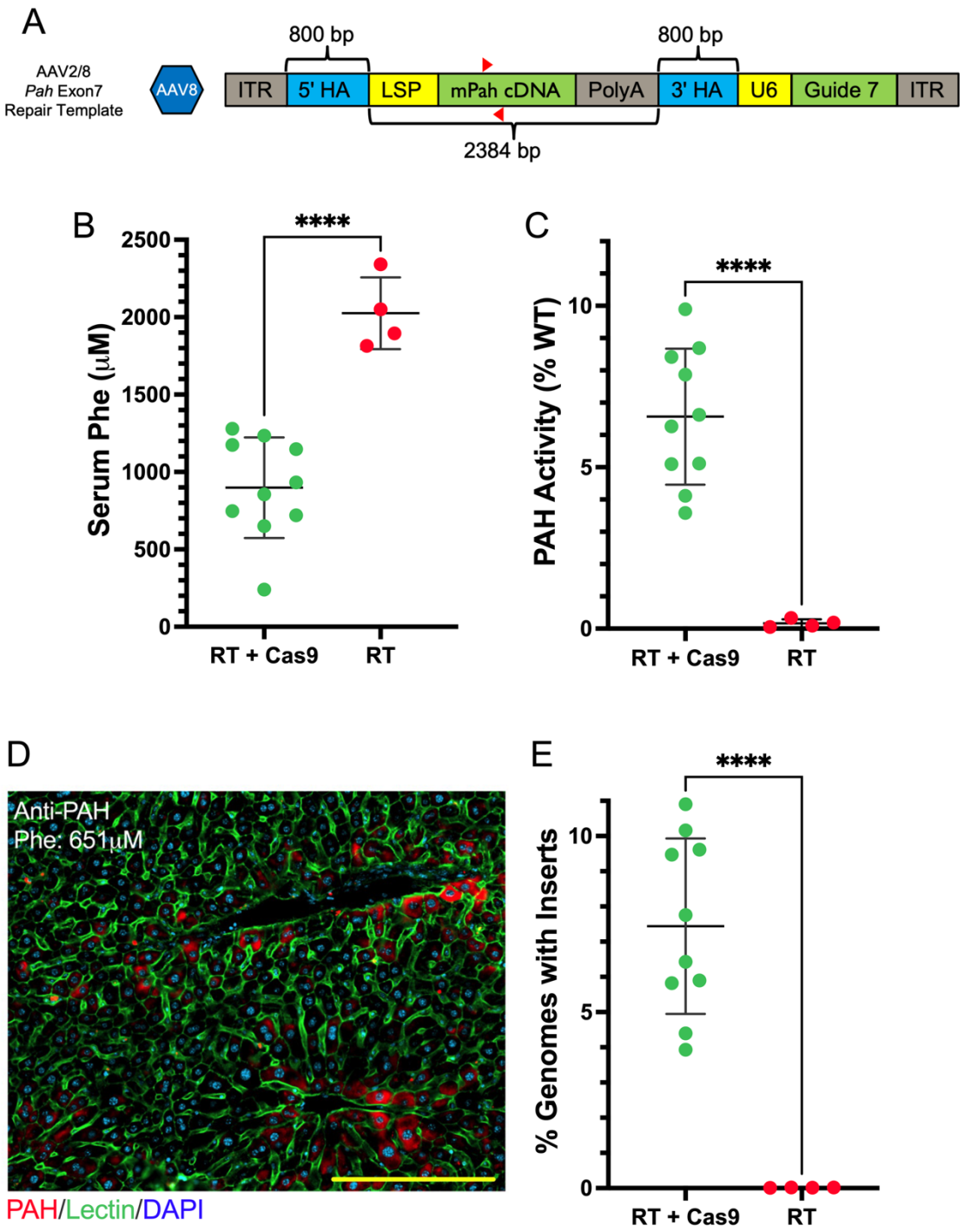

A
AAV2/8
Pah Exon7
Repair Template
AAV8
800 bp
800 bp
ITR
5' HA
LSP
mPah cDNA
PolyA
3' HA
U6
Guide 7
ITR
2384 bp
B
****
Serum Phe (μM)
2500
2000
1500
1000
500
0
RT + Cas9
RT
C
****
PAH Activity (% WT)
10
5
0
RT + Cas9
RT
D
Anti-PAH
Phe: 651μM
PAH/Lectin/DAPI
E
****
% Genomes with Inserts
10
5
0
RT + Cas9
RT

Figure 4-3. Insertion of the Transgene into *Pah* Exon 7. (**A**) Schematic of the repair template used to insert murine *Pah* cDNA into the *Pah* Exon 7 locus. Expression of sgRNA guide to this locus (Guide 7) under the expression of a U6 promoter (U6) and the adaptation of the 5' and 3' homology arms to the target locus are the major differences between Figure 1A. Animals were injected with a viral dose of 1.0 X 10^{14} vg/kg per vector and the same vanillin regime as in Figure 1. (**B**) Blood Phe concentrations in animals treated with both repair template (RT) and Cas9 carrying viral vectors (mean = 898 ± 324 μM, n = 10) versus those treated with only the RT (mean = 2026 ± 232 μM, n = 4). (**C**) PAH enzyme activity in RT + Cas9 treated animals (mean = 6.57 ± 2.11%) and RT treated animals (mean = 0.17 ± 0.12%). (**D**) Anti-Pah (Red, cytoplasmic), Anti-lectin (Green, cell membrane), and DAPI (Blue, nuclear) staining in liver section from RT + Cas9 treated animal. Scale bars, 100 μm. (**E**) Insertion frequency of the Pah cDNA (mean = 7.44 ± 2.49%) in treated animals, as determined by qPCR. ****$P < 0.0001$ by unpaired T-test. Data are means ± SD.

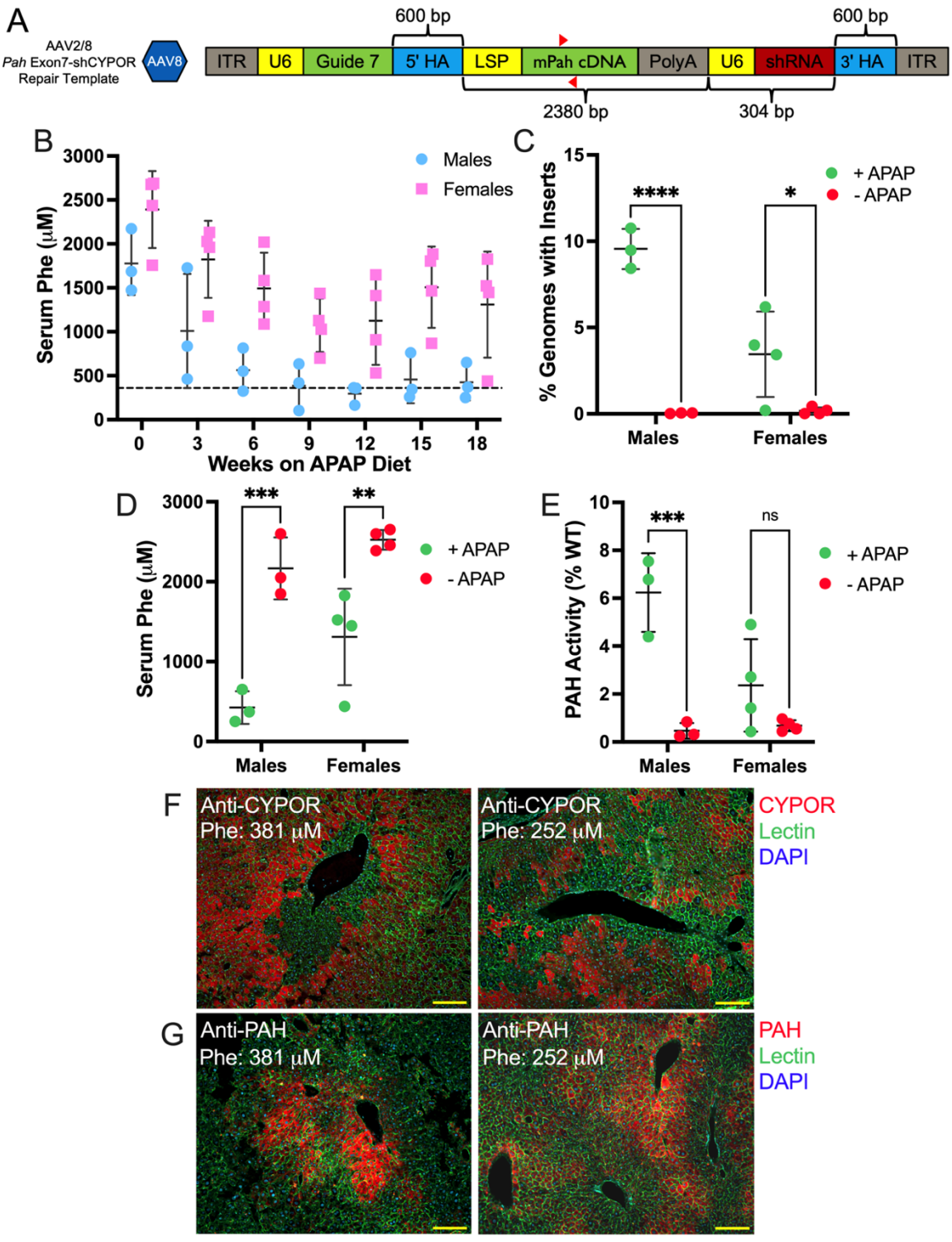

A
AAV2/8
Pah Exon7-shCYPOR
Repair Template
AAV8
ITR
U6
Guide 7
5' HA
LSP
mPah cDNA
PolyA
U6
shRNA
3' HA
ITR
600 bp
600 bp
2380 bp
304 bp
B
Serum Phe (μM)
Weeks on APAP Diet
Males
Females
C
% Genomes with Inserts
****
*
+ APAP
- APAP
Males
Females
D
Serum Phe (μM)
***
**
+ APAP
- APAP
Males
Females
E
PAH Activity (% WT)
***
ns
+ APAP
- APAP
Males
Females
F
Anti-CYPOR
Phe: 381 μM
Anti-CYPOR
Phe: 252 μM
CYPOR
Lectin
DAPI
G
Anti-PAH
Phe: 381 μM
Anti-PAH
Phe: 252 μM
PAH
Lectin
DAPI

Figure 4-4. Providing *Cypor* shRNA Induced Selective Advantage to *Pah* Transgene under APAP Exposure. (**A**) Schematic of dual-AAV vectors including 5' and 3' homology arms (HA), liver specific promoter (LSP) including a bikunin enhancer and human TBG promoter, murine Pah cDNA, HGH poly(A) signal (PolyA), U6 promoter (U6), *Cypor*-targeting shRNA (shRNA), and sgRNA guide for targeted Cas9 cleavage (Guide 7). (**B**) Blood Phe levels over 18 weeks of APAP diet in male and female Dexon1 mice initiated after weaning. (**C**) Transgene insertion frequency in treated males that received APAP diet (9.55 ± 1.16%, n = 3) and those that did not (0.04 ± 0.02%, n = 3), and female that received APAP diet (3.45 ± 2.48%, n = 4) and those that did not (0.17 ± 0.20%, n = 4) (*P < 0.0193 and ****P < 0.0001). (**D**) Terminal blood Phe concentrations between APAP treated males (425 ± 205 μM) and females (1309 ± 603 μM), as well as males (2165 ± 388 μM) and females (2524 ± 122 μM) that did not receive APAP selection but did receive viral vectors (***P <0.0005, and **P <0.0026). (**E**) PAH enzyme activity in treated males (6.24 ± 1.64%), treated females (2.36 ± 1.93%) and untreated males (0.46 ± 0.32%) and females (0.68 ± 0.23%) animals after 18 weeks of APAP induced hepatocyte selection (P < 0.1944 and ***P <0.0006). (**F**) anti-CYPOR (Red, cytoplasmic), anti-lectin (Green, cell membrane), and DAPI (Blue, nuclear) in treated animals. (**G**) anti-PAH (red), anti-lectin (green), and nuclear stain (blue) in respective treated animals. Scale bars, 100 μm. Bonferroni's multiple comparison test was used to calculate the significance of group differences. Significance calculated by two-way ANOVA with sex and treatment as variables. Data are means ± SD.

