



Unraveling the Genetic Code: Harnessing Precision Editing for Improper Cargo Sorting and Membrane Trafficking Within Inherited Cardiovascular Diseases

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Unraveling the Genetic Code: Harnessing Precision Editing for Improper Cargo Sorting and
Membrane Trafficking Within Inherited Cardiovascular Diseases

Chelsea L. Smith

A Thesis in the Field of Biotechnology
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Abstract

Background: Cardiovascular diseases affect over 80,000,000 individuals in the United States alone. Each disease has a broad range of debilitating consequences that affect not only the individual but, quite possibly, their offspring. For most cardiovascular diseases, inherited DNA sequences are influential in the genotype and phenotype (Kathiresan & Srivastava, 2012). As cardiovascular diseases remain the most common cause of death worldwide, the scientific community needs to utilize the most up-to-date technology, such as genetic editing techniques, to try and conquer these diseases. Myriad tools such as genome-wide association studies have recently been used to help identify previously unknown affected genes and variants, allowing for a further and more in-depth understanding of cardiovascular diseases (Vrablik et al., 2021). The scientific community already understands many inherited cardiovascular diseases, but the utilization of the newest technology has yet to make a significant appearance in reducing the number of affected individuals.

Hypotheses: I hypothesize that each inherited cardiovascular disease can be effectively treated using a specific genetic editing technique, tailored to the unique characteristics of each condition. The approaches are outlined as follows:

H1: The **CRISPR/Cas9** technique would successfully treat Familial Hypertrophic Cardiomyopathy (FHCM) because by gene correction, editing the faulty DNA sequences responsible for FHCM could potentially restore the normal function of the affected proteins.

H2: The **Cpf1 (Cas12a)** technique would successfully treat Danon Disease because Cpf1 offers extremely high precision in targeting genes, whether in gene silencing, insertion, or replacement, could offer complete restoration of the *LAMP2* gene and correct the LAMP2 protein function.

H3: The **MAGE** technique would successfully treat Pompe Disease because the MAGE technique not only offers to edit the mutated *GAA* gene but also could be beneficial by gene augmentation by providing increased production of the *GAA* gene, which could compensate for the defect and improving affected cells, in the breakdown of glycogen.

H4: The **Zinc Finger Nucleases (ZFNs)** technique would successfully treat Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C) because this technique targets with specificity and precision but it could not only correctly target the genes associated with one desmosomal protein, but the myriad involved with ARVD/C such as desmoglein (DSG), desmoplakin (DSP), and plakoglobin (JUP).

H5: The **Base Editing** technique would successfully treat Barth Syndrome because base editing allows for specific editing of individual nucleotides which would directly affect the mutation in the *TAZ* gene.

Methods: A multi-centric retrospective cohort review will be performed on individuals with a confirmed inherited diagnosis of Barth Syndrome, Arrhythmogenic Right Ventricular Dysplasia / Cardiomyopathy (ARVD/C), Pompe Disease, Danon Disease, and Familial Hypertrophic Cardiomyopathy (FHCM). Utilization of published articles, reports, trials, and journals between the years 2000-2023 will be used to gather information on the five above-listed inherited cardiovascular diseases and the specificity of each disease's cargo sorting membrane trafficking, and disease function.

Diagnosed individuals will be compared to others with the same diagnosis to confirm whether their disease presents the same genetically and establish a common baseline of genetic similarity. Those who have undergone treatment for the above-listed diseases will be analyzed to compare how their treatment has affected the disease and estimate whether genetic editing or engineering would be affected by such treatment.

To establish a baseline of genetic editing techniques, this study will solely focus on the following approaches: multiplex automated genome engineering (MAGE), Cpf1 (Cas12a), base editing, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9). As each of the above-listed techniques provides a unique approach, utilization of various journals and published articles will be analyzed to articulate the benefits and drawbacks of each genetic editing technique and conclude which approach would pose the greatest chance of influencing or curing each inherited cardiovascular disease.

Results: The initial hypotheses proposed that CRISPR/Cas9 would effectively treat FHCM by correcting faulty DNA sequences, Cpf1 (Cas12a) would restore *LAMP2* gene function in Danon Disease, and MAGE could change the outcomes of Pompe disease treatment by editing and augmenting the *GAA* gene. Additionally, ZFNs were hypothesized to provide precise correction of multiple desmosomal gene mutations in ARVD/C, while base editing was expected to correct point mutations in the *TAZ* gene for Barth Syndrome. The results confirmed the potential of CRISPR/Cas9 for FHCM and Cpf1 for Danon Disease, aligning with the original hypotheses. However, MAGE was found to be less viable for Pompe disease, with ZFNs emerging as a more effective

approach. Base editing also proved to be the most promising treatment method for both ARVD/C and Barth Syndrome due to its precision in correcting point mutations without introducing double-stranded breaks. These findings refine the understanding of gene-editing applications for inherited cardiovascular diseases and highlight how their therapeutic potentials can facilitate change in treatments for diseases.

Conclusion: This study evaluated the feasibility of various gene-editing techniques for treating inherited cardiovascular diseases, identifying the most promising approaches based on precision, efficiency, and safety. CRISPR/Cas9 demonstrated strong potential for treating FHCM due to its versatility and ability to generate targeted genomic modifications. Cpf1 (Cas12a) emerged as a superior option for Danon Disease, offering enhanced repair efficiency and reduced off-target effects compared to other nucleases. ZFNs showed the greatest promise for Pompe disease, leveraging their precision and established success in clinical applications. Base editing was identified as the most effective method for treating ARVD/C and Barth Syndrome, given its ability to precisely correct point mutations without causing double-stranded breaks, minimizing genomic instability. These findings highlight the strengths of each technique in addressing specific genetic disorders, paving the way for future advancements in gene therapy.

Author's Biographical Sketch

Growing up, I was surrounded by influences that instilled a strong sense of determination and resilience within me. Inspired to pursue a path in the medical field, I embarked on a journey that has been shaped by challenges, growth, and an unwavering commitment to research and biotechnology.

My undergraduate years at UC Irvine were marked by a dedication to Biological Sciences, despite facing challenges. These experiences tested my resilience, and I emerged stronger, equipped with a mantra of strength, work ethic, and commitment, reinforcing my dedication to scientific inquiry and a better understanding of medicine.

At UC Irvine, I joined a research lab focused on Autism, where I delved into the complexities of functional genomics. This experience allowed me to work with patients on a cellular level, providing a unique perspective on the diversity of Autism and the tailored challenges each patient faced.

Further pursuing my commitment to medicine, I volunteered with Global Medical Brigades in Nicaragua, where I provided essential medical assistance to underserved communities. This experience underscored the value of accessible healthcare and highlighted the potential for biotechnological advancements to transform patient care in resource-limited settings.

Today, my resilience, passion for research, and dedication to making a difference drive me forward as I pursue my ALM in Biotechnology, eager to contribute to meaningful advancements in the field.

Dedication

To my Incredible Parents and Grandmother,

Unwavering love, support, and belief in my successes have been my greatest strength throughout my life. The encouragement you give me to follow my dreams has given me the ability to surpass the obstacles in life. Enjoy the little things, the memories, and the foundation you have given me to succeed. This accomplishment would not have ever been possible without a question of an unknown answer. You have always recognized my ability to surpass difficulties and constantly resonate with the consistency of love. Devotion of time and effort will get you anywhere in life, thank you both for showing me the way.

“A job worth doing is a job worth doing well” -Philip Stanhope, the 4th Earl of Chesterfield..

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Chapter I.

Background

Genetic editing techniques have been ever-evolving in the world of medicine and pose a substantial recent benefit to the medical community. Genetic editing techniques are being utilized and expanded for their potential to cure diseases by precision targeting, potential for permanent correction, personalized medical treatments, and much more. As more genetic editing techniques are used for myriad diseases, a more comprehensive understanding of how these techniques can be directed to inherited cardiovascular diseases, specifically how they can be used to influence the genetic mutations that cause membrane trafficking and cargo sorting of lipids and proteins.

In determining which five inherited cardiovascular diseases will be involved in this analysis, a thorough background check of information through journals and published articles will ultimately determine individuality and uniqueness in development and overall genetic presentation within the genome of individuals. The following inherited cardiovascular genetic diseases being investigated each have unique disruptions in membrane trafficking and cargo sorting. These include Barth Syndrome, Arrhythmogenic Right Ventricular Dysplasia / Cardiomyopathy (ARVD/C), Pompe disease, Danon Disease, and Familial Hypertrophic Cardiomyopathy (FHCM).

To decipher which genetic editing technique may be best suited to successfully treat each inherited cardiovascular disease, six distinct techniques were selected that vary in their overall strategy and execution in the delivery of each method. There are various

genetic editing techniques, and to assess the compatibility for each noted disease, this proposal will be specifically researching multiplex automated genome engineering (MAGE), Cpf1 (Cas12), base editing, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) because of their wide variety of mechanisms of actions.

This thesis aims to decipher which genetic editing techniques, including MAGE, Cpf1 (Cas12), base editing, ZFNs, TALENs, and CRISPR/Cas9, can successfully alter or remove the functionality of inherited cardiovascular diseases, including Barth Syndrome, ARVD/C, Pompe disease, Danon Disease, and FHCM, in diagnosed individuals and possibly the next germline generation. Utilizing the retrospectively collected data will determine eligibility and overall compatibility between the disease orientation and the technique itself.

To conduct this research, a multicentric retrospective review will be performed on patients with a confirmed inherited cardiovascular diagnosis between 2000 and 2023. A comprehensive review of published articles, reports, trials, and journals will be analyzed to develop a comprehensive library of published materials on each disease and gene editing technique.

Genetic editing techniques first arose in the early 1970s, which allowed scientists to begin the forefront of succession into the manipulation of DNA (National Human Genome Research Institute, 2013). In 2009, CRISPR technology was developed, allowing for the possibility of changing the genome of germ line cells, offering a cheaper, more accurate and faster means of genetic editing. Since then, myriad techniques have

been developed that offer a wider selection of editing. This has enabled scientists to open the door for various gene therapies that can not only provide treatment to individuals but also prevent the disease from being passed onto offspring (National Human Genome Research Institute, 2019).