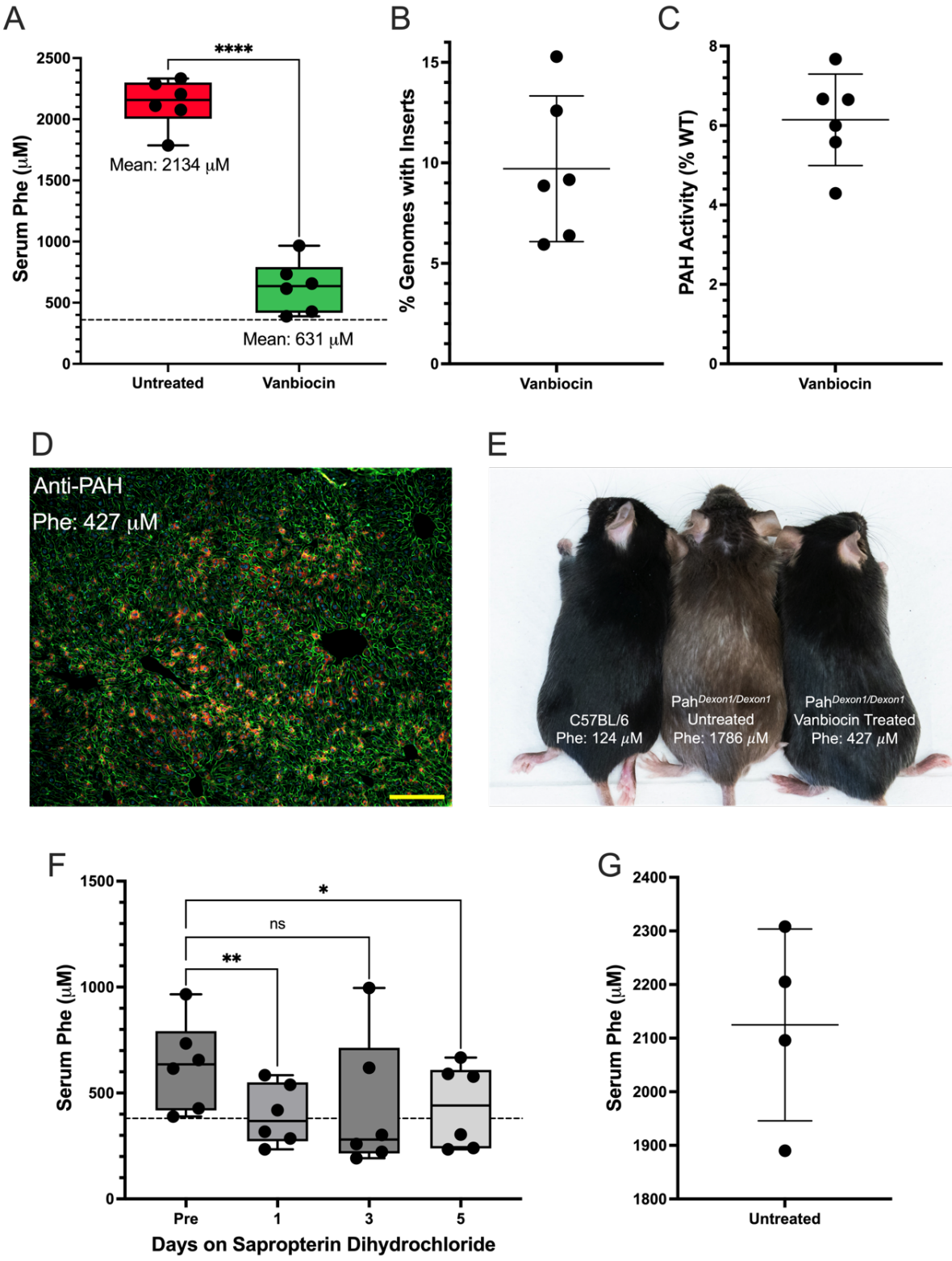

A
****
Serum Phe (μM)
2500
2000
1500
1000
500
0
Mean: 2134 μM
Mean: 631 μM
Untreated
Vanbiocin
B
% Genomes with Inserts
15
10
5
0
Vanbiocin
C
PAH Activity (% WT)
8
6
4
2
0
Vanbiocin
D
Anti-PAH
Phe: 427 μM
E
C57BL/6
Phe: 124 μM
Pah Dexon1/Dexon1
Untreated
Phe: 1786 μM
Pah Dexon1/Dexon1
Vanbiocin Treated
Phe: 427 μM
F
*
ns
**
Serum Phe (μM)
1500
1000
500
0
Pre
1
3
5
Days on Sapropterin Dihydrochloride
G
Serum Phe (μM)
2400
2300
2200
2100
2000
1900
1800
Untreated

Figure 4-5. Inhibition of NHEJ and MMEJ Increases Efficacy of Homology-Directed Recombination *in vivo*. (**A**) Blood Phe concentrations in untreated mice (mean = 2134 ± 197 μM, n = 6) and animals treated with Cas9 and RT AAV vectors with Vanbiocin (Vanillin and Novobiocin) (mean = 631 ± 211 μM, n = 6). Black dashed line indicates a therapeutic threshold of 360 μM. Data are means ± SD. ***P* <0.0027 and *****P* <0.0001 by paired t-test. (**B**) Insertion frequency of the mPah cDNA (mean = 9.71 ± 3.62%) in treated animals, as determined by qPCR. (**C**) PAH enzyme activity in Vanbiocin treated mice (mean = 6.14 ± 1.15%) measured as percent of wild-type PAH activity. (**D**) anti-PAH (Red), anti-lectin (green), and nuclear stain (blue) in treated animals. Scale bars, 100 μm. (**E**) Coat color rescue of wild-type C57BL/6, untreated Dexon1 mice, and Vanbiocin treated mice. Baseline blood Phe concentrations without sapropterin provided. (**F**) Blood Phe concentrations in Vanbiocin-treated animals was measured over a 5-day course of daily treatment with 100 mg/kg sapropterin dihydrochloride, administered via oral gavage 6 hours prior to retro-orbital blood collection. Black dashed line indicates a therapeutic threshold of 360 μM. *P* <0.1188, **P* <0.0242, and ***P* <0.0037 by one-way ANOVA. (**G**) Blood Phe concentrations in the progeny (mean = 2124, n = 4) of Vanbiocin treated female post-weaning. Dunnett's multiple comparison used with all ANOVAs. Data are means ± SD.

Supplemental Figures

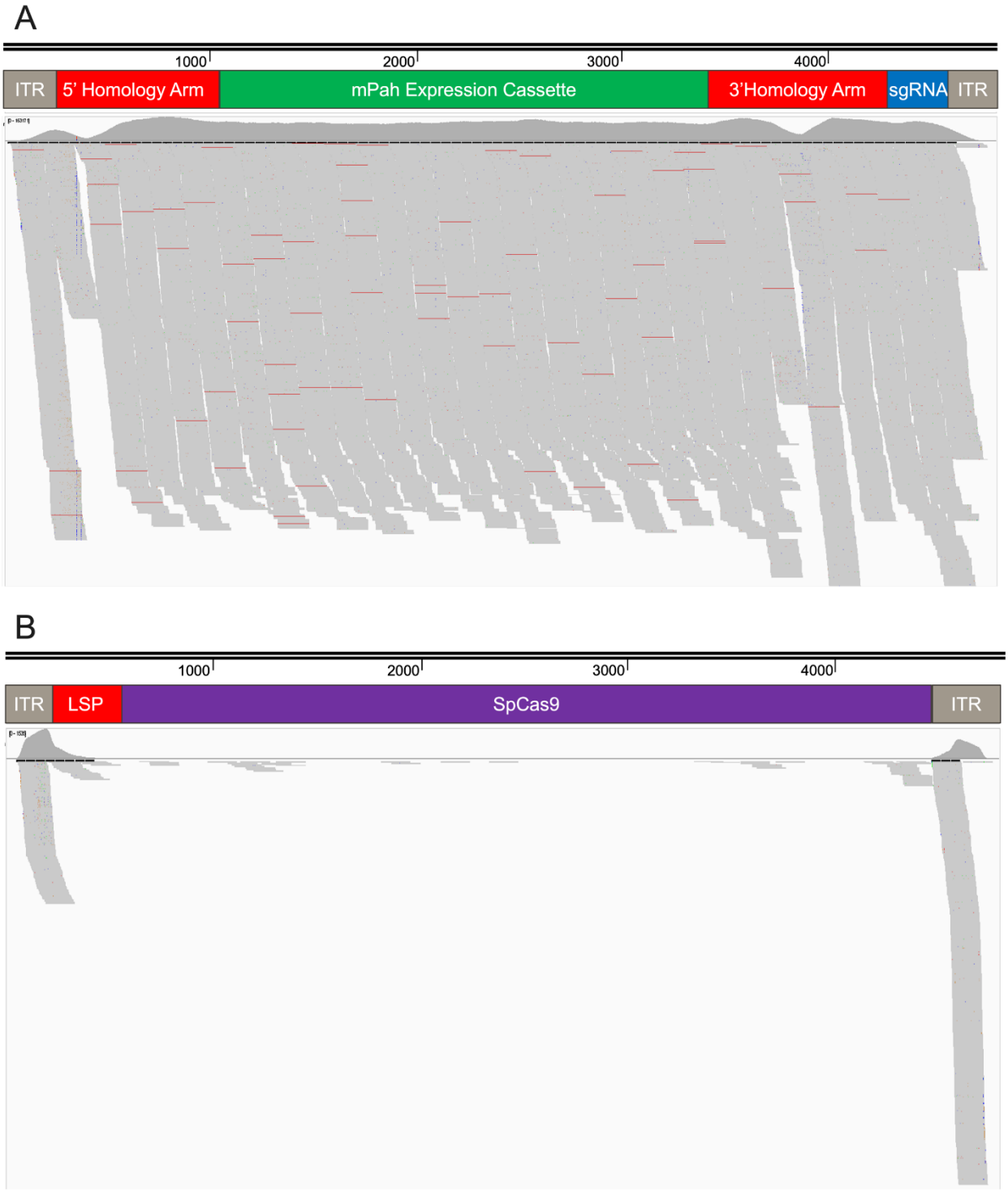


Figure 4-S1. Genome Annotation Depicting Coverage of Sequencing Reads. (**A**) Read coverage from enriched genomic DNA sequenced on Illumina next-generation sequencing (NGS) platform and aligned to annotated *Pah* Exon1 repair template. (**B**) Coverage of AAV2/8 spCas9 vector integrated at genomic target. Histogram indicates read coverage. Gray bars indicate number of aligned reads. Red lines indicate insertions-deletions (indels).

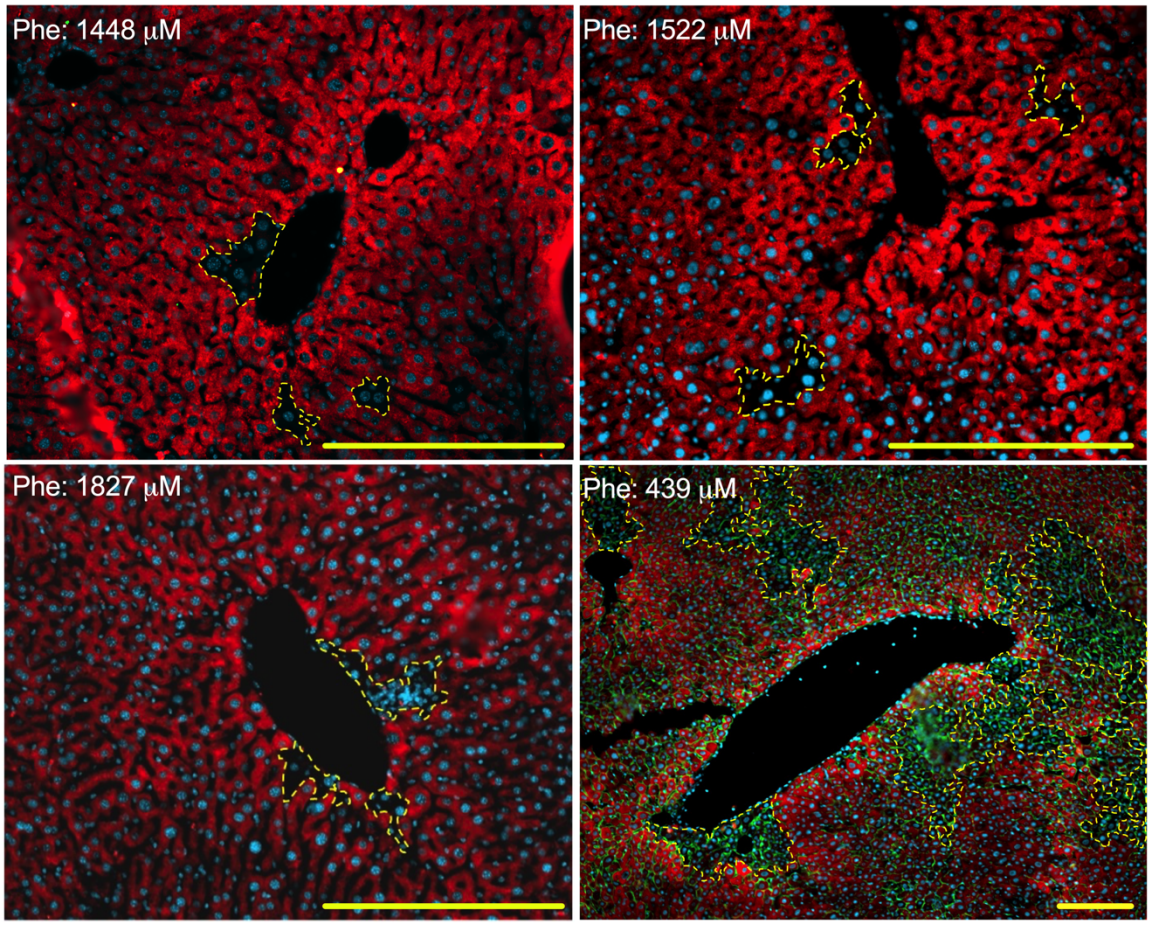


Figure 4-S2. Immunohistology of Exon7-shCYPOR Hepatocytes. Anti-CYPOR (Red, cytoplasmic) and anti-lectin (Green, cell membrane) immunostaining of female mice after injection of *Pah* Exon7-shCYPOR repair template and Cas9 AAV2/8 treatment and 18 weeks on 1.9% APAP diet. Dotted lines surround CYPOR negative colonies. Top, bottom right: 200x magnification; bottom right: 100x magnification. Scale bars, 100 μm.