The use of genetic editing holds tremendous potential to revolutionize medicine, offering a groundbreaking approach to solving some of humanity's most pressing challenges. Genetic editing and engineering pose both risks and benefits to society and the future of medicine. Some benefits include the ability to treat, prevent, and even cure certain inherited disorders, but also treat various cancers and infections. The risks associated with gene editing techniques include unanticipated mutations which can lead to cancers, damage to organs and or tissues, and other diseases such as neurological and immune system disorders. Many of the current advances in genetic therapies are safer than previously demonstrated, but scientists are still studying the risk factors involved especially when it comes down to alterations that would be passed down to offspring (National Heart, Lung, and Blood Institute, 2022). In 2017 the NASEM Human Genome Editing Consensus Report displayed openness to scenarios in which germline genetic editing may be justified and acceptable. Like the consensus report, in 2018, the Nuffield Council Report called for public debate as part of its recommendation, but both influential reports retained two commonalities: pillars of public engagement and establishment of guiding principles (Halpern et al., 2019).

The transformative potential of genetic editing techniques in the context of inherited cardiovascular diseases is profound, offering not only the promise of precision medicine and tailored therapies but also the possibility of permanent genetic correction.

This chapter has traced the historical evolution of genetic editing technologies, emphasizing their growing capacity to address the complex genetic underpinnings of inherited cardiovascular diseases. By focusing on the distinctive disruptions in membrane trafficking and cargo sorting implicated in the listed conditions, this research may establish a framework for evaluating how advanced genetic editing strategies could provide effective therapeutic interventions. The following chapter will delve into the specific genetic mutations associated with these diseases, elucidating the molecular mechanisms that drive their pathogenesis, and laying the groundwork for the application of genetic editing techniques in each case. Through this, a deeper understanding of the potential of genetic modification in the treatment of these disorders will be brought to light, ultimately setting the stage for further exploration in subsequent sections.

Chapter II.

Inherited Cardiovascular Diseases

Inherited cardiovascular diseases include a diverse group of conditions, each caused by unique genetic mutations and mechanisms. For instance, Barth Syndrome arises from mutations in the *TAZ* gene, affecting mitochondrial function. At the same time, ARVD/C involves the replacement of cardiac muscle with fatty or fibrous tissue. Pompe disease is marked by glycogen buildup in muscle cells due to an enzyme deficiency, whereas Danon Disease stems from a mutation in the *LAMP2* gene, leading to disrupted lysosome function. FHCM, on the other hand, involves heart muscle thickening caused by mutations affecting protein sorting. These differences exemplify the importance of evaluating specific genetic editing techniques for each condition. The subsequent sections of this chapter will provide a more thorough examination of each of these conditions.

Barth Syndrome

Barth Syndrome is classified as a rare X-linked recessive disorder that almost exclusively affects males and is characterized by cardiomyopathy (CMP), skeletal myopathy, neutropenia, growth retardation, and elevated urinary levels of 3-methylglutaconic acid (3-MGCA). Barth Syndrome is linked to mutations in the *TAZ* gene (G4.5) located on chromosome Xq28, which codes for the phospholipid transacylase tafazzin protein. These mutations in the *TAZ* gene are shown to lead to a deficiency in tafazzin, disrupting cardiolipin (CL) synthesis and ultimately affecting mitochondrial function (Jefferies, 2013). This deficiency has been shown to cause CMP,

lactic acidosis, neutropenia, skeletal muscle weakness, and other symptoms listed above (Pang J; Bao Y; Mitchell-Silbaugh K; Veevers J; Fang X). Barth Syndrome was initially recognized as a mitochondrial disorder in 1983 in a large Dutch pedigree with high infant mortality due to neutropenia, skeletal myopathy, and CMP (Barth et al., 1983). An earlier report in 1979 documented infants with heart failure, CMP, abnormal mitochondria in numerous tissues, including the liver, heart, and skeletal muscle, often succumbing to septicemia or heart failure (Neustein et al., 1979).

Barth Syndrome usually presents with a range of clinical features, most notably cardiomyopathy (CMP), which frequently manifests as left ventricular noncompaction (LVNC) or dilated CMP. These cardiac abnormalities have often shown to lead to heart failure and increase the risk of ventricular arrhythmias. Neutropenia is another key feature, significantly raising the risk of severe bacterial infections, with sepsis serving as the highest contributor to morbidity and mortality in affected individuals. Skeletal myopathy is another common finding, causing muscle weakness and delays in physical development. Growth retardation further highlights the syndrome's widespread systemic impact and mitochondrial dysfunction in Barth Syndrome is also evidenced by lactic acidosis, which is observed in some patients. While the condition itself is never fully consistent in its presentation, with some individuals displaying only a subset of the aforementioned symptoms, this emphasizes the heterogeneous nature of the disorder (Jefferies, 2013).

As Barth Syndrome is uniquely linked to the disruption of CL synthesis, a key process mediated by the tafazzin protein, CL plays an essential role in mitochondrial function, including energy metabolism, protein import, and maintaining mitochondrial

structural integrity (Paradies et al., 2019). Loss-of-function mutations in tafazzin result in defective CL remodeling, which contributes to the multisystemic manifestations of Barth Syndrome (Koshkin & Greenberg, 2002). Advances in being able to understand the genetic and molecular basis of Barth Syndrome have facilitated improved diagnostic and clinical management strategies, although understanding the true function of the *TAZ* gene on chromosome Xq28 is essential to finding treatment for proper CL remodeling.

The highly conserved *TAZ* gene is located in the q arm region of Xq28 and is responsible for Barth Syndrome (Bione et al., 1996). It spans approximately 11 kb and consists of 11 exons, with the first two being noncoding. Two distinct translation start sites drive alternative splicing, resulting in multiple transcript variants and highlighting the gene's functional complexity. To date, more than 160 mutations have been identified across all exons of the *TAZ* gene (Jefferies, 2013). These mutations are predominantly small insertions, deletions, and missense variants, although large deletions and truncating mutations have also been reported (Gonzalez, 2005).

The identified conserved *TAZ* gene is a phospholipid-lysophospholipid transacylase that is responsible for the remodeling of acyl chains of CL found in the inner mitochondria (Jefferies, 2013). The unique structure of CL is dimeric and consists of two phosphatidyl-moieties which are linked by a glycerol backbone (Fig.1) (Ghosh et al., 2019). In Barth Syndrome, remodeling of CL plays a crucial role in its maturation, involving the replacement of saturated acyl chains with unsaturated ones to produce the fully matured form of CL (Xu et al., 2006). In the heart and skeletal muscle where tissues are highly oxidative, CL is found normally to have four linoleoyl species typically found as tetralinoleoyl-CL and also known as L4-CL. Ultimately, mutations in the *TAZ* gene

lead to a reduced formation of L4-CL and an accumulation of the various intermediate forms of CL, known as monolysocardiolipins (MLCL), which contain three linoleoyl acyl groups. Tafazzin protein is responsible for remodeling immature CL into its mature form, primarily L4-CL. In Barth Syndrome, where tafazzin function is impaired, less mature CL is synthesized, resulting in an increased ratio of MLCL to L4-CL (Jefferies, 2013).

Barth Syndrome is known for its complicated pathology, and although there currently is no known cure, there has been extensive research that supports various types of treatments that have shown to produce longevity in diagnosed individuals. Managing Barth Syndrome is unique and requires an overall well-rounded approach because of the variety of medical issues that come with the disorder. A systematic review developed by Reynolds highlights various ways to successfully manage the symptoms of Barth Syndrome (Reynolds, 2015). Much of the available research that supports this review comes from case reports and expert opinions rather than clinical trials. While these reports provide useful information, they aren't always easy to apply to every patient. Still, there is a general consensus that treatments like cardiac medications, granulocyte colony-stimulating factor (G-CSF) therapy, and physical therapy can help ease symptoms and improve outcomes for those with Barth Syndrome (Reynolds, 2015). As responses to treatment can vary and major side effects can occur, the importance of tailoring the management of each patient to their individual needs is essential.

Individuals with Barth Syndrome can benefit from the utilization of cardiac medications, including β -blockers. Angiotensin-converting enzyme inhibitors have also been reported to improve symptoms of heart failure and extend life expectancy. G-CSF has shown promise in addressing neutropenia, with several studies noting increases in

circulating neutrophils following treatment [(Aprikyan & Khuchua, 2013), (Bachou et al., 2009), (Huhta et al., 2005)]. However, some patients still experience severe infections while on G-CSF, exemplifying the need for close monitoring (Rigaud et al., 2013). Surgical interventions such as cardiac transplants have shown some success, but the literature lacks data on the long-term survival rates of these patients. On the other hand, nonmedical treatments, especially those focused on feeding and nutrition, haven't been studied much, and there is little research on successful supplements and/or feeding methods (Reynolds, 2015). Although there are numerous other approaches to therapy for Barth Syndrome, gene editing therapies have only suggested candidacy rather than promise.

Arrhythmogenic Right Ventricular Dysplasia / Cardiomyopathy (ARVD/C)

ARVD is also known as Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) and is a complex genetic condition that is characterized by abnormalities in the structure and function of the myocardium, mainly affecting the right ventricle (Riasat et al., 2022). ARVD is defined by specific electrical, functional, and structural changes, notably including fatty infiltration of the myocardium (Groeneweg et al., 2015). One of the main hallmark features is regional right ventricular dilation that can progress to other bodily involvement, although in various cases, the left ventricle's lateral and posterior walls have also been shown to be affected. The typical presentation of this disease is usually presented to patients around the age of 30, and most commonly, it follows an autosomal dominant inheritance pattern, but a recessive form associated with cutaneous manifestations also exists. Most of the gene mutations involve desmosomes, which are

crucial to the mechanical integrity of tissues, especially cardiac tissues (Riasat et al., 2022). To confirm that the desmosomal gene mutations are frequently involved, Basso, C. conducted a study on current gene mutations encoding intercalated disc proteins in ARVD/C patients. Utilizing electron microscopy, it was found that the gene mutations did involve desmosomes in the right ventricles of 21 ARVD/C probands (Basso, 2006).

Diagnosing ARVD/C has proven to be challenging because of its non-specific presentation, but individuals should be considered if they show unexplained right precordial ECG abnormalities, such as T-wave inversion in V1-V3, frequent premature ventricular contractions, ventricular tachycardia (VT) with left bundle branch block (LBBB) morphology, or sudden cardiac death (SCD), especially when one is performing exercise activities (Dalal et al., 2005; Niroomand, 2002). Many of the diagnostic tools for ARVD/C include ECG, transthoracic echocardiogram (TTE), and cardiovascular magnetic resonance imaging (CMR). A notable feature that is important in diagnosing the disease is in identifying the disease in the epsilon wave, where a small positive deflection occurs at the end of the QRS complex (Dalal et al., 2005).

Although there are still numerous identifiers that could indicate the presence of ARVD/C, for those that have been diagnosed, the management of the disease maintains focus on preventing SCD, incorporating activity restrictions, implantable cardioverter defibrillators (ICDs), and beta-blockers. In some advanced cases, a cardiac transplant may eventually be required, but the main identifier remains to be family screening as first-degree relatives are at higher risk (Marcus et al., 2010).

As ARVD is a disease classified by the replacement of heart muscle with fibrous or fatty tissue, it is important to understand what is causing the buildup of unwanted fatty tissues. Mutations in genes such as *Desmoplakin (DSP)*, *Plankophilin-2 (PKP2)*, and others that have been linked to ARVD can disrupt the sorting of proteins that are integral in maintaining the structural integrity of cardiac cells (Riasat M;Khan A;Meghrajani V;Gaikwad M;Gill R;). Other genes including *Desmoglein-2 (DSG2)*, *Desmocollin-2 (DSC2)*, *Plakoglobin (JUP)*, and *Transmembrane protein 43* along with *DSP* and *PKP2* account for ARVD/C in up to 50% of the patients screened. (*Genetics of Arrhythmogenic Right Ventricular Dysplasia / Cardiomyopathy (ARVDC)*, n.d.). Although there are multiple variables that seem to contribute to a diagnosis of ARVD/C, it's notable that the most common associations are coming specifically from *DSP* and *PKP2*.

The *DSP* gene encodes desmoplakin which is a crucial protein in desmosomes that links transmembrane cadherins and associated proteins to the cytoplasmic intermediate filament cytoskeleton (Green et al., 2019). *DSP* and *DSC2* play a significant role in the pathogenesis of ARVC and are mainly associated with structural and functional abnormalities in cardiac tissue, but *DSP* mutations often lead to left ventricular involvement, extending the classical understanding of ARVC as a primarily right ventricular disease. Like *DSP*, mutations in *DSC2* have been linked to the development of ARVC (McNally et al., 1993). Additionally, *DSP* mutations are associated with an elevated risk of arrhythmias, further distinguishing their clinical impact from the classical right ventricular involvement caused by *PKP2* mutations (Smith et al., 2020). Genetic testing for desmosomal mutations is an essential component of ARVD/C diagnosis.

In ARVD/C the desmosomal gene *PKP2* is the most implicated gene, making it the dominant associated genetic contributor. Other desmosomal genes, mentioned above, are far less frequently represented (Groeneweg et al., 2015). Research into the clinical manifestations of mutations in *DSP* has revealed significant differences in its disease profile compared to *PKP2* (Selgrade et al., 2024).

These *PKP2* mutations are strongly associated with the classical ARVC phenotype, which primarily involves the right ventricle and is characterized by distinct arrhythmias (Gandjbakhch et al. 2018; te et al., 2013). The *PKP2* gene encodes plakophilin-2, a crucial desmosomal protein that plays a key role in stabilizing cell-cell adhesion within the myocardium by linking cadherins to the cytoskeleton. The majority of *PKP2* mutations are truncating variants, which account for over 90% of pathogenic mutations, according to databases such as ClinVar (Biernacka et al., 2021). Patients that have been shown to contain *PKP2* mutations tend to experience earlier disease onset compared to those without such mutations. As mentioned previously, T-wave inversions in the V1–V3 leads are more commonly observed in *PKP2*-positive individuals (Xu et al. 2017). Also, patients with *PKP2* mutations tend to have better left ventricular function, slower disease progression, and a lower incidence of heart failure symptoms compared to those without *PKP2* mutations. When compared to mutations noted in *DSP*, these are more often associated with left ventricular or biventricular involvement and carry a higher risk of end-stage heart failure (Biernacka et al., 2021). Highlighting the unique roles of *PKP2* as not only the most prevalent genetic contributor to ARVC/D but also a driver of distinct clinical characteristics compared to *DSP* is essential to note, especially when it comes to treatments.

One of the more recent treatments that has shown potential in treating individuals with a *PKP2* mutation associated with ARVC is gene therapy. A study that came out in 2024 investigated the potential of adeno-associated virus 9 (AAV9)-mediated gene therapy to restore *PKP2* expression in a cardiac-specific knockout mouse model of *PKP2*. The researchers demonstrate that a single dose of AAV9:*PKP2* not only prevents the development of cardiomyopathy before its onset but also slows disease progression after cardiomyopathy has been established. Restoring the *PKP2* expression enhanced the structural integrity of desmosomes and gap junctions, improved heart function by maintaining left ventricular ejection fraction, prevented right ventricular dilation, reduced arrhythmia frequency and severity, and prevented fibrotic remodeling. RNA sequencing revealed that restoring *PKP2* expression corrects broader transcriptional disturbances beyond desmosome dysfunction, providing insights into the underlying causes of ARVC. The study suggests that cardiac-selective AAV9:*PKP2* gene therapy could be an effective therapeutic strategy for treating ARVC patients with *PKP2* mutations (Wu et al., 2024).

Pompe Disease

Pompe Disease is a disorder known to be caused by a mutation in the *GAA* gene which leads to the enzyme acid alpha-glucosidase deficiency. The acid alpha-glucosidase enzymatic deficiency leads to the accumulation of glycogen specifically within lysosomes, organs, and tissues in the body. Due to the lack of glycogen breakdown, glycogen accumulation occurs, which ultimately leads to improper cargo sorting ultimately leads to improper cargo sorting in cells with high demand, such as muscle cells (Fayssoil, 2008).

Pompe disease was originally discovered by a Dutch pathologist named Johannes Cassianus Pompe, after whom the disease is named. Dr. Pompe described an autopsy of a 7-month-old girl who had been diagnosed with "idiopathic myocardial hypertrophy" (which is a condition where the heart muscle becomes overly enlarged without an obvious cause) and widespread muscle weakness. He observed that the cause of the illness was due to the buildup of large amounts of glycogen in almost all the body's tissues (Kohler et al., 2018).

Pompe disease is now also known as Type II glycogen storage disease (GSDII) or acid maltase deficiency. It is a severe and rare metabolic myopathy caused by mutations in the *GAA* gene that encodes acid alpha-glucosidase, an enzyme crucial for breaking down glycogen within the lysosome. In normal conditions, acid alpha-glucosidase degrades glycogen into glucose in the acidic environment of the lysosome. This degradation allows the glucose to be released and utilized as an energy source. Although, when acid alpha-glucosidase is deficient or absent due to genetic mutations, glycogen accumulates in the lysosome instead of being properly broken down. This accumulation leads to cellular dysfunction and damage, particularly in cardiac and skeletal muscles. These two muscle groups are the most severely affected tissues in Pompe disease. As the glycogen builds up, it impairs the normal function of these tissues, leading to the characteristic symptoms of the disease, such as progressive muscle weakness, respiratory difficulties, and heart problems. Pompe disease is often grouped into two main diagnoses: the infantile-onset form, which manifests in the first few months of life, progressing rapidly to early mortality, and the late-onset form, which progresses at a slower pace and may not appear until later in childhood or adulthood. The severity of symptoms and the

age of onset can vary widely depending on the degree of acid alpha-glucosidase enzyme deficiency (Kohler et al., 2018).

Pompe disease has unique genetics that reveal a complex set of mutations and variations in the gene *GAA*, not only influencing enzyme function, disease severity, but also diagnostic challenges across different populations and geographical areas (Chien et al., 2013). *GAA* is located on chromosome 17q25 and spans approximately 28 kilobases (kb) and includes 20 exons, with the first exon being untranslated and separated by the second exon by a 2.7-kb intron. *GAA* produces a 952-amino acid peptide with a molecular weight of 110 kDa, which undergoes post-translational modifications such as glycosylation and proteolytic processing to form the mature 76-kDa and 70-kDa acid alpha-glucosidase proteins (Chien et al., 2013). Over 250 mutations have been identified in *GAA* and are seen to be distributed throughout its sequence (Kroos et al., 2012). Common mutations are found within various ethnic groups, and this includes the c.-32-13T>G splicing mutation commonly found in Caucasian patients, the p.R854X mutation observed in African American patients, and the p.D645E mutation frequently identified in Chinese patients who originate from Taiwan. These differences in mutations are not only correlated to various phenotypes but also to disease severity (Chien et al., 2013).

Comprehending the pathophysiology of Pompe disease is essential to understanding the complex processes that drive the main contribution of muscle damage. The muscle damage and weakness seen in Pompe disease have conventionally been attributed to the continuous enlargement of glycogen-filled lysosomes that eventually rupture and release glycogen into the cytoplasm and cause a shifting in myofibrils. Although this explanation doesn't entirely explain the full contributions of Pompe

disease, it fails to account for the secondary events triggered by the accumulation of unmetabolized substrates inside lysosomes (Kohler et al., 2018). Recent research has revealed that other pathogenic mechanisms, such as impaired autophagy, disruptions in calcium homeostasis, oxidative stress, and mitochondrial abnormalities, play a main role in tissue damage as well as other lysosomal storage disorders (LSDs) (Griffin, 1984; Thurberg et al., 2006). These additional mechanisms provide a deeper insight into the Pompe disease's complexity and its overall impact on muscle function.

Macroautophagy, also known as autophagy, is the cellular process of recycling damaged components occurring in lysosomes and is largely disrupted in Pompe disease. There are three known autophagic pathways: microautophagy, chaperone-mediated autophagy, and macroautophagy which is the most predominant and relevant to Pompe disease (Kohler et al., 2018). Macroautophagy mostly involves sequestration of different cytosolic constituents into newly developed double-membrane vesicles, also known as autophagosomes. These fuse and eliminate their content into lysosomes, typically for breakdown and recycling (Yang & Klionsky, 2010). In Pompe disease the severely dysregulated macroautophagy leads to the accumulation of undigested debris, and autophagosomes contribute heavily to the muscle weakness seen in patients (Kohler et al., 2018).