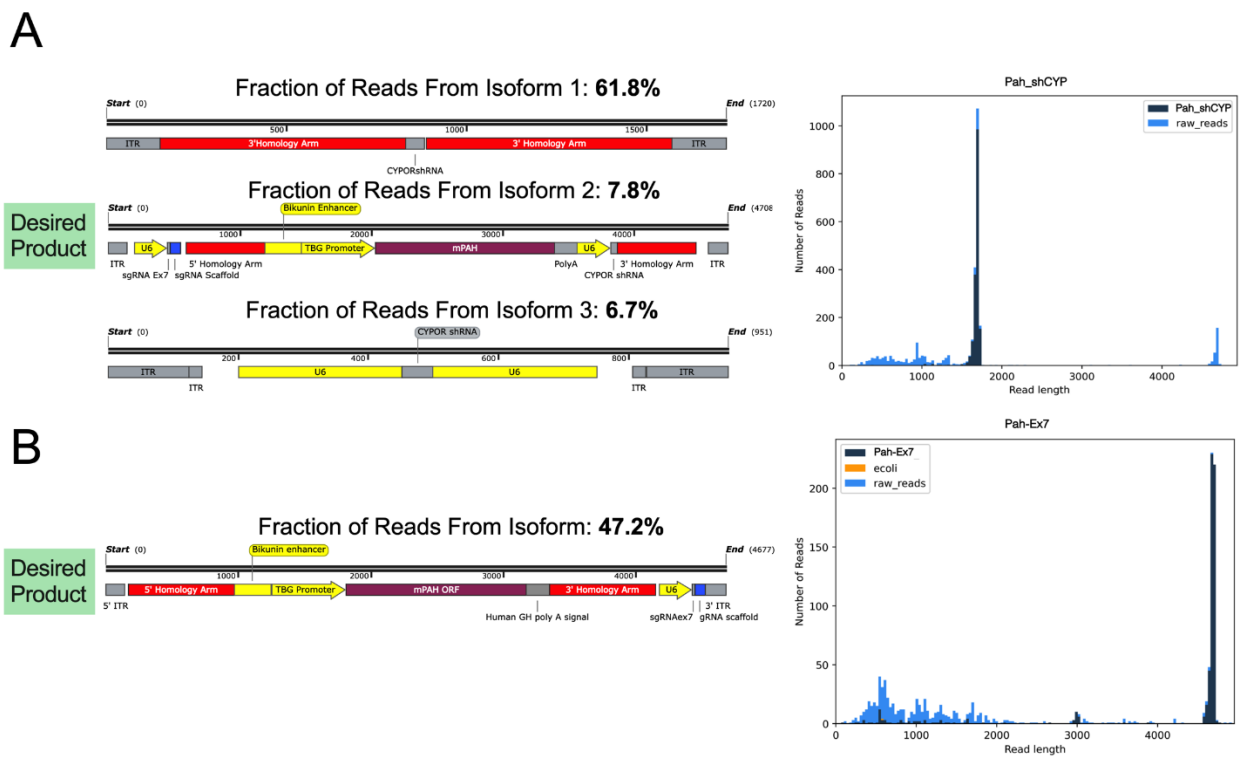


Figure 4-S3. *Pah* Exon7-shCYPOR Vector Genome Sequencing. (**A**) Oxford Nanopore sequencing of *Pah* Exon7-shCYPOR AAV2/8 vector genome. Consensus sequences of three isoforms listed in order of prevalence. Right: Histogram illustrating size of all sequenced reads. (**B**) Oxford Nanopore sequencing of Pah Exon7 AAV2/8 vector genome with only one consensus isoform sequence. Right: Histogram illustrating size of all sequenced reads.

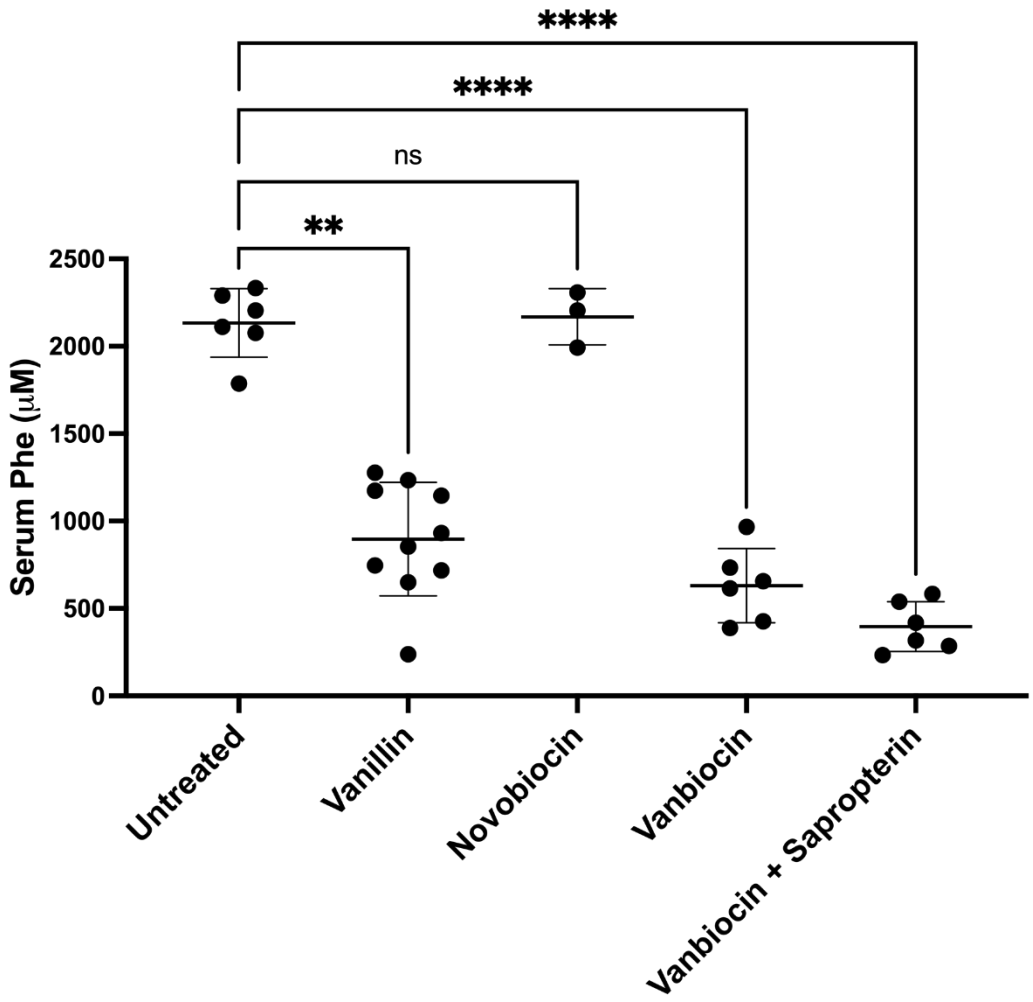
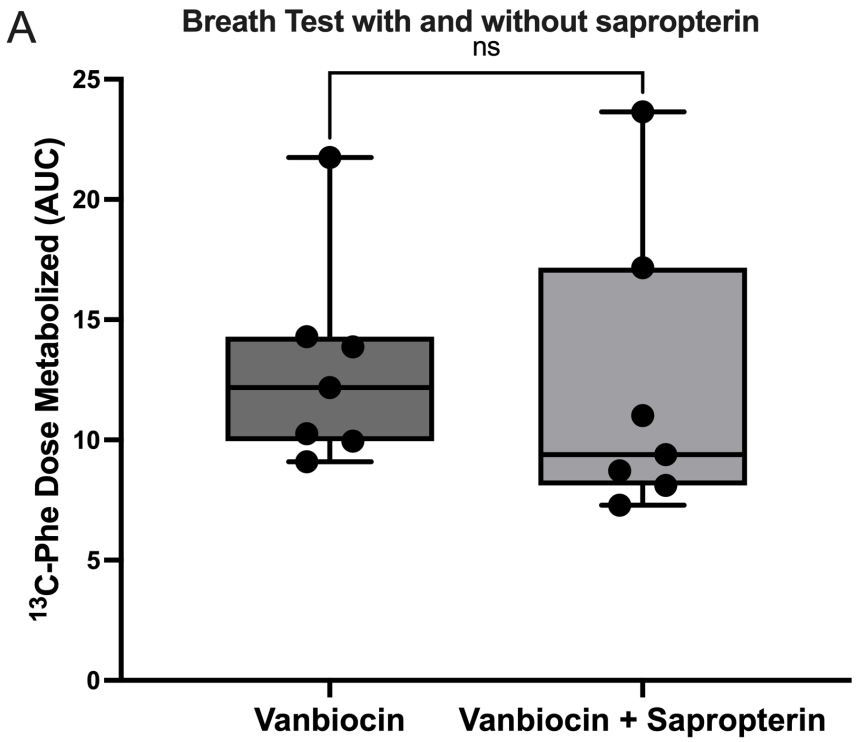


Figure 4-S4. Combinatorial Effect of Small Molecules on HDR. Serum phenylalanine concentrations after Cas9/*Pah* Exon7 AAV2/8 treatment with differing small molecule treatment. ****P <0.0001, **P = 0.0013, and P = 0.9869 by one-way ANOVA with Dunnett's multiple comparison test. Data are means ± SD.


A
Breath Test with and without sapropterin
ns
25
20
15
10
5
0
^{13}C-Phe Dose Metabolized (AUC)
Vanbiocin
Vanbiocin + Sapropterin

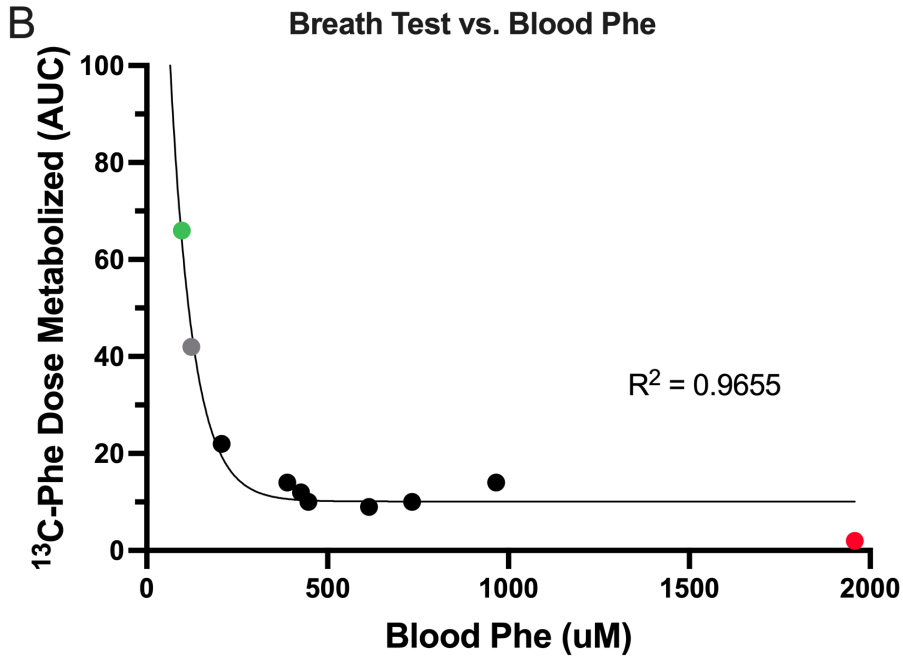

B
Breath Test vs. Blood Phe
100
80
60
40
20
0
^{13}C-Phe Dose Metabolized (AUC)
R^2 = 0.9655
0
500
1000
1500
2000
Blood Phe (uM)

Figure 4-S5. Stable Isotope Breath Test Data. (**A**) A stable isotope breath test, measuring the area under the curve (AUC) of exhaled radiolabeled $^{13}CO_2$ over 30 minutes following IP administration of 1-^{13}C-phenylalanine, was conducted on Vanbiocin-treated mice, with and without sapropterin gavage administered 4 hours prior to the test. *P* = 0.7612 by unpaired T-test. (**B**) Stable isotope breath data of all Vanbiocin treated animals (Y-axis, AUC), including those heterozygous for the *Pah*$^{\Delta exon1}$ mutation, versus blood phenylalanine concentrations. A single untreated wild-type (Green, Phe = 97 μM), *Pah*$^{\Delta exon1/+}$ (Grey, Phe = 124 μM), and *Pah*$^{\Delta exon1/\Delta exon1}$ (Red, Phe = 1958 μM) mouse were included in this study. Best fit to exponential curve (R^2 = 0.9655). Data are means ± SD.