Autophagic dysfunction in Pompe disease has shown a close relation to mitochondrial abnormalities, as damaged mitochondria are typically cleared by another form of autophagy called mitophagy. The mitochondrial defects in Pompe disease appear in forms such as calcium overload, reduced membrane potential, and increased oxidative stress which have been reported in both animal models and patient muscle cells (Lim et

al., 2015). These defects are intensified by the accumulation of the waste product lipofuscin, which reduces lysosomal capacity and sustains oxidative stress. Additionally, the disease disrupts the mTOR signaling pathway which is a key regulator of muscle mass and repair, further contributing to muscle wasting. The combined effects of lysosomal dysfunction, mitochondrial impairment, and autophagic failure create a dangerous cycle that highlights the progressive muscle function and damage decline in Pompe disease (Kohler et al., 2018). Understanding these interconnected pathways shows the importance of targeting multiple mechanisms for effective treatment strategies.

Pompe disease treatments have been shown to help reduce and slow symptoms, but there still isn't a cure. Enzyme replacement therapy (ERT) has been shown to produce some treatment for Pompe disease, but other therapies, such as gene therapy, offer alternatives that address the limitations of others like ERT. Gene therapy involves delivering a functional copy of *GAA* utilizing viral vectors, with adeno-associated virus vectors emerging as the preferred choice due to their low immunogenicity and ability to target specific tissues (Athanasopoulos et al., 2017). Preclinical studies have demonstrated that systemic delivery of adeno-associated virus vectors, such as AAV8 targeting the liver, can enhance the production and secretion of acid alpha-glucosidase, allowing for glycogen reduction and improved muscle function in knockout (KO) mice (Sun et al., 2010; Puzzo et al., 2017). Additionally, liver-targeted approaches have shown the potential to induce immune tolerance, reducing immune responses to the recombinant enzyme and enabling more effective cross-correction in distant organs (Bond et al., 2019; Kohler et al., 2018). A clinical trial utilizing liver-directed adeno-associated virus therapy is underway to evaluate its safety and efficacy. While challenges such as immune

reactions to the adeno-associated virus capsid and transgene remain, strategies like immune modulation with rituximab have shown promise in controlling these responses during clinical trials (Corti et al., 2014). Essentially, the abilities of gene therapy to deliver sustained *GAA* expression and its potential for a one-time treatment, suggest it could be a more beneficial treatment compared to ERT, especially if it is conducted early in the diagnosis.

Danon Disease

Danon Disease is a rare X-linked dominant genetic disorder caused by mutations in the lysosomal-associated membrane protein 2 in the *LAMP2* gene. Overall *LAMP2* is responsible for the production of the LAMP2 protein, which is important for the correct functioning of lysosomes that are responsible for cargo sorting and waste removal. The reduction of this protein leads to an accumulation of autophagic material, lysosomal dysfunction, and glycogen in cardiac and skeletal muscle (Rowland et al., 2016) The condition is primarily characterized by cardiomyopathy, myopathy, and intellectual disability (D'souza et al., 2014). Cardiomyopathy is known to be present in all affected individuals, with 84% exhibiting cardiac hypertrophy, and intellectual disability is observed in 70% of males and 6% of females (Sugie et al., 2002). Symptoms typically emerge before the age of 20 in males, while females tend to develop symptoms later in adulthood (Arad et al., 2005). Key clinical features include hypertrophic cardiomyopathy, elevated creatine kinase levels, skeletal muscle weakness, and intellectual disability. Symptoms are often subtle and can be misdiagnosed as isolated hypertrophic cardiomyopathy. Heart failure is also known as a major factor influencing disease prognosis (Konrad et al., 2017). Muscle pathology in Danon disease reveals the presence

of multiple membranous autophagy vacuoles, reflecting underlying lysosomal dysfunction (Zhou et al., 2019).

As Danon disease is X-linked, dominant, and a heritable condition, it is important to understand genetically how and why patients who are diagnosed exhibit symptoms. Zhou et al. conducted a study aiming to identify the *LAMP2* mutation in a family with Danon disease by using whole exome sequencing (Zhou et al., 2019). The study was conducted on a proband of an 18-year-old male identified in pedigree as (III-1) in Figure 1a. At the age of three, (III-1) was diagnosed with cardiomyopathy and delays in some physical developments were observed early in life. The patient's intelligence was noted as average, and they were able to complete normal daily activities and elementary calculations. A year before this study was conducted, the patient began experiencing a variety of symptoms such as dizziness, fatigue, shortness of breath and mainly chest tightening after repeated activities. A blood test revealed exceedingly high levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), CK-MB, CK-MM, cardiac troponin, and N-terminal pro-b-type natriuretic peptide (Zhou et al., 2019).

Cardiac magnetic resonance imaging (MRI) of the patient was also collected (Figure 2b, 2c) and demonstrated hypertrophy of the left and right ventricular walls, reduced overall contractile activity, left atrioventricular enlargement, and a decreased myocardial T1 value (Zhou et al., 2019). MRI plays an essential role in identifying cardiomyopathy by indirectly assessing myocardial tissue composition and pathological changes through the measurement of the longitudinal relaxation time (T1 time) of the myocardium. Various conditions, including myocardial infarction, hypertrophic

cardiomyopathy, and dilated cardiomyopathy, are known to be associated with an increased initial myocardial T1 value (Stokes et al., 2016). Although, diseases with reduced initial T1 values, such as metabolic cardiomyopathies are relatively rare, distinguishing other conditions from classical hypertrophic cardiomyopathy. In this patient, the reduced T1 value strongly suggested the presence of metabolic cardiomyopathy and the patient's electromyographic report revealed electrophysiological evidence of active myogenic damage involving the extremity muscles (Zhou et al., 2019).

Sanger sequencing, an electrocardiogram, and an echocardiogram were taken for the patient's mother (II-2), patient's father (II-1), the patient (III-1), and the patient's brother (III-2) shown in Figure 3. The patient's results revealed frequent atrial premature beats, ventricular pre-excitation, complete left bundle branch block, and double ventricular hypertrophy. Echocardiography of patient showed an increase in the left ventricle, thickening of the left and right ventricular walls, and abnormal myocardial echo, with reduced overall systolic activity (left ventricular ejection fraction [LVEF], 30%; tricuspid annular plane systolic excursion [TAPSE], 15 mm). Sanger sequencing confirmed that the proband of patient (III-1), his mother (II-2), and his younger brother (III-2) all carried the *LAMP2* c.741+1G>T mutation, while the patient's father (II-1) did not carry the mutation. Based on the findings from the evaluation of the index patient and his family, it was suspected the disease was likely caused by a hereditary genetic mutation (Zhou et al., 2019).

To confirm the hypothesis of a hereditary genetic mutation, whole exome sequencing genetic testing on the family was conducted and identification of a novel pathogenic mutation in *LAMP2*: c.741+1G>T was confirmed in III-1, II-2, and III-2.

LAMP2 is made of nine exons encoding the LAMP2 protein. This protein consists of a total of 410 amino acids and includes three domains: lysosomal, transmembrane, and cytoplasmic. Exons 1–8 encode the intra-lysosomal domain, while exon 9 encodes the transmembrane and cytoplasmic regions (Zhou et al., 2019). A muscle biopsy from III-1 was also taken, followed by RNA extraction, reverse transcription to generate cDNA, and subsequent PCR and sequencing analysis. The results of the muscle biopsy revealed that the c.741+1G>T mutation led to the insertion of six nucleotides in the mRNA, with the last three forming a stop codon. This mutation does not cause exon loss in the mRNA, but it actually introduces six additional nucleotides, the second of which creates a stop codon. This alteration leads to the complete loss of the protein encoded after exon 5, which includes the essential transmembrane domain. This change is assumed to affect the fusion of lysosomes and autophagosomes, impairing cellular autophagy function and leading to clinical manifestations. All these tests confirmed the diagnosis of Danon disease (Zhou et al., 2019).

To date there is no current cure for Danon disease and although intermediate treatments can help with cardiac strength, the most common and effective treatment is a heart transplant (Sugie et al., 2016). It's recommended that cardiac function in male patients be evaluated every three to six months, with consideration for cardiac transplantation because of the rapid progression of the disease. Genetic counseling should also be utilized to help patients with Danon disease and their families to help reduce the risk of transmission to future generations (Bottillo et al., 2016). As the understanding of Danon disease's pathogenesis improves and advancements in gene therapy and cell

transplantation techniques continue, there is hope for the development of effective treatments for this in the future (Zhou et al., 2019).

Familial Hypertrophic Cardiomyopathy (FHCM)

FHCM is an autosomal dominant disease and a genetically inherited subtype of Hypertrophic Cardiomyopathy (HCM), which is a condition that leads to the thickening of heart muscles, specifically the left ventricle. Mutations in genes, including but not limited to *MYH7* and *MYBPC*, are responsible for low-density lipoproteins (LDL) directly influencing and disrupting the correct sorting of sarcomeric proteins in the heart muscles. This disruption leads to irregular muscle contraction, which is attributed to the thickening of the heart walls (Taylor et al., 2004). The efforts to establish a clear genotype-phenotype correlation for the identified mutations have been largely unsuccessful. Other genetic factors, along with non-genetic influences such as lifestyle, gender, and age, have also been found to impact the clinical presentation of FHCM. The mechanisms by which each mutation leads to hypertrophy and/or myofibrillar disarray remains unclear (Bashyam et al., 2003).

Of recent, there have been at least 270 distinct mutations in nine sarcomeric protein genes that have been associated with FHCM, signifying the importance of genetic diversity that parallels the clinical variability of the condition. Although the ultimate clinical phenotype in FHCM patients is known to be influenced by multiple factors, including modifier genes, environmental factors, and genotype, early screening studies suggested that specific gene mutations might be linked to distinct diagnoses. As the sarcomeric genes associated with FHCM code for proteins with previously known identified functions, a wide range of biochemical, biophysical, and physiological

techniques have been used to explore the molecular mechanisms underlying the pathogenesis of this complex cardiovascular disease (Tardiff, 2005).