Supplemental Movie 4-1. Motor dysfunction in AAV2/8 Pah Dexon7-shCYPOR treated litter. Mice treated with AAV2/8 vectors containing *Cypor* shRNA and Cas9, along with IP injections of 100 mg/kg of vanillin, at ~P16.

Supplemental Movie 4-2. Motor dysfunction in AAV2/8 Pah Dexon7-shCYPOR treated individual. Mouse treated with AAV2/8 vectors containing *Cypor* shRNA and Cas9, along with IP injections of 100 mg/kg of vanillin, at ~P16.

Discussion

Our results demonstrate that, through small molecule inhibition of NHEJ and MMEJ, large transgenes can be inserted into the genome of animals at a physiologically relevant frequency using HDR-dependent editing. The results presented here demonstrated an increased *in vivo* editing efficacy over the gene correction strategy we previously described[192], but with the advantage of inserting a full *Pah* transgene. If further refined, this treatment strategy could provide the same therapeutic relief as has been observed in AAV-mediated gene addition trials for humans with PKU[159,160,164,213,214] with the advantage of being a long-term treatment that is not dependent upon of the survival of viral episomes. As we have presented, this technique is not overly dependent upon homology arm length or a unique locus, suggesting this form of HDR has the potential to treat, not only PKU, but also a wide variety of other liver-based genetic diseases.

To enhance the efficacy of HDR-mediated transgene insertion, we provided edited hepatocytes with a selective advantage conferred via shRNA inhibition of CYPOR. However, we have found that the presence of shRNA in a repair template dramatically inhibits HDR-mediated gene editing when delivered by AAV2/8 vectors by both dramatically reducing the effective viral titer and providing competitive viral isoforms in the form of 'snapback' genomes. Future research that could deliver shRNAs in viral vectors without inducing titer heterogeneity may be able to rescue the integration frequency we observed in our earlier experiments (**Fig. 1,2**), while conferring edited cells with a selective advantage that will require far less time and APAP toxicity to provide a

therapeutic effect. Furthermore, expression of *Cypor* shRNA from AAV2/8 in neonatal mice appears toxic to the nervous system.

We found greater success through pharmacologic inhibition of NHEJ and MMEJ, the two DNA repair pathways that predominantly compete with the planned HDR event. Vanbiocin treatment resulted in mean transgene insertion frequency of almost 10% of hepatocyte genomes and 70.6% reduction in blood Phe concentration, which is to our knowledge the best efficacy from whole transgene insertion reported to date in PAH-deficient mice. Furthermore, sapropterin supplementation reduced mean blood Phe even further to 396 μM, just above the target lifelong treatment threshold for humans with PKU[277]. In humans with sapropterin-responsive PAH deficiency, sapropterin treatment may act through three possible molecular mechanisms: 1) stabilization of misfolded variant PAH monomers, 2) allosteric activation of PAH activity, or 3) rescue of *PAH* variants that alter the K_M for binding of the native tetrahydrobiopterin cofactor[143]. Given that Dexon1 mice do not produce any native PAH protein, the sapropterin supplement would only be acting on wild-type PAH expressed from the integrated transgenes: folding and kinetics of the transgene-expressed PAH protein was expected to be normal. Therefore, sapropterin responsiveness in Vanbiocin mice was unexpected. Sapropterin-mediated allosteric activation of transgene-expressed PAH is the likely mechanism to explain this observation. However, we were unable to demonstrate a significant increase in whole body 1-^{13}C-Phe oxidation following sapropterin treatment. So, the mechanism of Phe lowering following sapropterin treatment in Vanbiocin mice remains unexplained.

However, the results suggest that PAH-deficient humans exhibiting a partial response to clinical gene therapy may benefit from sapropterin treatment and experience further blood Phe reduction.

CRISPR/Cas9 nuclease treatment has been shown to produce off-target mutations across the genome in major regulatory segments and cause major translocation events due to aberrant repair[278]. However, one of the benefits of performing HDR in neonates, as we have done in this study, is that viral episomes responsible for Cas9 expression are rapidly lost during liver development, preventing long-term exposure to Cas9 and reducing the risk of genotoxicity[169,171,279]. In our previous work using the same *Pah* exon 7 sgRNA, we sought insertions and deletions at several of the most likely predicted off-target sites in genomic liver DNA and found these to be below the limits of detection for the amplicon based assay employed[192]. We have not repeated a search for off-target edits here, but a more exhaustive analysis for off-target genotoxicity will be necessary before this approach, with human genome directed reagents, could be advanced to the clinic.

HDR is dependent upon active cell division, as HR is active predominantly in late S/G2 phase of the cell cycle. Therefore, this treatment approach is maximally effective in neonatal or infantile animals with rapidly growing livers. Pharmacologic inhibition of NHEJ and MMEJ may not overcome the lack of HR activity in post-mitotic cells of adult animals; further studies are needed to understand whether pharmacologic enhancement of HR-mediated editing will be effective in adult animals.

While these promising results represent significant progress in HDR as a treatment option, in our opinion, we have not demonstrated sufficient efficacy to justify bringing this treatment design to human clinical trials. Individuals who are heterozygous for a *Pah* variant frequently exhibit a dominant-negative phenotype[33], wherein mutant PAH monomers form tetramers with wild-type monomers, leading to reduced enzymatic activity. As such, a greater transgene insertion frequency may be necessary both to disrupt mutant PAH monomer production and to restore wild-type PAH activity, thereby lowering blood Phe into the normal physiologic range. Therefore, further optimization is necessary to consistently achieve robust disease correction in murine and larger animal disease models. Targeted inhibition of other proteins involved in non-HR repair pathways, promotion of factors required in HR, or artificially promoting cell-cycle turnover could enhance the frequency of site-specific gene insertion and improve the physiologic relevance of this treatment strategy. In conclusion, while significant progress has been made, realizing the full potential of HR-mediated whole gene insertion for therapeutic applications will require a deeper understanding of the molecular mechanisms involved as well as strategic interventions to enhance precision and efficiency.

Methods

Animal husbandry

Animal care and experimentation were performed in accordance with the guidelines of the Department of Comparative Medicine, Oregon Health & Science University, and the NIH Guide for the Care and Use of Laboratory Animals. C57BL/6-*Pah*$^{\Delta exon1/\Delta exon1}$ mice (hereafter Dexon1 mice), which are homozygous for a deletion of exon 1 in the murine *Pah* gene, lack production of any PAH monomer and are completely deficient in liver PAH activity[9]. *Dexon1* mice exhibit severe hyperphenylalaninemia on an unrestricted diet. Neonatal Dexon1 mice for these experiments were generated through breeding of *Pah*$^{\Delta exon1/\Delta exon1}$ sires to *Pah*$^{\Delta exon1/+}$ dams. All mice were fed tap water and standard mouse chow (LabDiet Picolab Rodent Diet 5LOD, St. Louis, MO, USA) *ad libitum*, with the exception of mice on APAP diet, providing approximately 24% protein and 1.04% L-Phe by weight, except the breeders that received high-energy chow (LabDiet Rodent High Energy Diet 5058, St. Louis, MO, USA), providing approximately 22% protein and 0.99% L-Phe. Given that adult mice consume approximately 5 g chow per day, daily L-Phe intake was estimated to be approximately 50 mg/day. The animals were housed under a standard 12-h:12-h on-off light cycle. All surgical procedures were carried out with inhaled isoflurane general anesthesia to minimize pain. Mice that exceeded 20% weight loss were given 2 days on a high fat diet to regain weight. Animals were euthanized through CO_2 or isoflurane inhalation followed by cardiac puncture.

Genotyping of Dexon1 mice

To assess whether mice were homozygous for the Dexon1 deletion a PCR of Pah exon 1 was performed (5'-*GCAGGGAACAGTCATCCTTAAT-3'* forward primer, *5'-ACCAATGAGCCACCCAAAG-3'* reverse primer)[258]. An allele with the Dexon1 deletion will yield a product of 498 bp, while a wild-type allele will yield a product of 757 bp.

Stable isotope breath test

1-^{13}C-L-Phenylalanine (Cambridge Isotope Laboratories, Tewksbury, MA) was diluted to 5 mg/ml in sterile H_20. Each mouse received 100 ul injections IP and placed in custom breath collection chamber with CO_2 analyzer. Exetainer collection tubes (Labco, Lampeter, Ceredigion UK) were used to collect 5 ml of breath sample every five minutes from baseline to 30 minutes. CO_2 was measured in ppm simultaneously with each breath collection. The concentration of radiolabeled CO_2 was analyzed (Metabolic Solutions LLC, Nashua, NH) and used to calculate metabolic turnover of L-Phenylalanine as previously reported[280]. Data are reported as % 1-^{13}C-L-phenylalanine dose metabolized.

Vector design and viral production

Plasmids were designed in Snapgene (Version 7.0.1). Whole plasmid sequencing was performed by Plasmidsaurus using Oxford Nanopore Technology to confirm intact ITRs and correct plasmid structure. The AAV2/8 vectors used to express PAH and/or *Cypor* shRNA after insertion into *Pah* exon 7 in our study pAAV[Exp]-U6>sgRNAex7-5'HA and pAAV[Exp]-U6>gRNA-mPAH-

Ex7 were constructed and packaged by VectorBuilder (Chicago, IL). Viral vectors were purified via cesium chloride gradient ultracentrifugation and titers were estimated using qPCR-based quantification of ITR sequences. The vector IDs for these constructs were VB221216-1108hwe and VB220601-1003sae respectively, which can be used to retrieve detailed information about the vector on www.vectorbuilder.com and synthesized and packaged into AAV2/8 by VectorBuilder.

The OHSU Molecular Virology Support Core produced large-scale preparations of recombinant AAV2/8 virus for the insertion of *Pah* into Exon 1, referenced as AAV2/8 *Pah* Exon1 Repair Template, using standard triple plasmid transfection procedures into cultured HEK293 cells and purified by iodixanol gradient ultracentrifugation. AAV titers were determined using ITR-based qPCR analysis.

Delivery of Small Molecules and Viral Vectors

Vanillin was dissolved in saline (0.9% w/v) and heated in 50˚C water bath with frequent vortexing. Novobiocin sodium salt (Thermo Fisher Scientific, Waltham, MA) was dissolved in 400 uL of DMSO and heated in a 50˚C water bath before slowly adding 600 μL of saline preheated to 50˚C. Viral vectors, diluted in saline, were injected into the facial vein of neonatal mice at either P3 or P5. An IP injection of the desired small molecule cocktail was performed immediately after facial vein injection and would continue for 3-5 days.

Serum phenylalanine

Serum phenylalanine was determined using an established fluorometric protocol[253].

PAH enzyme activity

PAH enzyme activity was determined using a previously reported radioactive chromatography assay, with modifications[281].

Immunohistochemistry

Liver tissue was immediately placed in 4% paraformaldehyde after organ harvest and kept overnight. Tissue was then placed in a gradient of 10, 20, and 30% sucrose. Tissue was then frozen imbedded in OCT and 8–10-micron sections were collected using cyrostat (Tanner Scientific, Sarasota, FL) and transferred to slides (Superfrost Plus, Fisher, Pittsburgh, PA). Slides were permeabilized in 0.1-0.25% triton X-100, diluted in PBS, for 12 minutes, followed by a five-minute wash with PBS that was repeated three times. Blocking buffer of 10% normal goat serum (Sigma-Aldrich, Burlington, MA) was added to slide for 30 minutes. PAH antibody (Bioworld, BS3704) was diluted 1:500 in blocking buffer and incubated on slides overnight at 4 °C. Slides were again washed three times with PBS for five minutes. Alexa 594/555 goat-anti-rabbit antibody (Invitrogen, Eugene, OR) was diluted 1:500 in blocking buffer in addition to lectin fluorescein conjugated antibody (Vector Laboratories, FL-1171) at the same dilution. Secondary antibodies were incubated for 2 hours at room temperature and washed three times with PBS for five minutes. If staining for CYPOR was

desired anti-CYPOR antibody (Abcam: ab252084) was diluted 1:200 in blocking buffer and incubated overnight at 4C. Slides were washed 3x5 in PBS for a final time before Dapi-Fluoromount G (Southern Biotech) was added to sections and cover slips were mounted to the slides and sealed with nail polish. Slides were imaged using a Ziess ApoTome 2 microscope.

Image analysis

Image analysis for the quantification of PAH positive hepatocytes (**Fig. 4-2D**) was performed in ImageJ.

APAP preparation and delivery

To produce 1.9% (w/w) APAP chow, acetaminophen (6.84 g) was dissolved in 200 proof ethanol (50 ml). The APAP/ethanol solution was added to high fat chow (LabDiet Picolab Rodent Diet 5LOD, St. Louis, MO, USA) (360 g) and shaken until fully absorbed; the chow was then allowed to dry overnight in a fume hood. To induce selection of CYPOR-negative hepatocytes, gene edited mice were placed on 1.9% APAP treated chow for at least three weeks beginning after weaning. As APAP toxicity is associated with liver dysfunction which can artifactually elevate blood Phe, measurement of blood Phe concentrations was carried out in experimental animals only after APAP chow had been discontinued and standard chow substituted for at least one week prior to phlebotomy for measurement of blood Phe.

Insertion Frequency via qPCR copy number assay

The percentage of haploid liver genomes containing PAH cDNA insertions in edited mouse livers was measured by real-time quantitative polymerase chain reaction (qPCR) using a primer set complementary to *Pah* exons 7 and 8, that spans an intron boundary and therefore will not amplify the genomic *Pah* gene in a PCR protocol with a brief amplification cycle. The primers used span exon-exon boundaries between exon 7 and 8, with the as 5'-*GCTGGCTTACTGTCGTCTCG*-3' the forward primer and 5'-*CATGTCCCAAGAGTTCATGACAG*-3' as the reverse primer, with a final product size of 136 bp. A standard curve was created by diluting a plasmid carrying *Pah* cDNA to calculate the insertion frequency of the transgene in treated liver samples.

Target enrichment and NGS sequencing

Custom probes specific to the *Pah* expression cassette and 400 bp flanking the genomic cut site were produced (101001, Twist Biosciences, San Francisco, CA). Using the 'Twist Target Enrichment Standard Hybridization v1' protocol target sciences were enriched and read sequenced on Illumina NovaSeq 6000.

NGS data analysis

NGS data was aligned to the murine genome, and other genomes of interest (see **Supplementary Figure 4-1**) using the QuasR pipeline (Version 1.44.0). Aligned data was visualized using the Integrated Genomics Viewer desktop application.

AAV genome sequencing

Single stranded AAV sequencing was performed by Plasmidsaurus using Oxford Nanopore Technology with custom analysis and annotation shown in **Supplemental Figure 4-1**.

Qualitative assessment of urinary phenylpyruvate excretion

During the stable isotope breath testing, urine samples were collected passively over 30 minutes by placing Whatman 3MM Chr filter paper on the floor of the breath collection chamber. Areas of fecal contamination on the filter paper were excluded by its yellow appearance on long wave UV illumination. Urine spots that appeared purple on UV illumination due to the presence of urea were excised from the filter paper and eluted with 2 ml 0.01 M NH_4OH. Semi-quantitative organic acid analysis was performed by trimethylsilyl (TMS) derivatization followed by gas chromatography-mass spectrometry (GC-MS) according to the method of Hoffman and Sweetman[51] with modifications. In this assay, the concentration of the TMS derivative of phenylpyruvate was measured relative to the added internal standard, undecanedioic acid[282]. In this comparison, relative phenylpyruvate concentrations were not corrected for urine creatinine excretion.

Graphical illustrations

Graphical illustration of DNA repair and experimental workflow shown in **Figure 4-1** was created in www.biorender.com.

Statistical analysis

Significance of **Figure 4-2**, **Figure 4-3**, **Figure 4-4C**, and Supplemental Figure A were analyzed by unpaired T-test. An Ordinary one-way ANOVA was used to analyze significant in Figure **4-4D**, **4-4E**, **4-5A** and **4-5B**. The significance of supplemental **Figure 4-3B** was performed by one-way ANOVA. A post-hoc Dunnetts multiple comparison test to establish significant differences between groups. All statistical analysis was performed using Graphpad Prism version 10.2.2 for Mac (GraphPad Software, La, Jolla, California, www.graphpad.com). Full data and analysis results can be found in **Supplemental Table 4-1**.

Contributions

M.A.M. designed, executed, and analyzed data from experiments in this work, and wrote this manuscript with assistance from all authors. D.Y.R. designed and executed initial experiments targeting *Pah* Exon 1. S.R.W. performed facial vein injections, tissue processing, and performed PAH protein activity assessment in later experiments. A.M.B. performed NGS data analysis and visualization. A.V advised the experimental design and analysis for experiments utilizing Cypor shRNA-mediated selective advantage. S.D. and L.H. bred and genotyped Dexon1 mice. C.O.H. assisted in the design of all experiments in this manuscript as well as its writing.

Data availability statement

Datasets generated or analyzed during the current study are available from the corresponding author upon request.

Acknowledgements

We thank Biomarin and Dr. Brian Whitlock at The University of Tennessee for providing the Kuvan (sapropterin dihydrochloride) used in this study. We thank the OHSU viral production core for AAV production and tittering. We also thank Aaron Bieleck for assistance photographing mouse coat color. Finally, we would like to thank Dr. Charles Venditti for providing equipment to execute $^{13}CO_2$ breath testing.

Funding

All work funded by a National PKU Alliance (NPKUA) Research Award to COH.

Chapter 5: The Ethics of Gene Editing

Intelligence and education that hasn't been tempered by human affection isn't worth a damn.

- Daniel Keyes, *Flowers for Algernon*

Introduction

I am not, by any traditional measure, a philosopher of morals or ethics. In some ways, it's as senseless for me to write about ethics as it would be for an ethicist to write about the influences facilitating one DNA repair pathway over another. But they don't live in a laboratory, and I do live in a world with ethical consequences. Most scientists shy away from these discussions, and I can't say that I blame them. It's difficult enough to assess the ethical dilemmas of the present without concerning ourselves with the ethics of 'what-could-be'. Especially when your livelihood is based upon making that 'what-could-be' a reality. However, what we are trying to achieve in this work could have unimaginable societal consequences. If successful, the practical development of gene editing would unleash an incredible power for society, and I believe that it is our responsibility, as its creators, to consider the consequences *before* it is fully developed, and not after. After all, once a new discovery is made it is almost never the scientists who maintain control of it. Fermi, Einstein, and Oppenheimer can all attest to that. Therefore, if we are to have any say regarding how this technology should or should not be used, we should say it now.

I believe the continued dereliction of ethical discourse in scientific spaces will foment the repression and misuse of gene editing; creating opportunities for dogmatic or misinformed opponents of scientific research to impose restrictions—restrictions that frequently result in cruel, illogical policies that hinder innovation and medical care. For example, President George W. Bush, after banning federal funding for embryonic stem cell research, called for prohibiting "the most egregious abuses of medical research," including viable animal-human

hybrids[283]. This statement revealed a fearful ignorance that conflated legitimate stem cell research with transgressive and fictional human-animal experimentation. Policymakers Andrea L. Bonnicksen and Bernard Rollin refer to this as “bad ethical thinking”, which they define as “a confusion of whatever disturbs people with genuine ethical issues”[284,285]. They suggest that “if scientists do not look much into the ethical implications of their research, this leaves a vacuum about ethical implications”[284], which is soon “filled by doomsdayers, sectarian theologians, politicians, and others with claims that tend to be thrilling, lurid, and pretentious but are devoid of genuine ethical content”[285]. It is my belief that such a vacuum exists in the discussion of human gene editing today, and by the time the technology achieves practical applicability it will be born into a hailstorm of scientific distrust, misinformation, and conspiracy theories.

As scientists, our role is not to render ethical judgments alongside our discoveries; it never was, and hopefully, it never will be. Our mission remains, as it always has been, to distinguish the substantive from the sensational, the genuine from the false. Usually this requires forming testable hypotheses and then rigorously testing them, but it also means giving thoughtful attention to legitimate societal concerns—asking hard and uncomfortable questions that often defy an objective resolution. And asking our society and policy makers to be, as Kurt Vonnegut once said, “kinder and more responsible than they often are”[286]. As such, I have included this ethics section to take a personal role in weighing the practical and moral dangers of my research. With the hope that the

scientific community, and ultimately society at large, can better understand and address the broader implications of my research when it eventually bears fruit.

Technical & Societal Ramifications of Germline and Somatic Editing

Somatic vs Germline Cells

Somatic cells do not participate in reproduction. These are, by far, the most plentiful cells in the body and perform all of the functions that keep us breathing, walking, and thinking. Germline cells are those involved in reproduction, such as sperm and ovum. Unlike somatic gene editing, changes to the DNA of germline cells can be readily passed down to future progeny.

Germline Gene Editing

The research reported in this thesis has developed methods for somatic gene editing, which does not carry the same dangers as germline editing. The ability for our viral vector (AAV8) to transduce and edit germline cells is minimal, if not nil, as demonstrated in **Figure 4-5.** However much of the mechanistic implications of our research are directly transferable to germline editing. As such, we should ask what our intentions for gene editing, and for medicine as a whole, are. Furthermore, we must consider how our research will be used when it inevitably falls into the hands of those who do not share our values.

As a thought experiment, let's consider the consequences of using germline editing with the best of intentions. Let us say that we hope to achieve one of science's greatest goals by curing cancer, and we've decided to borrow a trick from elephants to do so. Elephants, despite their large size and long life-span, seem to be less prone to developing cancer than humans and other mammals[287,288]. Some researchers suspect this cancer resistance is due to

multiple copies of tumor protein P53 (*TP53*). *TP53* is a gene that expresses the tumor suppressing protein p53, capable of triggering DNA repair[289], arresting cell development and division[290], and initiating apoptosis[291]. While humans have only a single copy of *TP53* in their genome elephants have at least 20[292]. This *TP53* copy number variation (CNV) is hypothesized to confer cancer resistance in these animals, with evidence suggesting that evolutionary pressure to increase body size coincided with *TP53* duplication[292]. Therefore, if we wanted to use gene editing to defeat cancer, the so-called "Emperor of All Maladies", might we recreate this *TP53* CNV in human embryos?

It simply wouldn't be feasible to implant an edited embryo for every single new birth. However, if each nation created a program to sponsor embryonic editing in a fraction of the population, then, after a few generations, the population with one or more copies of the variation may expand. If those with the *TP53* CNV are resistant to cancer they will likely be less of a burden on that nation's health care system. As such, the government would have a financial incentive to encourage those with the *TP53* CNV to have more children than those without the CNV. This could come in the form of direct payments, tax deductions, sponsored childcare, or even by restricting the number of births for those lacking the variation. With these advantages, the presence of such a copy number variation may become the norm for newborns within a few hundred years.

This extreme example may resemble the sensational and unsettling hypotheticals described by Rollins and Bonnicksen, but my purpose in presenting

it is not to provoke fear; rather, it is to explore whether it is even possible to perform safe and ethical germline editing of any stripe. And I would challenge the reader to seriously consider this example, and examples like it, as we discuss the societal and health risks below.

Societal Implications

Editing of germline cells as a public health policy will almost certainly require governmental legalization, if not endorsement, and it will be easy to unintentionally create policies that benefit one “genetic class” of people over another. Even with the best of intentions, an attempt to eradicate genetic diseases would require a government approved elimination of “bad genes” and promotion of “good genes”. This may not only lead to some kind of socio-genetic caste system, wherein people cohabit, marry, and work in isolation based on genetic differences, but may also create a state-sponsored endorsement of a favored genetic class. In other terms: there may not be a way to separate germline editing from eugenics. Even if all 195 governments of the world restrain themselves from actively sponsoring such programs, the mere legalization of germline editing could signal an acquiescence to creating genetic value judgements. Judgements that may be adopted culturally to disastrous effect.

How then can we parse our social goals from our medical goals? “The Goals of Medicine” by Mark J. Hanson and Daniel Callahan suggests that there are four fundamental objectives in modern medical care: “prevention of disease, relief of suffering, care of the ill, and avoidance of premature death”[293]. The only reason why germline editing would be medically used is as a prophylactic treatment for coming generations. However, I do not believe this truly qualifies as

“prevention of disease” because that goal was intended to be applied to existing individuals with a justifiable risk of contracting a disease. Therefore, when you begin to actively alter germline tissue you’re making an enhancement, not providing a treatment. However, there are already those who crow about the great opportunities gene editing provides for enhancing humankind[294,295], and companies are cropping up promising to select embryos for *in vitro* fertilization that have the greatest potential IQ[296,297]. In my view, all of these interventions blur the line between disease prevention (a medical goal) and genetic enhancement (a social goal). Moreover, the very concept of genetic "enhancement" is inherently shaped by societal value judgments rather than the principles of evolutionary fitness.

Health Risks

One of the major risks is that germline editing does not simply insert or remove a genetic variation from an individual, but from the gene pool. Some argue that this is the only way to truly eliminate genetic diseases from plaguing humanity. However, unlike somatic editing, it has the potential to harm, not only a given patient, but all of the progeny of that patient. Therefore, the possibility of eliminating genetic diseases stands shoulder-to-shoulder with the possibility of creating new genetic maladies.