In 2014 Guo et al. conducted a study on a FHCM family of twelve individuals, five of whom were affected and seven unaffected, to study their genetic and clinical backgrounds seen in the pedigree of Figure 4. Echocardiography, electrocardiography, and physical examinations were conducted for all family members. The diagnosis of FHCM was primarily based on echocardiographic findings, specifically an interventricular septal diameter exceeding 1.2 cm, along with electrocardiographic results and family history (Guo et al., 2014). Symptoms first appeared in most patients during their twenties, although the proband (III-22) experienced an earlier onset at 14 years of age and presented with a severe phenotype in their echocardiogram. Common symptoms exhibited among the family included fatigue, palpitations, angina, dyspnea, and limb swelling, with the severity varying between individuals, but no knowledge of sudden death in this family has been reported in this instance of FHCM.

To further understand the studied family, exome sequencing, analysis, and mutation detection using Sanger sequencing was utilized to confirm the candidates' variants. Sanger sequencing was used to confirm the candidates' variants, focusing on exon 3 of JPH2, and exon 13 of MYH7 (Guo et al., 2014).

In total, whole-exome sequencing of the patient produced 78,282,594 reads, with 56,599,099 reads (72.3%) mapped to the human genome, resulting in a mappable yield of 5,615,031,704 bp. The coverage analysis revealed that 96.1% of the target was covered at 1x and 84.9% at 10x. A total of 10,133 exonic variations were identified and were filtered to 61 variants: 59 nonsynonymous single nucleotide variants (SNVs) and two stop gain

SNVs across 46 genes. This was based on a minimum depth of 20x and a frequency below 5%, although none of the above listed variants were associated with FHCM. In order to refine the analysis, a list of genes that are frequently implicated in FHCM was generated from OMIM, which included *MYH7*, *ACTC*, *CSRP3*, *TNNC1*, *MYH6*, *VCL*, *MYOZ2*, *JPH2*, *PLN*, *CALR2*, and *NEXN* (Home - OMIM - NCBI, 2019). Comparison of these genes with the patient's exome sequencing revealed the following two mutations *MYH7* p.G407C and *JPH2* p.A396T (Figure 5.). Subsequent testing of 100 normal DNA samples confirmed that *JPH2* p.A396T was a polymorphism, while *MYH7* p.G407C was found to be pathogenic. Exon 13 of *MYH7* was then amplified and sequenced in the family, confirming the proband (III-22) carried a pathogenic *MYH7* p.G407C mutation. Family members 4, 7, 9, and 13 also were found to have the mutation, while members 6, 8, 17, 18, 21, 23, and 24 did not (Guo et al., 2014).

The importance of the *MYH7* p.G407C relates to a single base substitution (guanine to thymine) in the codon 407, changing the amino acid from glycine to cysteine. This mutation serves as a key pathogenic variant in this study, making it a crucial finding for understanding the genetic basis of FHCM in the proband's family, as there has only ever been one other report of a patient with this mutation who suffered sudden cardiac arrest (Smaniotto et al., 2009).

This mutation replaces a nonpolar, hydrophobic glycine residue with a polar, hydrophilic cysteine residue, significantly altering the protein's properties. Glycine, the most flexible amino acid, is essential for maintaining protein structure and function, especially in regions requiring specific backbone conformations. The p.G407C mutation occurs at a highly conserved position within an inverse gamma and a beta turn,

suggesting its importance in developing proper protein movement and keeping structural integrity. The substitution introduces an additional hydrogen bond between the sulfur atom of mutant Cys407 and the nitrogen atom of Asn408, which likely disrupts the formation of the protein's secondary structure. The resulting structural and functional impairment is strongly associated with myocardial hypertrophy observed in the affected family members, emphasizing the mutation's prominent significance in the pathogenesis of FHCM in this study (Guo et al., 2014).

Another finding from this study confirmed a phenotype-genotype correlation which presented in all affected family members as a heterozygous mutation. The echocardiography of the affected patients showed an obstruction in the outflow tract which showed the symptoms including lower levels of physical activity, palpitations, and fatigue (Table 1.) (Guo et al., 2014). It has been noted that certain mutations of *MYH7* have normally been correlated to significantly shortened life spans with HCM (Watkins et al., 1992), but most of the affected family members were not diagnosed until adulthood. This exemplifies that the p.G407C mutation has shown to be aligned with a somewhat benign course of the disease in the heterozygous state, but the 14-year-old studied proband suggests that early onset of FHCM is also possible (Guo et al., 2014).

As an uncured disease, FHCM has become somewhat manageable with various medical and interventional therapies. Patients who have begun to show symptoms usually are placed on beta-blockers or non-dihydropyridine calcium channel blockers to improve diastolic filling of the heart and symptom control. Cardiac myosin inhibitors are also utilized to reduce sarcomeric force generation and can improve LVOT obstruction, but these results are not guaranteed. Gene therapies, including gene replacement, base

editing, and allele silencing, have been conducted in animal models and have shown promise (Litt et al., 2023). FHCM is a disease that affects individuals worldwide and with the potential of genetic editing as a treatment patients can potentially avoid detrimental outcomes, especially sudden cardiac arrest.

Chapter III.

Gene Editing Technologies

Genetic editing techniques have transformed the ability to precisely modify DNA, each technique offering identifiable capabilities that are tailored to specific research and therapeutic needs. Multiplex Automated Genome Engineering (MAGE) is distinct in its ability to simultaneously make multiple precise genetic changes using oligonucleotides. This approach enables targeted mutations, insertions, or deletions without relying on nucleases, making it invaluable for high-throughput studies of gene function and trait engineering (Wang et al., 2009). In contrast, CRISPR/Cas9, renowned for its simplicity and efficiency, uses a guide RNA (gRNA) and the Cas9 enzyme to locate, cut, and edit specific genes. This system is highly versatile and forms the foundation for innovations like Cpf1 (Cas12a), which differs by recognizing a T-rich protospacer adjacent motif (PAM) sequence and cleaving DNA with distinct precision, broadening the range of genomic targets (Safari et al., 2019).

Base editing, unlike CRISPR/Cas9 and Cpf1, focuses on converting single DNA base pairs without creating double-stranded breaks, this enables base editing to be a safer and more consistent option for correcting point mutations linked to genetic diseases (Rees & Liu, 2018). In comparison, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) rely on FokI nuclease domains to induce double-stranded breaks but differ in their DNA recognition systems. ZFNs utilize zinc finger motifs for sequence specificity, requiring two separate ZFNs to bind adjacent to the target site to activate FokI (Carroll, 2011). TALENs, on the other hand, use TAL effector

proteins with repeat arrays corresponding to individual base pairs, offering high customizability but with potential off-target effects (Sun & Zhao, 2013). Together, these tools provide complementary approaches, each uniquely suited for advancing genomic research, engineering, and therapeutic applications.

Multiplex Automated Genome Engineering (MAGE)

Multiplex automated genome engineering (MAGE) is a high-powered tool utilized for large-scale genetic programming and cellular evolution. It allows simultaneous modifications at multiple chromosomal locations within a single cell or across a population, generating extensive combinatorial genomic diversity. MAGE uses synthetic oligonucleotides, which target specifically selected DNA sequences to introduce precise mutations, deletions, or insertions, facilitating the rapid exploration of gene functions and the creation of engineered traits for research. The process of MAGE is cyclical and scalable, with prototype devices automating this technique to accelerate the generation of genetic variants (Wang et al., 2009).

To demonstrate the success of MAGE, Wang et al. conducted a study that describes the simultaneous modification of 24 different genetic components. Wang et al. validate the utilization of MAGE by enhancing the 1-deoxy-d-xylulose-5-phosphate (DXP) biosynthesis pathway found in *Escherichia coli* (*E. Coli*). MAGE's modification of the 24 genetic components produced over 4.3 billion genomic variants each day and achieved more than a fivefold increase in lycopene production within three days. MAGE offers an efficient and automated method for modifying multiple genomic sites, including

genes and regulatory regions, at various scales ranging from individual nucleotides to the entire genome all at the same time (Figure. 6)(Wang et al., 2009).

The MAGE process was tested using a modified strain of *E. coli* (EcNR2) and allelic replacement was facilitated by the bacteriophage λ -Red single-stranded DNA (ssDNA)-binding protein β . This protein directs ssDNA or oligonucleotides (oligos) to the lagging strand of the replication fork during DNA replication allowing for precise genetic modifications. It was observed that optimization of key parameters seen in Figure 7, established maximum efficiency of oligo-mediated allelic replacement, enabling genetic modifications to be introduced in over 30% of the cell population every 2–2.5 hours under optimal conditions (Figure 7d.) (Wang et al., 2009).

To generate genetic diversity, a pool of targeting oligonucleotides is repeatedly introduced into cells to create sequence diversity in any chromosomal region via allelic replacement. The efficiency of introducing mismatches, insertions, or deletions depends on specific factors. For mismatch and insertions, a higher efficiency correlates with greater homology between the oligo and the chromosomal target (Fig. 7a., b.), and for deletions, the efficiency is dependent on the size of the deletion being introduced (Fig. 7c). The ΔG value, which predicts two-state hybridization free energy between the oligo and the target sequence, is a strong indicator of allelic replacement success (Fig. 7d.) (Wang et al., 2009). In a pool of oligonucleotides with differing degrees of homology, those seen with higher sequence similarity to the target site are incorporated more often into the chromosome. This feature of MAGE allows the controlled generation of genetic diversity, ensuring researchers direct evolution along favorable pathways by tuning the oligonucleotide design (Wang et al., 2009).

To determine the efficiency of this technique in generating sequence diversity, Wang et al. produced an experimental set up where MAGE was tested by targeting the *lacZ* gene using three different 90-mer oligos. These included cN6 Oligo, which contained six consecutive degenerate bases; cN30 Oligo, which was made up of 30 consecutive degenerate bases; and iN6 Oligo, which had six degenerate bases spread across a 30-bp region (Figure 8). These oligos were introduced into three separate cell populations and after 2, 5, 10, and 15 MAGE cycles, the targeted *lacZ* region was sequenced in 96 random clonal isolates to assess genotypic variation (Wang et al., 2009).

The results from the successive MAGE cycles showed increased divergence from the wild-type *lacZ* sequence as exemplified by the results from cN6 and cN30. The cN6 results showed that by five cycles, an average of 3.1 base pair (bp) changes per cell was observed (Fig. 8 blue), equating to over 4.3 billion bp variations per day across the population (based on 3.1 bp changes/five cycles in 7×10^8 cells with 10 cycles/day). After 15 cycles, all possible N6 genotype combinations were generated using either cN6 or iN6 oligonucleotides. The cN30 results displayed that due to lower replacement efficiency for a 30-bp mismatching oligo (1.5% from Figure 7a.), only 21.8% of the cN30 cell population underwent allelic replacement after 15 cycles. However, this population did show an average mean of 5.6 bp changes per cell from the wild-type sequence (Figure 8 orange). Overall, the MAGE technique effectively creates diverse chromosomal sequences in a targeted region, with efficiency impacted by oligo design. The shorter or interspersed degenerate bases (cN6 and iN6) generate broader variation faster, while the longer mismatching oligos (cN30) have shown lower replacement rates but still contribute to population diversity (Wang et al., 2009).

The above results highlight MAGE's scalability and precision in generating targeted genetic variation. MAGE is also a fast-paced evolution tool that allows for the repeated introduction and persistence of many neutral (or harmful) mutations in a cell population. Normally, these mutations would disappear due to genetic drift or natural selection, but this targeted and high-throughput approach speeds up their buildup in individual cells, making it more likely to discover sets of mutations that work together to create unexpectedly beneficial traits (Wang et al., 2009).

MAGE has shown immense promise, but its application is mostly limited to *E. Coli* due to the challenges of using short oligonucleotides for homologous recombination in other species. One example of this is Yeast Oligo-Mediated Genome Engineering (YOGE), which adapted MAGE for *Saccharomyces cerevisiae*, but it struggled with low efficiency outcome (DiCarlo, Conley, et al., 2013). Despite improvements in strain engineering and oligos design, double modifications remained below 0.01% after three cycles, limiting its effectiveness (DiCarlo et al., 2013). Even with additional strategies like phenotypic screening or site-specific nucleases, YOGE still faces significant hurdles in multiplexed genome engineering (Bao et al., 2015).

Even though MAGE does show limitations that still need time to be perfected, it does offer a powerful method for the introduction of multiple genetic modifications simultaneously, all the while enabling rapid genetic diversity within cell populations. Its unique ability to target specific genomic locations and generate a wide variety of genetic changes has proven valuable in research and biotechnology applications. Even though various challenges remain, such as its limited success in certain microorganisms and the need for further optimization in non-model organisms, MAGE ability to push

evolutionary processes and facilitate precise genetic alterations makes it a promising tool for engineering organisms with desirable traits (Singh & Braddick, 2015). As technology continues to improve MAGE holds influential potential for advancement in synthetic biology, metabolic engineering, and other biotechnology.

CRISPR-Cas9

Clustered regularly interspaced palindromic repeats (CRISPR) is a sequence in the bacterial genome that acts like a genetic memory of past infections. Cas9 is an enzyme that acts like molecular scissors. Together they make up CRISPR/Cas9, and altogether, this system can locate, cut, and edit the selected gene of choice (Wang et al., 2016). CRISPR/Cas9 has shown to become one of the revolutionary gene-editing technologies that have transformed biomedical research by enabling the correction of genetic errors and the ability to activate or deactivate genes in cells and organisms efficiently, affordably, and with minimal complexity. Its versatile applications include the swift creation of cellular and animal models, functional genomic screening, and real-time imaging of the genome within cells (Hsu et al., 2014).

An overview of CRISPR/Cas9 is demonstrated in Figure 9. CRISPR are sequences found in bacterial genomes that provide protection against invading viruses when paired with CRISPR-associated (Cas) proteins. One of these associated proteins, known as Cas9, functions as an endonuclease and is capable of cleaving both strands of DNA. Cas9 is guided to its target by a specific RNA segment, which can be synthesized as a single strand known as a synthetic single guide RNA (sgRNA) (Redman et al., 2016). The RNA section that binds to the genomic DNA is around or between 18–20 nucleotides in length. For Cas9 to cut the DNA, a specific DNA sequence, typically 2–5

nucleotides in length (depending on the bacterial source of the Cas9 protein), must be present at the 3' end of the guide RNA. This sequence is called the protospacer adjacent motif (PAM). After the DNA is cut, repair can proceed in two pathways, one is a non-homologous end joining, that often results in error prone random insertions or deletions of DNA, or two, homology-directed repair, that utilizes a homologous DNA template for repair. The homology-directed repair allows for more precise genome editing by delivering a homologous DNA segment with the desired sequence change alongside the Cas9 nuclease and sgRNA, enabling edits as specific as a single base-pair (Redman et al., 2016).

CRISPR/Cas9 system offers significant potential in translational medicine, but it has been noted that the off-target effects it can cause remain a major challenge. These effects occur when protein Cas9 unintentionally targets non-specific genomic sites, causing cleavages in DNA that can lead to undesirable changes in the DNA. The likelihood of off-target activity is largely influenced by the sgRNA, as Cas9 can tolerate up to three mismatches between the sgRNA and the genomic DNA, which increases the chances of unintended edits (Guo et al., 2023).

CRISPR/Cas9 has immense potential to help correct and eliminate many disorders and diseases including HIV, cystic fibrosis, cancer immunotherapy, manipulations of cancer genome and epigenome, eliminations or inactivation's of carcinogenic viral infections, genetic disorders caused by single gene mutations such as Duchenne muscular dystrophy (DMD), haemoglobinopathies and much more. In one study, Tabebordbar et al. utilized an adeno-associated virus to deliver CRISPR/Cas9 endonucleases to a mouse model which had DMD. By deleting the exon that held the mutation, they restored

dystrophin expression which resulted in a truncated but functional protein. Treated DMD mice displayed partial recovery of muscle function. Importantly, the study demonstrated that the dystrophin gene was edited in muscle stem cells, which regenerate mature muscle tissue, ensuring the therapeutic effects were long-lasting (Tabebordbar et al., 2016; Redman et al., 2016). Similarly, other studies have used CRISPR/Cas9 *in vivo* to enhance dystrophin expression and improve muscle function in mouse models of DMD. *In vitro* research has also shown success in targeting exon duplications in the human dystrophin gene, enabling the production of full-length dystrophin in myotubes derived from individuals with DMD (Redman et al., 2016).

Although the advantages of using adeno-associated virus with Cas9 include not only extremely low risks of carcinogenesis, low immunogenicity, and serotype associated target cell specificity, utilizing adeno-associated virus-mediated Cas9 system of delivery has been shown to carry out very well in a laboratory setting, but in a clinical setting it has more limitations. adeno-associated virus has a max capacity of 4.7kb, and with the Cas9 system made up of 4-7kb, the potential amount of DNA to be carried enclosed in the virus is very limited (Naso et al., 2017).

Another main target for CRISPR/Cas9 is the treatment of human immunodeficiency virus (HIV). While antiretroviral therapy effectively treats HIV, a cure remains to be developed because the virus integrates permanently into the host genome. Research conducted by Hu et al. demonstrated that the CRISPR/Cas9 system could target and disrupt HIV-1 genome activity (Hu et al., 2014). This model successfully inactivated HIV gene expression and replication in various cell types capable of latent infection and did so without causing toxic effects. Additionally, the study showed that cells could be

immunized against HIV-1 infection, offering a convincing step toward eliminating the virus. With further development and refinement, the researchers proposed that their findings could carve a path for gene therapies or the transplantation of genetically modified bone marrow stem cells or inducible pluripotent stem cells as a potential strategy to eradicate HIV infection (Redman et al., 2016).

CRISPR/Cas9 technology offers significant potential for advancing not only cancer therapies but also treating genetic diseases by enabling precise genome editing. Despite challenges such as off-target effects and ethical concerns, ongoing advancements in efficiency and delivery methods are extending the therapeutic application of this technique. Limitations may occur from considerations of ethical boundaries, but this technique offers limitless potential in curing what was thought to be incurable diseases.

Cpf1 (Cas12)

Cpf1 also known as Cas12a is considered to be a Class 2 Type V CRISPR protein. Similar type V CRISPR interference proteins are known to activate non-specific single stranded DNAase activity when it is bound to DNA in a gRNA-dependent manner. This makes Cas12a useful for fast and precise nucleic acid detection (Chen et al., 2018). There are a multitude of similarities between Cas9 and Cpf1, for instance Cas12a also relies upon a gRNA to be complementary to find the target DNA choice creating a double-stranded break in the DNA. Cpf1 then recognizes a T-rich PAM sequence, unlike Cas9, which recognizes a G-rich PAM sequence, and after that point, the DNA is cleaved, similar to Cas9 cleavage (Safari et al., 2019).

In analyzing the CRISPR technology, it is imperative to understand the differences between Cas9 and Cas12a (Cpf1) and how they are utilized. They are both highly effective genome-editing tools but are different in several crucial ways that make each system unique. Cas9 is derived from *Streptococcus pyogenes*, and is part of the Class 2, Type II CRISPR system (Jinek et al., 2012). It requires two RNA molecules for processing which include tracrRNA and crRNA. Cas9 recognizes a 5'-NGG-3' PAM sequence located on the non-template DNA strand and induces double-stranded breaks by cleaving both DNA strands at specific locations and creates blunt double-stranded breaks typically 3 base pairs upstream of the PAM (Anders et al., 2014). The mechanism of action involves crRNA hybridizing with the target DNA, while tracrRNA helps process the crRNA with host RNase III (Deltcheva et al., 2011). This identifiable property makes Cas9 ideal for gene knockout, genome editing, and gene regulation applications, where precise and predictable double-stranded break creation is critical (Paul & Montoya, 2020).

On the other hand, Cas12a is derived from *Francisella novicida* and is classified as a Class 2, Type V CRISPR system, as mentioned above. Cas12a requires only a single crRNA for RNA processing, simplifying the system by eliminating the need for tracrRNA or RNase III (Deltcheva et al., 2011). Cpf1 recognizes a more flexible 5'-TTTN-3' PAM sequence, which allows for wider targeting compared to Cas9's PAM recognition. As stated above, Cas9 generates blunt cuts, but Cas12a creates staggered double-stranded breaks with 5 nucleotide overhangs, which is shown to improve precision in DNA repair processes (Zetsche et al., 2015). Cas12a also can process its own crRNA, which reduces experimental complications. Another feature of Cas12a is its

capability to degrade ssDNA indiscriminately once activated, which could be advantageous for certain applications, although this may also pose risks to genomic stability if it is not carefully controlled (Chen et al., 2018). The auto-processing of crRNA makes Cas12a especially versatile for multiplex gene regulation, allowing for the simultaneous targeting of multiple genomic loci without requiring additional steps (Jasin & Rothstein, 2013). This makes Cpf1 a great option for complex gene editing tasks, including gene repression and precise genome modifications (Paul & Montoya, 2020). Comparisons of Cas 9 and Cas12a are distinctly described in Figure 10.