Allowing germline editing to become a routine medical procedure would inevitably introduce unique genetic variations into the population. While the immediate effects may not be apparent, these changes could manifest late in life or lead to further structural rearrangements of the genome through generations. For example, the creation of multiple duplications of a sequence is associated

with diseases like Huntington's. So, if we were to insert CNVs into our genome we would have no way of knowing, and even less way of controlling, how that variation would further evolve or interact with other genetic elements. Indeed, much of our working knowledge of DNA expression, replication, structure, and expression remains woefully incomplete. Over the course of my own education, I have seen trivial "junk DNA" transform into critical "non-coding DNA", and the discovery of topologically associated domain (TAD) boundaries revolutionize how we think about gene expression. Given this, it's clear that we cannot accurately assess the safety of emerging germline editing technologies without a much deeper understanding of genomics and evolution. Therefore, it is my opinion that we should restrain ourselves from performing germline editing or risk the health and welfare of untold future generations.

Case Study: Embryonic CCR5 Editing in Humans

In 2018, a Chinese scientist by the name of He Jiankui shocked the world by announcing that he had used CRISPR-Cas9 to knock-out the *CCR5* gene in two newborn twins. The *CCR5* gene has garnered great interest among some researchers as its inhibition has been correlated with immunity to HIV[298]. The scientific community's condemnation came swiftly, with researchers[299], educators[300], and even the NIH director, Francis Collins[301], expressing their revulsion at the immense irresponsibility of this experiment.

The controversy stemmed from both how and why this experiment was performed. It came to light that He had used the popular Chinese social media application "WeChat" to recruit Chinese couples with HIV (infected men, and uninfected women) for his so-called "clinical trial"[302]. He did so outside of the

purview of any regulatory body that would provide review or oversight; without approval from the Chinese government, which expressly outlawed germline gene editing[303], and without providing third-party education or representation to his patients (*i.e*. test subjects). He had lured desperate and ill couples seeking to have a healthy child by promising them an opportunity to be a part of an historic experiment that would allow them to reproduce without government restriction. What's more, this procedure was entirely unnecessary: a technique employed by He called sperm washing was sufficient to eliminate the risk of HIV transmission to the child. Moreover, *CCR5* depletion is not without its own dangers, it has been linked to increased susceptibility to infections such as West Nile Virus and influenza[304]. Despite the lack of any clear medical need, there were significant risks that He largely ignored. He had implanted embryos into the mother despite sequencing data showing mosaicism. This means that at the time of transplantation He could not have known if his desired edit would be fully conferred (indeed, one of the twins ended up being heterozygous for the intended deletion) or the extent of possible off-target mutations.

Not only was this experiment poorly executed, but the reasons for attempting it in the first place were equally bankrupt. Even if successful, the modification would not have represented a scientific breakthrough: every technique He employed had already been developed by better scientists. Neither would it provide a new avenue for public health, as there already existed much less expensive means of preventing HIV in newborns. The only possible benefit to these infants is enhanced intelligence and mental plasticity correlated with

CCR5 silencing mutations[305,306] Therefore, He's primary interest was likely not in conferring HIV immunity at all, but in enhancing intelligence and placing himself at the forefront of a new age in human gene editing. As the Guangdong Provincial Health Commission would report in January of 2019, He Jiankui had conducted this work "in pursuit of personal fame and fortune, with self-raised funds and deliberate evasion of supervision and private recruitment of related personnel[307]." In other words, He had purposefully evaded regulation and jeopardized multiple lives to fulfill his own vainglorious attempt to be the first scientist to perform germline editing on a human. For this, He was sentenced to a three-year prison sentence in China. Since his release, He has become the director at the Institute of Genetic Medicine at Wuchang Technical College and remains active at fundraising and communication of his goals on Twitter and WeChat[308].

This incident marked the first time someone performed germline gene editing on a human. The question the world wondered was, "What are the chances that it would be the last time?" The answer to that question, we realized with dread: vanishingly small. What's more is that the research developed by thousands of scientists with good intentions, research like that found in this dissertation, could be used for these purposes. In fact, this episode unmasked a small yet dedicated contingency of scientists, politicians, and oligarchs actively promoting these goals. Therefore, I believe the He Jiankui incident should act as a clarion call for open and robust ethical discourse. Beyond discourse, we need

regulation alongside a clear understanding of the values we support and the goals scientific research should strive to achieve.

Case Study: Homology Medicines' Clinical Gene Integration Trial

The dangers of somatic gene editing may not hold the same scope as germline editing, but there are some unique factors that are worthy of consideration. Gene therapy that relies on viral vectors will inherently cause a near permanent change in the immune system for those treated. As discussed in **Chapter 1**, a single dose of AAV is sufficient to provide long-term immunity to that vector. As such, when a patient in a clinical trial is given an AAV injection that has no possibility of providing therapeutic relief, it deprives them the opportunity to be effectively treated with that vector in the future. This creates an ethical quandary: how do you correctly control patient studies with randomized trials without endangering the health and welfare of the patients. This problem isn't entirely unique to gene therapy, disease research has long grappled with this problem, but the complexities mount when the field is almost entirely dominated by small private corporations.

Homology Medicines Incorporated (hereafter Homology Medicines) was a biotechnology company developing a means to treat PKU by inserting a functional phenylalanine hydroxylase gene, with a liver specific promoter, into patients. Initially, they had made claims to developing a unique AAV serotype (AAVHSC15) that could provide long-term expression of the gene in murine models of PKU[309]. In my personal conversations with representatives from Homology Medicines, they believed the transgene was in fact inserted into the genome, without a double-strand break, due to the presence of the homology

arms flanking their transgene. Despite the dearth of evidence that their vector would work as claimed, and the unsupported notion that homology arms are sufficient for transgene insertion, Homology Medicines began a clinical trial based on their research.

As discussed in **Chapter 2**, Homology Medicines was providing a form of gene addition that would cause an acute drop in blood phenylalanine levels due to expression of viral episomal vectors. Previous studies had shown that after a few years, liver-directed episomes would become diluted with the liver due to cell turnover, making such treatments temporary[310,311]. Unsurprisingly, Homology Medicines reported an initial drop of serum phenylalanine in their clinical subjects[312], but these patients failed to demonstrate consistent permanent expression of the vector. In 2022, Homology Medicines was sued by its investors for misrepresentation of the efficacy and commercial viability of their clinical trial[313]. While this suit proved unsuccessful the loss of investor satisfaction led to lay-offs of 87% of Homology Medicines' workforce by 2024, and the selling of its intellectual property to Q32 Bio[314]. As a result of their insolvency and subsequent merger, Homology Medicines is no longer required to provide *post hoc* analysis or treatment for potential hepatocellular carcinoma in the patients they treated. Bankruptcy has even allowed Homology Medicines to evade the FDA requirement for providing a 15-year follow-up after a gene therapy trial since there is no way to enforce that requirement on any successor. The subjects and their metabolic clinics are simply left drifting.

To review, the patients treated were brought into a clinical trial and told there was a possibility they would be cured, which was not supported by the established literature. When this proved false and Homology Medicines essentially dissolved, they abandoned their patients who were now potentially immune to any future AAV cure, and without monitoring the potential long-term side effects from the trial.

It's easy to cast down scorn for failed experiments and theories, when, in truth, failure is part-and-parcel to the scientific enterprise. However, there is a fine line between ignorance and reckless incompetence, and this line can be easily crossed when there are inherent and lifelong side-affects in a given treatment approach. Providing a sick person with a viral vector without a possibility of successfully treatment, whether that's an empty vector or a poorly designed one, causes direct harm to the patient by denying them the future possibility of an AAV-mediated cure. As such, I believe we should seriously reconsider any such study design and adopt the direct of The Hippocratic Oath when conducting clinical trials: due no harm. This may require adjust or abandoning pure control groups or, at the very least, provide informed consent to those in them if there is any realistic negative consequence. Additionally, corporations evading their responsibility to patients through bankruptcy may be legal, but it is wholly unethical. It is my opinion that the leaders of such companies should be held financially responsible for fulfilling the responsibilities that they had agreed to. This is especially important when the translational application of research is dominated by private equity.

Ownership and Access

Gene therapy treatments have longed been criticized by their high individual cost. Zolgensma, which is an AAV-directed gene therapy treatment for spinal muscular atrophy, costs $2.125 million per dose, as of 2022[315]. I can say from personal experience that the cost of a given treatment is one of the first things those with genetic disease ask about when discussing potential options. Many question whether the high cost of these nascent treatments excludes all but the wealthiest from gaining access to them, and if it is a way to exploit an exorbitant amount of money from a population with no other alternatives.

While I do consider these concerns regarding cost important to consider, it is also difficult to limit individual cost while developing a new technology. After all, if we had held personal computing to the same standards as gene therapy we may have been infuriated at the multi-million dollar, 30-ton, monstrosities produced by Hewlett-Packard fifty-years ago. However, the costs for the treatments, much like the cost of computers, will improve as the technology's efficiency improves. The current high price for these treatments is largely due to the extreme costs to develop and produce them: the production, purification, and quality control for the viral vectors alone can cost millions. However, as development of these treatments becomes more and more popular the economy of scale should reduce these production costs. Additionally, I think health insurers may be incentivized to cover such treatments; two or three million dollars for a curative treatment may save a health insurer ten million dollars for the lifelong treatment of those with a disease like PKU. However, the question of when

people outside of our healthcare system will have access to these treatments is unclear.

With that said, as long as ownership is in the hands of a small group of individuals the possibility for exploitation remains high. As does the possibility that promising avenues of research will be abandoned based, not on clinical efficacy, but on its competition with more profitable therapeutics. Currently, there are approximately 15 gene therapy treatments currently approved by the FDA for a wide variety of diseases[316]. Each one of these treatments was developed using publicly funded research, and several of them were conceived and initially validated in NIH funded labs. However, all of them are now the property of corporations whose primary legal obligation is to reap the most profit to its owners and shareholders. While we live in a capitalist society that encourages for-profit healthcare, I think it bears considering if it is ethical to have a singular pathway for translational development; a pathway that hands the breakthroughs in medical research away from the people who have funded it for decades, and into the hands of corporations seeking to extract the most profit from them.

This conversation is particularly relevant in the field of gene therapy. As there is no clear mechanism to bring breakthroughs in gene editing from the lab into the clinic that doesn't go through private equity. What then is the goal of scientific research, and the scientists who build it, if not to enrich biotechnology investors?

We grapple with these questions in all areas of healthcare, but in regards to the research in this dissertation I wonder how feasible it will be to patent some

gene editing technologies. If we developed a method for enhancing HDR with common small molecules, would it even be possible for a corporation to own and thereby profit from such a process? Without the ability to own and patent the technology, is there any mechanism that would fund its ascent through clinical trials and into the clinic? I have offered more question marks than conclusions on this subject, but that is a part of my point. I do not recall a discussion in the scientific community that sought to create this current system. Nor have I observed scientists questioning the morality of it. Instead, as if through entropy, the ownership of our scientific labor is falling into the hands of those driven purely by a profit motive. And I am deeply concerned that patients' access to treatments may be limited by this competing interest.

As an alternative, patient advocacy groups, such as the National PKU Alliance (NPKUA), may be able to raise money from donors within its membership, and from limited external investing, to foster treatments that can be owned collectively by the patient population itself. There would be many challenges with this strategy, but it may behoove us, as scientists, to reach out to such organizations when we have developed a promising treatment option. Whether through this mechanism or others, it is my hope that we explore alternative funding pathways for our scientific achievements. Otherwise, we risk creating an environment in which no invention may benefit mankind before it first enriches the wealthiest individuals.

Conclusion: A Regulatory Balance

While these ethical discussions are important, their purpose, outside of a doctoral dissertation, is to assist in the creation of rational and value-based

regulations. Much of the conversation around the ownership and access of potential gene therapy treatments is occurring as a debate around healthcare as a whole. As such, this section will discuss the regulations on scientific research with an emphasis on germline editing, which I believe to be the primary concern for my own research. To begin, let's briefly explore the global regulatory landscape for this technology.

The current laws surrounding germline editing in humans is largely based upon transgressive human experimentation that has occurred in the past. The infamous Tuskegee syphilis study as well as Nazi human experimentation led to the near world-wide adoption of the ethical principles enumerated in the Belmont report, the Nuremberg Code, and the Declaration of Helsinki[315]. These documents emphasize scientific medical research that provides "respect for the individual, informed consent of the patient, understanding of the risks and benefits, voluntary participation, fairness in the conduct of experiments, maximum professionalism of the researchers, etc."[315]. However it wasn't until 1997, that the first binding international treaty (exclusively among European countries) was signed, which prohibited the misuse of biomedical research and protected inherent human rights[300,317]. The Oviedo Convention prohibits human genome editing of all stripes unless there is a medical cause for its use[318]. It also provides regulations regarding the consent, privacy, and education that protect the dignity and welfare of patients during biomedical research. Between the Oviedo Convention and domestic legislation, heritable human genome editing is

currently banned in at-least 70 countries, with more nuanced restrictions in others[319].