To sum up, Cpf1 and Cas9 can be used in similar experimental situations, and each contributes to the diversity of CRISPR-based technologies. Cas12a offers several advantages over Cas9, and this includes Cpf1's ability to perform multiplex genome editing, its production of staggered double-stranded breaks, which promote homology-directed repair, non-homologous end joining, and its use of a simpler crRNA system for RNA processing (Paul & Montoya, 2020). Cas12a also has a multi-checkpoint mechanism that reduces off-target effects, even though the degradation of ssDNA remains in question for both Cas9 and Cas12a. Despite all the recent advancements, CRISPR Cas technology still faces various challenges, particularly its reliance on host cell DNA repair machinery, which has been shown to influence the efficiency of gene editing in non-dividing cells, such as neurons. Although, developments like CRISPR-associated transposase (CAST) systems composed of Tn7 like transposase and CRISPR effectors (TypeV-K), which do not rely on the host repair machinery, seem to be able to offer different potential solutions for more precise and efficient gene editing (Peters et al., 2017). Both Cas9 and Cas12a remain foundational tools in the field of genome editing,

and ongoing research aims to enhance their capabilities for broader applications in life sciences (Paul & Montoya, 2020).

Base Editing

Base editing represents another groundbreaking technology that facilitates the precise, programmable conversion of single nucleotides. Base editing is known to rely on the use of a single guided (sgRNA), although it is different from the CRISPR/Cas9 technology as base editing is primarily utilized to convert one DNA base pair into another without causing a double-stranded break in the DNA, which helps avoid unwanted mutations. This precision makes base editing an invaluable tool for correcting point mutations associated with certain genetic diseases. By avoiding double-stranded breaks, it minimizes the risk of unintended genomic alterations, enhancing its safety and reliability in therapeutic applications (Rees & Liu, 2018).

Base editing's system is composed of Cas9D10A, which is a partially active Cas9 nuclease connected to either a natural or synthetic DNA modifying enzyme. Early development of base editing showed transformative potential in translational and basic research, allowing for the creation or repair of diseased alleles in various cell types and organisms (Schatoff et al., 2019). Schatoff et al. describe that in 2016, two different research groups individually reported the development of base editing enzymes that combined the genome-targeting abilities of Cas9 with the direct DNA modifying activity of cytidine deaminases, such as APOBEC or AID. In this approach, they demonstrated that Cas9 bound to the target locus and separated the DNA strands, which allowed the deaminase enzymes to mediate cytosine to thymine (C>T) transitions within a narrow 4-5 nucleotide editing window at the 5' end of the sgRNA target sequence. More recently,

synthetic DNA editing enzymes have been developed, which have expanded the scope of base editing to also include adenine-to-guanine (A>G) transitions (Schatoff et al., 2019).

Base editing allows for advantages over technologies like CRISPR knockout methods by facilitating specific single-nucleotide changes, allowing it to create both missense and nonsense mutations that can modify, truncate, or disable genes. This capability makes base editing well-suited for generating disease-relevant alleles because many human genetic disorders are caused by single nucleotide variants (Schatoff et al., 2019). For example, base editing could introduce mutations that inactivate oncogenes or disrupt tumor suppressor genes. It is noted that by using existing enzymes, more than half of all cancer-associated mutations could hypothetically be engineered using base editors (Zafra et al., 2018).

Before the development of base editing, the most effective method for generating targeted single nucleotide variants relied on homology-directed repair mediated editing, using the cell's endogenous DNA repair mechanisms to integrate an exogenous DNA template into the genome. On the other hand, homology-directed repair is inherently inefficient in many cell types due to its dependency on the cell cycle, being most active during the G2/M phases, and requiring efficient delivery of donor DNA in either single or double-stranded form. In contrast, base editing enables targeted mutagenesis without the need for a DNA template and is effective in both dividing and non-dividing cells (Yeh et al., 2018). As base editing's genome targeting is driven by sgRNA, the possibility of engineering multiple mutations at the same time can be accomplished by delivering multiple sgRNAs. This approach has been successfully applied in *ex vivo* cultured organoids and fertilized zygotes to generate complex genetic models (Zafra et al., 2018).

In comparison, introducing multiple modifications using homology-directed repair remains to be a challenge because the efficiency is often very low to be viable (Schatoff et al., 2019).

The ability to use base editing in different clinical and laboratory settings has shown to be efficient especially with mitochondrial DNA (mtDNA). Yi et al. conducted a study that hypothesized that by introducing ssDNA around mtDNA target loci could allow for accurate base editing. This would address the challenges of correcting pathogenic point mutations found in mitochondrial diseases. CRISPR-based base editing is ineffective for mtDNA due to the difficulties seen in gRNA delivery, requiring alternative methods. Yi et al. developed mitochondrial DNA base editors (mitoBEs) by integrating transcription activator-like effector (TALE)-linked nickases with deaminases to create targeted base substitutions.

The linked nickases that were used in this study are TALE–MutH and TALE–Nt.BspD6I(C). TALE–MutH is based on the bacterial MutH enzyme, which typically recognizes hemimethylated DNA and introduces nicks at specific sites. TALE–Nt.BspD6I(C) is derived from the nicking endonuclease Nt.BspD6I, which introduces targeted single-strand cuts without generating double-strand breaks (Yi et al., 2023). These enzymes were specifically designed to generate single-stranded DNA (ssDNA) regions within the mitochondrial genome, as traditional CRISPR-based approaches have shown inefficiency due to the lack of effective double-stranded break repair mechanisms in mitochondria. By leveraging these nickases to create ssDNA regions and pairing them with the adenine deaminase TadA8e or the cytosine deaminase ABOBEC1, Yi et al. achieved highly efficient A-to-G and C-to-T conversions with up to 77% accuracy. The

strand-biased editing mechanism allowed the modifications to be selectively retained, providing a promising tool for studying and potentially treating mitochondrial genetic diseases (Mok et al. 2020; Gabsalilow et al. 2021).

Base editing was further enhanced by integrating nickase and deaminase domains within the same TALE arrays, leading to monomeric mitoBEs, which demonstrated comparable or superior efficiency to dimeric systems. The utilization of nickases enabled targeted deamination without inducing double-strand breaks, preventing the rapid degradation of mtDNA. The study also demonstrated the successful application of mitoBEs in patient-derived cells using circular RNA delivery, confirming their therapeutic potential. The findings of this study established that mitoBEs are not only precise but also are an efficient base editing system capable of correcting mtDNA mutations without as many effects as other gene therapy options (Yi et al., 2023).

Zinc-Finger Nucleases (ZFNs)

Zinc-finger nucleases (ZFNs) are a class of engineered DNA cleavage reagents that have made their impact as powerful tools for gene editing. These nucleases are synthetic and consist of two distinct functional domains, including a zinc-finger protein (ZFP), which provides sequence-specific DNA binding, and a FokI endonuclease domain that induces double-stranded breaks at specified targeted sites. The customizable structural nature of ZFPs allows them to be tailored to recognize specific DNA sequences, making ZFNs an effective approach for genome modification (Carroll, 2011). When the endonuclease FokI domain dimerizes upon DNA binding, they introduce precise breaks in the genome. This break has shown repairability by the cell's

endogenous mechanisms, and this repair process often occurs through non-homologous end joining, which can lead to small insertions or deletions that can disrupt gene function, or HR, which allows for the precise insertion of new genetic material (Carroll, 2011). These properties make ZFNs particularly useful for targeted gene replacement and mutagenesis in different biological applications.

Davis & Stokoe conducted a review that explored the development and application of ZFPs as sequence-specific tools. The primary focus was on the fusion of the FokI nuclease to ZFPs and the resulting ability of the fusion protein, known as ZFNs, to manipulate genes of interest at the genomic level. They also discussed the use of other functional domains, such as transcriptional activators and repressors highlighting their roles as tools for discovery and therapy (Davis & Stokoe, 2010). Since the initial proof of concept studies, major progress has been made in designing highly functional ZFPs. The first-generation ZFP design involved a modular assembly approach, where individual zinc fingers were optimized against target triplet DNA sequences and combined to form three or four ZFPs to target 9 or 12 bp sequences. Although the modular assembly led to successful ZFP applications, it was noted that this approach had a high fail rate (Ramirez et al., 2008). This deficiency is likely because of the influence of neighboring zinc fingers on the sequence specificity of a given zinc finger, which led to the development of selection-guided assemblies (Davis & Stokoe, 2010).

ZFNs have also been implicated in recent studies demonstrating functionality and promise with human diseases. In 2024, Lessard et al. conducted a study on sickle cell disease (SCD) involving ZFN-mediated gene editing in hematopoietic stem cells, resulting in the reactivation of fetal hemoglobin. This study focused on BIVV003, which

is a gene-edited autologous cell therapy that is currently under clinical development for the potential treatment of SCD. The therapy involves genetically modifying hematopoietic stem cells (HSC) with mRNA encoding ZFNs which target and disrupt a specific GATAA motif in the BCL11A erythroid specific enhancer (ESE). The use of ZFNs to generate double-stranded breaks at the defined genomic locations, followed by repair through non-homologous end joining or microhomology mediated end joining, resulted in effective gene disruption and HbF reactivation in erythroid cells, showing no adverse effects on terminal erythroid differentiation and is a key factor in mitigating the clinical symptoms and provides a potential treatment for SCD (Lessard et al., 2024).

This study significantly advances the public's understanding of ZFNs by demonstrating their high efficiency in editing HSCs which can lead to long-term multilineage engraftment and sustained HbF expression. Lessard et al. also highlight the potential of ZFN-mediated gene editing in clinical applications, providing instruction to treat genetic disorders like SCD. Through clonal analysis, this study also showed enriched biallelic editing, offering insight into the effectiveness and precision of ZFNs in gene therapy. Furthermore, the data, along with preliminary clinical studies, suggest that BIVV003 could be a promising new cell therapy for SCD, further enhancing the potential of ZFNs in clinical gene editing (Lessard et al., 2024).