These legal restrictions are a valuable safeguard against medical malfeasance. However, there remains a continued interest and research into heritable germline editing. If this isn't properly addressed within the scientific community such human experimentation may continue in nations that have no such restrictions, such as South Africa or Egypt. If we wait for such an event to occur again the legislative backlash may very well stymie legitimate biomedical research. Therefore, I believe we should form a world-wide convention of scientists to specifically decide when research should be avoided due to the unknown risks. This type of restriction is known as the "precautionary principle," and it has been previously instituted within the scientific community to pause certain lines of research until the risks can be reasonably assessed[320]. This kind of agreement was famously instituted after the discovery of recombinant DNA, when attendees of the Asilomar Conference of 1975 proposed a moratorium on molecular cloning until the risks could be better understood[321,322]. A similar moratorium was proposed, and widely adopted, for the editing of human embryonic cells, when, in 2015, a group of highly established biotechnologists met in Napa after the advent of CRISPR technology[323]. However, just a few years later, He Jiankui performed CRISPR-mediated gene editing on two implanted embryos, bringing widespread condemnation and renewed appeals for a moratorium of such experiments[324,325].

These attempts at enforcing the precautionary principle may have been well intentioned, but they proved ineffective in a number of ways: the discourse behind these restrictions was typically between a self-selected group of elite American scientists with much of the global, and even national, scientific community excluded; the discussions and meetings occurred intermittently, if not spontaneously, with no real follow-up or structure; and their rationale and decisions failed to make an impact with society at large. This is why we need to form an organized, world-wide, scientific convention that can discuss the ethical and practical dangers within the research community and create regulations that are humane, intelligent, and reflective of our fundamental values. I have listed proposed guidelines for utilizing the precautionary principle below:

Guidelines for Applying the Precautionary Principal:

1. Must be in response to a danger to public health, not individual health
2. Should be instituted by a large, international, group of scientists
3. Should clearly and publicly explain rationale
4. Must actively work to investigate unknown dangers to the best of their ability
5. Must be re-evaluated at regular, predetermined, intervals

Finally, as we attempt to reach compromises and consensus within the scientific community, we will need to consider how to make a long-term impact on public thinking. If future generations took rational and safe precautions for granted, they may ignore the long-term dangers of genetic interventions. Unfortunately, public discourse is generally ill-suited to forward thinking. Future

generations are pure abstraction, and many believe, as they have for millennia, that humanity or, at the very least, the knowledge of our civilization, will not survive very far past our own lifetimes. Therefore, whatever genie we take out of the proverbial bottle won't be around for long. However, I strongly disagree with this view. The general trend of humanity is towards survival, and if humanity has any merit, it is our ability to retain the knowledge of the past. Therefore, if we do not establish publicly accepted and understood regulations for this technology it is inevitable that it will eventually be misused on a broad scale.

More than anything else, it is my hope that we, as a scientific community and as a society, actively scrutinize whether our actions hold true to the values that we claim. My greatest fear isn't that the wrong decisions will be made, but that no decision will be made at all; that we will stumble into the future, filling whatever empty space we detect and being all the more vacuous for it. If our research is handed to a society that operates in this way, I believe it will be used to satisfy our most avaricious tendencies, and not to aid the most vulnerable members of our society. For my own contribution to this discussion, I would echo the sentiments of another, far more eloquent, Portlander:

"*We are frequently told that greed for endless increase of material goods is natural and universal – as is greed for endless life. We are all supposed to agree that you can't be too rich or live too long. The desire to live is certainly natural and universal, since it's the basic directive of living creatures: once born, our job and our desire is to try to stay alive. But is that the same as the desire to stay alive forever, to be immortal? Or is it just that we can't imagine not being, so we*

invent an endless existence called immortality? … The idea of individual immortality, an endless ego-existence, is more dreadful to me than the idea of letting go the self in death to rejoin shared, eternal being. I see life as a shared gift, received from others and passed on to others, and living and dying as one process, in which lies both our suffering and our reward. Without mortality to purchase it how can we have the consciousness of eternity? I think the price is worth paying."

- Ursula K. Le Guin[326]

Chapter 6: Discussion and Future Directions

Current *in vivo* Editing is Improved Yet Insufficient

These results have demonstrated that the dual inhibition of NHEJ and MMEJ significantly enhances the efficiency of HDR. This is likely caused by an increase in the frequency of HR for the repair of double-strand breaks. Using AAV-delivered Cas9 and a PAH repair template, we were successful in lowering the blood phenylalanine concentration in murine models of PKU from ~2,000 μM to an average of 631 μM. We found that these levels could be further lowered to 396 μM, very near the threshold for hyperphenylalaninemia, by providing oral supplementation of sapropterin dihydrochloride, a co-factor of PAH.

As of the time of this writing, these results represent the most efficacious treatment of murine PKU using homology-based gene editing ever achieved. Unlike untreated Dexon1 mice, our treated mice successfully gave birth to a litter of pups and sustained them through weaning (**Fig. 4-5**). Furthermore, the coat color of affected mice, which is brown due to phenylalanine's inhibition of melanin production, was restored to black (**Fig. 4-5**). However, even with this progress, our treatment strategy remains insufficient for providing a full cure for PKU and should not be advanced into human clinical trials. Humans with similar blood Phe concentrations experience adverse mental and behavioral symptoms that necessitate dietary restrictions. This is supported by the significantly decreased brain weights among our treated mice in comparison to their unaffected siblings (**Fig. 6.1**). Finally, while our demonstration of sapropterin as an enhancer post-*Pah* insertion may benefit future treatments, it is only relevant in diseases whose pathophysiology involves aromatic amino acid hydroxylation.

Therefore, further research into DNA repair modulation and Cas9-mediated HDR is required to develop a treatment that can fully mollify phenylalanine concentrations in individuals with classical PKU. Our research validates a combinatorial small molecule approach for enhancing HR and illustrates the untapped potential of HDR. Finally, unless we have taken manipulation of DNA repair to its utmost extreme, this work suggests that a cure for a multitude of genetic disease is very possible using Cas9-mediated *in vivo* gene editing.

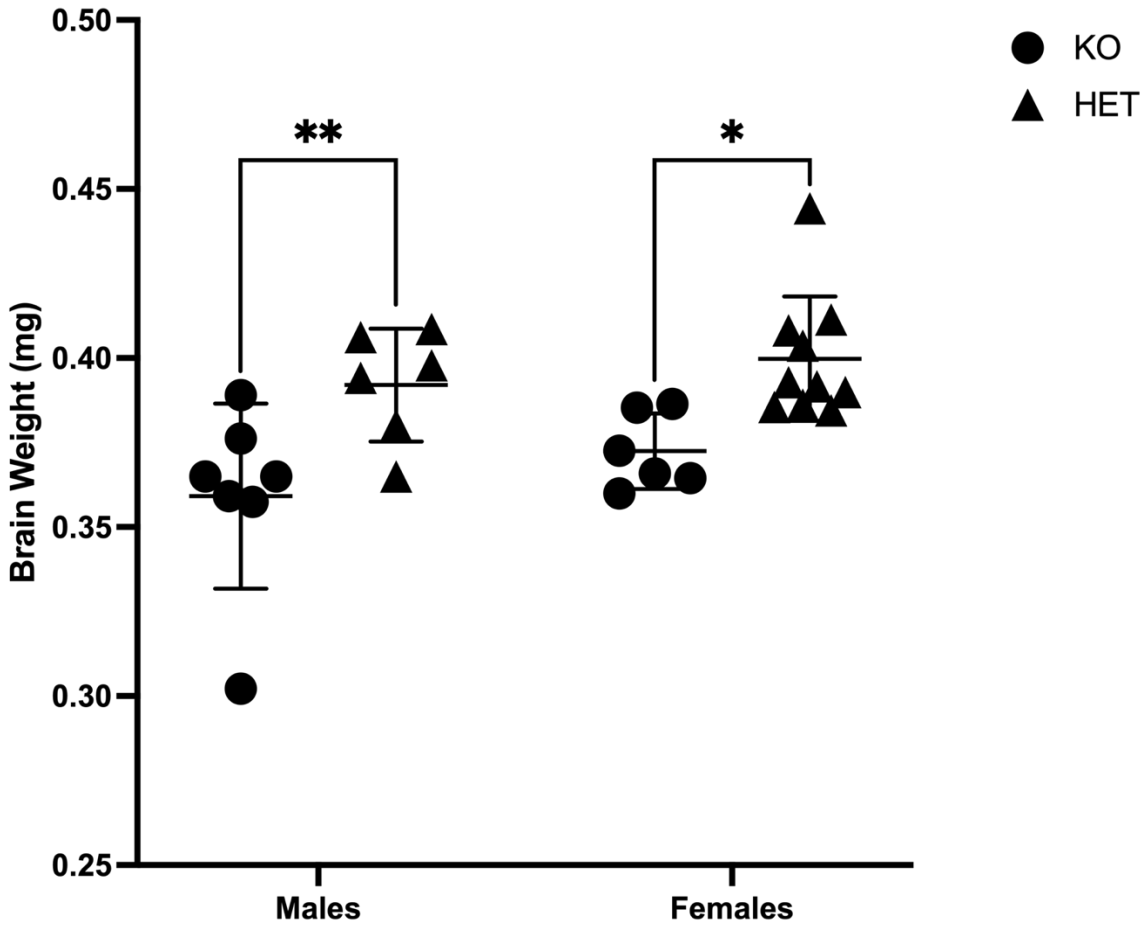


Figure 6-1. Brain Weights of *Pah* Exon1 Gene Integration. Brain weights of animals treated with AAV2/8 Pah Exon1 (see **Figure 4-2**) 4 weeks post-weaning (***P*=0.0057, **P*=0.0119). Two-way Anova (Sex, Treatment).

High-Dose Neonatal AAV-Treatment Poses Neurologic Risks

Our work using *Cypor* shRNA failed to provide a tangible solution for our low editing efficiency. However, it did provide a number of surprising observations that may prove useful in future AAV-mediated treatments. These are enumerated below:

1. The incidence of snap-back genomes and titer heterogeneity in AAV vectors with an shRNA
2. Death and severe motor dysfunction following AAV injections that included shRNA
3. Sex-based response to acetaminophen hepatocyte selection

Future studies may attempt to separate the desired vector from truncated snap-back genomes to rescue the effective titer and prevent competitory repair templates during HR. Regardless, the death and motor dysfunction of these mice suggests that viral transfection of the brain or other parts of the nervous system is occurring: we just don't observe it using other vectors because there's no deleterious effect as there is when producing shRNA. We have suggested this may be due to a partially formed blood brain barrier in newborn mice. However, it remains to be seen if high-dose AAV treatment ($>1x10^{13}$ vg/kg) can penetrate or perturb the nervous system of grown animals. There is a single documented occurrence of an adult human developing motor dysfunction after high-dose AAV treatment in the literature. Due to the infrequency of this observation these neurological risks have gone largely unstudied. However, the adverse effects when using Cypor shRNA may in fact provide a model to future researchers as a

means to isolate, identify, and resolve the factors at play. Our own Nissl staining in the treated brains of these mice yielded no observable pathology (**Fig. 6-2**); though perhaps future neuroscientists can perform more thorough investigations.

Finally, the sex-based difference in our Cypor study seems contradictory; we have not seen a sex-based difference in our other gene insertion experiments, and these differences were only observable after acetaminophen selection began. Baseline phenylalanine concentrations are lower in males than in females and transduction frequencies of some viral vectors are more efficient in males as well, but this would not explain why we observe a sex-based difference here and not in our previous editing experiments. Additionally, a single female mouse did respond well to acetaminophen selection, which may indicate that sex was not the determining factor in this treatment. It is possible that the mice that responded to treatment, predominantly males, simply consumed more food and were therefore exposed to more of the drug. Females, due to their improved sense of smell and slightly smaller size, may have been less likely to consume food laced with such a bitter drug[327]. However, due to the small sample size of this study, we cannot ignore the possibility that this variance is simply a random occurrence that is not based on sex at all.

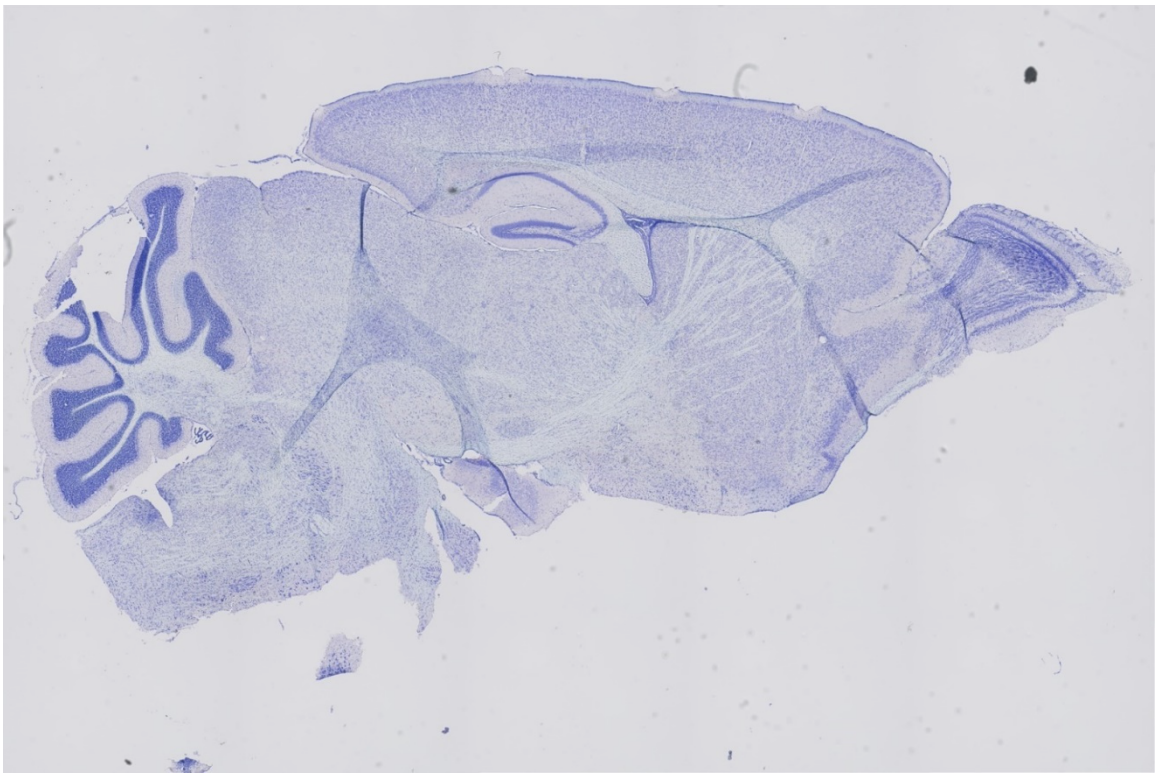


Figure 6-2. Nissl Staining in AAV2/8 *Pah* Exon7 Cypor Pup.

PASTE Editing

The work in this dissertation primarily concerns itself with homology-based gene editing. However, there are several other forms of genetic manipulation. One advancement has been in the use of Programmable addition via site-specific targeting elements (PASTE) editing. PASTE editing uses a prime editor to create the targeted insertion of a small “beacon” sequence. A transposase or integrase can then be delivered into the target cell and integrate a large transgene with a “receptor” sequence directly into the new genomic beacon. A recent paper, published by the now defunct company, Tome Biosciences, demonstrated that the presence of AAV vectors during the prime edit caused insertions into >20% of hepatocytes in non-human primates[328].

This system has yet to tested in an *in vivo* model of disease, and our Dexon1 mouse would be prime testing grounds for this technology. However, instead of depending upon HR, there may be a dependence on NHEJ, or other pathways, that resolve insertions of inverted terminal repeats. Therefore, our lab may be poised to take our practical experience in applying gene repair modulators into a system that is not dependent upon DSBs to elucidate an integration system that is largely unexplored.

Our future experiments will attempt to build upon our protein and small molecule discoveries to find improved methods for enhancing gene repair. In addition, we will explore alternative methods of gene integration, such as PASTE editing and the dual-flap integrations reported in Tome’s previous work.

Characterization of Motor Dysfunction Phenotype Post AAV-shRNA Treatment

One of the more surprising results from this dataset is the motor dysfunction and viral heterogeneity/truncation observed when delivering a CYPOR shRNA. This presents two avenues for future research: (1) elucidation of the exact cause of the AAV8-induced motor dysfunction, and (2) alternative approaches for CYPOR inhibition during transgene integration.

To investigate the first avenue of research, it may prove useful to deliver an AAV8 vector expressing GFP to neonatal mice followed by histological analysis of GFP in the brain and spinal tissue. The goal of this experiment would be to confirm that AAV8 is capable of crossing the BBB and help ascertain the exact cell types causing this side effect. As mentioned previously, prior studies have shown DRG pathology post-AAV8 inoculation, but, due to the sensory role of DRGs this does not adequately explain the dysfunction we, and others, observed when delivering shRNA in an AAV vector. This research will be vital before treating human newborns with such vectors, as it may demonstrate that the nascent BBB is insufficient to fully protect the brain from high-dose viral infection. Such a discovery may also provide benefits to treating genetic disease affecting the brain by providing a developmental window in which the brain can be efficiently targeted with intravenous inoculation of such viral vectors.

CYPOR Knockout via Transgene Insertion

The second avenue of research may benefit from attempting to insert a transgene with a terminator sequence (transcriptional stop signal) at its end, and then inserting that into one of the introns of the CYPOR gene. This would ensure

that the targeted integration of the transgene is linked with the truncation, and silencing, of CYPOR. Unfortunately, both alleles of a diploid hepatocyte would require the targeted insertion to fully benefit from the protective effects of CYPOR inhibition. Though it is possible that partial knockdown of a single allele will still provide some level of APAP-mediated selective advantage.

This strategy holds significant benefits over attempting to simultaneously knockout CYPOR using a sgRNA while inserting the *Pah* transgene. As this approach would allow hepatocytes without integrated transgenes to become APAP resistant, which may create a PAH-deficient population to compete against the fully edited cells during selective growth. Additionally, these data suggest that ~8% of hepatocytes are receiving the transgene that has been provided, and previous data using the *Pah* exon 7 sgRNA with vanillin measured on-target indels at a frequency of ~6%[192]. This would suggest that to the success of both events (transgene integration and indel production) would occur in approximately 0.48% of hepatocytes. Therefore, the majority of hepatocytes that received the Pah integration would not carry the desired CYPOR mutation and be just as exposed to APAP toxicity as unedited cells.

Investigating Viral Packaging of i53 AAV Vectors

Despite doubling *in vitro* HDR frequency by two-fold, the addition of i53, a 53BP1 specific ubiquitin variant, demonstrated no benefit in the *in vivo* model. It is possible that episomal expression of i53 was detrimental to viral packaging. Previous studies have shown that inhibition of 53BP1 significantly diminishes production of the Epstein Barr virus (herpesvirus 4)[329]. However, this is likely due

to the interaction between 53BP1 and a protein called Zta, which acts as a viral switch governing viral latency and replication[329]. While this exact mechanism is not utilized in GMP grade viral production, it isn't difficult to imagine that the inhibition of 53BP1 could severely impact the replication and packaging of viral DNA. Therefore, sequencing of viral genomes packaged with this construct should be performed to assess the content of these vectors as well as digital-droplet quantification to accurately assess viral titers. If it is found that viral packaging of i53 is complicated by expression of its own transgene an alternative approach would be to split i53 into two vectors as an intein fusion protein or to control i53 expression using an inducible promoter.

Regulatory Solution: Formation of the National Genetics Administration

As I consider the current scientific landscape, I find myself looking towards other great public works. In this way, I have become another victim whose imagination has been caught by La Sagrada Familia. This structure stands as one of the grandest and most awe-inspiring cathedrals in the world. Millions flock to Barcelona for the opportunity to gawk at the soaring honeycomb spires and kaleidoscope stained-glass, which resemble a fantasy wilderness as much as a church. Despite the hundreds of sculptures that adorn its façade and the meticulous detail dripping from every square inch of its masonry La Sagrada Familia remains incomplete, as it has since its construction first broke ground in 1882. At that time, a young Antoni Gaudi was still in the process of fully designing his magnum opus, the construction of which would have been impossible to realize with his current technology. Despite this, Gaudi and his benefactors laid a foundation and formulated a quest so intricate and complex that it wouldn't, and couldn't, be completed within 150 years.

I find myself thinking of this often and marveling at the patience and prescience of Gaudi to begin an endeavor that he would never live to see completed. Nothing could be further from the state of modern disease research. Startups design business plans that predict a handful of years before typically going bankrupt. NIH-funded labs are only marginally better—often churning out enough immediate results to publish in respectable journals, allowing them to renew grants and continue their research in the never-ending, hamster-wheel race for academic survival. In this way, small steps are made in our

understanding of biology and disease. As is the way of scientific progress. Small steps.

A notable, and fairly recent exception to this script is the human genome project, which was first proposed in 1984 and finally "completed" in January of 2022. This massive international undertaking would employ thousands of scientists and dozens of institutions to knit together a single complete reference of the human genome. This is a masterwork of science, which has created entirely new fields and has perhaps done more to open the door for the identification and cure of genetic diseases than any other single project. With this in mind, it's interesting to look back at some of the original discussions surrounding the project. The feasibility of sequencing the entire genome with high fidelity was uncertain, the costs exorbitant, and, as Charles DeLisi would note, there was a general concern that "the technology of the day was not appropriate for the complexity of the task"[330]. However, the benefits of such a project were undeniable and, correctly predicting that the technology of the day would develop as the task progressed, the project was eventually funded with a completion date set decades in the future.

It seems inevitable in retrospect, yet projects like this are very rare. They typically only progress if they are vital for national defense or pass through a gauntlet of scrutiny from the scientific community and, most importantly, funding bodies. However, this project would likely not have been completed without the strong political connections of the leading scientists and the economic boom that

the United States experienced at the end of the century. And it is not realistic to expect these same factors to converge for the next grand research project.

We are, as has been noted by popular science magazines the world over, in the midst of an extremely exciting revolution in the study of genetics; the advent of CRISPR affiliated gene-editing tools and the explosion in genetic and epigenetic analysis has provided us with completely new ways of reading, understanding, and manipulating DNA. Despite this, there has not been a complementary change in the funding or administrative practices to propel this revolution. We have largely been content to let venture capitalists and the meager funding of the NIH fuel one of the greatest works of human exploration, both of which are insufficient for the realization and democratization of these technologies. Indeed, private industry will inevitably seek to commodify, and therefore control, the fruits of this labor, which may have disturbing consequences.

How then should we both encourage these advances while also making them widely available? The last time we encountered a similar scientific challenge we founded a separate administration, NASA, to put leading scientists in collaboration with one another for a collective goal. Independently none of those scientists could obtain the funding to land on the moon, and the inherently competitive nature of research would have inhibited immediate communication of their research. Meaning that the incremental gains that are part-and-parcel to the scientific endeavor would be retread by various independent groups before being widely disseminated. As such, I think it is time to establish a National Genetics

Administration to conduct large-scale projects for the next great frontier of discovery. This time, instead of studying the largest and grandest objects in the universe, we will be studying one of the very smallest: the double-stranded helix that is the sole source of all known life.

The chief aim of the National Genetics Administration would be to improve the health and well-being of the people. An acute goal would be to improve upon and accelerate gene therapy treatments for sickle-cell anemia, hemophilia B, and phenylketonuria. Such an administration could conduct the long-term studies that will be required to fully evaluate the safety and prolonged efficacy of gene editing and gene addition treatments, which will take several decades and require significant investment. Few such long-term studies are currently in the works, and, to my knowledge, none is seriously being planned, largely due to the high financial costs that will not merit a commensurate product.

Additionally, this initiative would create a space where leading scientists could directly collaborate on ambitious and innovative projects, such as developing animal organ donors, modifying plants to produce valuable commodities and medicines, and discovering cures for genetic diseases. While these are all grand and exciting outcomes, it is important to note that the purpose of the National Genetics Administration wouldn't necessarily be to fund moonshot projects, but to fill fundamental gaps in our understanding to achieve specific goals.

Whether the project is large or small, the scientific method inherently leads to incremental advances in knowledge—small step by small step. However, by

sharing resources, fostering collaboration over competition, embracing long-term projects that may take decades or even centuries to bear fruit, and accelerating the dissemination of experimental results, we can take those steps more quickly and efficiently. In doing so, we will not only advance science but also provide a direct benefit to people along the way.

Conclusion

This thesis has explored the fundamentals of metabolic disease, the history and pathophysiology of PKU, and gene editing techniques, both new and old, to cure it. The data herein demonstrate the utility of using the cell's native repair mechanisms to insert whole genes. More specifically, we have demonstrated that inhibition of polymerase theta, Ku70/80, and PARP-1 can dramatically enhance the frequency of HDR *in vitro* and, in the case of polymerase theta and Ku70/80, *in vivo*. These factors play a vital role in either NHEJ or MMEJ, suggesting that inhibition of these pathways causes a concomitant increase in the utilization of HR.

Our success integrating a complete transgene highlights the broad utility of this research; carrying significant implications in not only disease research, but also as a means for interrogating genetic structure and function. With continued research and optimization there is hope that our group, or another in the field, will be able to hurdle the obstacles standing between patients and an efficacious treatment. I thank you for your careful consideration of these ideas, and hope that they may be of use to you as a scientist, patient, or student.

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