Preclinical results from the study indicated that gene-edited HSCs from both SCD and healthy donors showed high biallelic editing efficiency, long-term multilineage engraftment, and the reactivation of HbF, with therapeutic benefits such as reduced sickling in erythroid progeny. ZFN-mediated editing also showed no detrimental effects on the function or potency of hematopoietic progenitors and displayed high precision

with small indels within the BCL11A ESE. The interim results from the Phase 1/2 study further support the therapeutic potential of BIVV003, with sustained increases in total hemoglobin and HbF levels in participants, leading to clinical benefits such as the prevention of severe vaso-occlusive crises. Despite some challenges with the manufacturing process, the study highlights the efficacy of ZFN-mediated gene editing and its promise as a novel approach to SCD therapy. The data suggests that BIVV003 and its potential for long-term therapeutic benefits, should continue its clinical development as a viable treatment for SCD (Lessard et al., 2024).

Transcription Activator-Like Effector Nucleases (TALENs)

Transcription activator-like effector nucleases (TALENs) have proven their capability as an influential genome editing tool, which has been widely utilized for precise genetic modifications. TALENs consist of a customizable DNA-binding domain derived from transcription activator-like effectors (TALEs) fused to the nuclease domain of FokI, enabling site-specific double-stranded breaks in DNA. These targeted double-stranded breaks are repaired by either non-homologous end joining, which introduces insertions or deletions, or homology-directed repair, which allows for accurate sequence modifications. TALENs offer several advantages that include high specificity, modularity, and minimal off-target effects making them suitable for applications in gene therapy (Sun & Zhao, 2013). Their ability to efficiently edit complex genomes has allowed them to become a valuable alternative to other genome-editing technologies such as zinc finger nucleases (ZFNs) and CRISPR-Cas systems.

Following behind ZFNs, TALENs marked a significant milestone in genome editing and are a main driving contributor of precise genetic modifications. TALENs were the first genome editing tools that could be easily designed, and they enabled targeted modifications with high precision and efficiency. TALENs were quickly adopted for applications in different organisms, including livestock, crops, and model species, because of their versatility and reliability (Becker & Boch, 2021). This technology played a major role in the first genome-editing treatment to save human lives by curing cancer in 2015. In this unique situation TALENs were utilized to modify T cells by creating CAR19 T cells. These modified T cells were specifically designed to seek and attack cancerous B cells in two infants who had leukemia and who had relapsed after multiple standard treatments including chemotherapy and stem cell transplants. By removing some of the natural features of the T cells including TCR and CD52 scientists made sure the engineered cells would survive in the patients' bodies without being attacked by their immune systems. As a result, these powerful cancer-fighting cells were able to effectively locate and destroy the cancer cells, leading the infants into remission with no detectable cancer remaining in the patients (Qasim et al., 2017).

Genome editing extends the fundamental concept of recombinant DNA technology, which was first realized in the 1970s when scientists recognized that any DNA could be combined. While early genome editing efforts in the 1990s relied on meganucleases and ZFNs, their complexity and technical challenges limited worldwide adoption. However, in 2010 the development of TALENs revolutionized the field by offering a simpler and more accessible tool (Becker & Boch, 2021). The emergence of another gene editing technique, CRISPR/Cas9, was developed in 2012 and further

transformed genome editing by offering a more easily used system, leading to its widespread adoption as a routine laboratory technique and surpassing TALENs. TALENs require protein engineering for each target sequence, but CRISPR/Cas9 utilizes a gRNA to direct the Cas9 enzyme to specific genomic loci, making it more adaptable and scalable (Pickar-Oliver & Gersbach, 2019). The simplicity and versatility of CRISPR/Cas9 contributed to its incredibly fast dominance in the field, but despite the fast expansion, TALENs still remain a valuable tool, particularly in applications requiring precise and efficient genome modifications without the risk of off-target effects associated with CRISPR (Becker & Boch, 2021).

While both CRISPR/Cas9 and base-editing approaches have introduced new methods for achieving targeted edits described in the above sections, TALENs continue to offer advantages in certain contexts, such as higher efficiency in modifying complex genomic regions. Additionally, newer genome editing techniques, including base editing and prime editing, are expanding the capabilities of precise genetic modification, though their efficiency remains variable (Anzalone et al., 2020). Even though TALENs have taken a backseat to gene editing, they offer a much wider range of utility especially when combined with other techniques such as combining TALEs and CRISPR systems (Becker & Boch, 2021).

A study conducted by Mussolino et al. investigated the activity, toxicity, and specificity of TALENs to assess their full potential for therapeutic applications. Due to TALENs high cleavage activity and low cytotoxicity, it makes them a competitive alternative to ZFNs and CRISPR/Cas9 systems (Joung & Sander, 2012). While ZFNs and CRISPR/Cas9 have been successfully utilized for gene editing, including many studies

and clinical trials for HIV-resistant T cells, both techniques exhibit off-target mutagenesis that may limit their therapeutic potential (Perez et al., 2008). TALENs on the other hand have appeared to be better tolerated in human cells and animal models, though their off-target activity has not been extensively characterized (Tesson et al., 2011). Existing high-throughput methods for assessing off-target cleavage are either technically complex or not robust enough for routine use. Given that TALENs are gaining traction for therapeutic application, a more detailed analysis of their potential and negative effects is necessary which is portrayed in this study (Mussolino et al., 2014). The study compared the efficacy, specificity, and cytotoxicity of TALENs and ZFNs in targeting the three different human loci: AAVS1, CCR5, and IL2RG. TALEN pairs were successfully developed and tested using an optimized scaffold to generate functional nucleases. High-throughput sequencing was employed to assess off-target mutations, and the PROGNOS bioinformatics tool was used to predict potential off-target sites. TALEN and ZFN cytotoxicity were evaluated by measuring apoptosis and cell cycle arrest in human cell lines (Mussolino et al., 2014).

It was found that TALENs demonstrated comparable effectiveness to benchmark ZFNs in gene disruption in primary human cells. Mutation efficiency was highest at AAVS1 (100%) and CCR5 (90%), while IL2RG had lower efficiency (33%), likely due to CpG dinucleotides affecting monomer binding. TALENs exhibited high specificity, as evidenced by low cleavage activity at predicted off-target sites. ZFNs, in contrast, induced higher apoptosis and cell cycle arrest, suggesting greater cytotoxicity due to off-target effects. TALENs showed significantly fewer off-target mutations than ZFNs, particularly at CCR5, where TALENs achieved a higher specificity factor (up to 60:1)

compared to ZFNs (1:2) (Mussolino et al., 2014). The findings supported TALENs as a more precise and safer genome editing tool than ZFNs in the aforementioned loci.

TALENs were associated with lower cytotoxicity, as was demonstrated by the absence of cell cycle abnormalities and minimal genomic rearrangements at the CCR2/CCR5 loci.

Mussolino et al. identified CpG dinucleotides as potential inhibitors of TALEN activity and highlighted the importance of optimizing target site selection to enhance specificity.

The study also validated the predictive accuracy of the PROGNOS bioinformatics tool (Fine et al., 2013) while also introducing a refined TALEN scaffold with a short linker that improved specificity.

Different design strategies, such as avoiding CpG sites and using obligate heterodimeric FokI domains, could further enhance TALEN specificity, making them a promising tool for future therapeutic use (Mussolino et al., 2014).

Chapter IV.

Gene Therapies and Target Diseases

Gene editing technologies are rapidly emerging as transformative tools in the treatment of genetic diseases by offering unprecedented precision in modifying DNA sequences to correct mutations at their source. These advances in genome engineering have led to the development of techniques such as CRISPR/Cas9, base editing, ZFNs, MAGE, TALENs, and Cpf1 (Cas12a), each providing unique capabilities for targeting and repairing defective genes. These approaches are gaining increasing recognition for their potential to treat a wide range of inherited and acquired disorders, surpassing traditional therapies that primarily focus on symptom management rather than addressing the root cause of disease.

This chapter examines the proposed hypotheses by evaluating how different gene editing techniques could be used to treat inherited cardiovascular diseases. Each hypothesis explores a different genome-editing approach, assessing its efficacy in targeting and correcting mutations associated with conditions such as Barth Syndrome, ARVD/C, Pompe disease, Danon Disease, and FHCM. Additionally, this analysis will compare these techniques to existing treatments to determine which offer the most precise and effective therapeutic potential. While the hypotheses highlight promising gene editing approaches for each disease, other techniques will also be examined to understand why they may or may not be suitable. This comprehensive evaluation will provide insight into the strengths and limitations of various gene editing strategies, ultimately identifying the most viable options for future therapeutic applications. By

thoroughly analyzing these approaches, this chapter aims to provide a clearer understanding of the potentials and challenges of gene editing technologies in treating inherited cardiovascular diseases.

Hypothesis I - CRISPR-Cas9 and FHCM

The original hypothesis proposed that the CRISPR/Cas9 technique could successfully treat FHCM by correcting faulty DNA sequences, potentially restoring the normal function of the affected proteins. In the following discussion, we will evaluate the feasibility of this approach and assess whether CRISPR/Cas9 can effectively achieve this therapeutic outcome, or if the other techniques can provide a more efficient outcome.

CRISPR/Cas9 and FHCM

The CRISPR/Cas9 system holds significant potential for the treatment of FHCM because of its ability to directly target and edit specific mutations responsible for the disease. FHCM is primarily caused by mutations in genes like MYH7, which encodes for cardiac myosin and is a key protein in the heart muscle responsible for muscle contraction. Specifically, the MYH7 p.G407C mutation, which changes a glycine residue to cysteine residue at a crucial position in the protein structure, significantly changes protein function and contributes to the pathogenesis of FHCM. The CRISPR/Cas9 system could potentially be used to precisely edit this mutation by employing a gRNA to direct the Cas9 endonuclease to the exact DNA sequence containing the mutation. Cas9 would then create a double-strand break in the DNA, followed by homology-directed repair allowing for the introduction of a corrected sequence that restores the proper function of

the MYH7 gene. In the case of FHCM, correcting the MYH7 mutation at the genetic level could prevent the thickening of the heart muscle and alleviate the symptoms associated with the disease.

In FHCM, CRISPR/Cas9 could also allow for precise modifications, which is significant given that FHCM is caused by various mutations across different genes, with MYH7 being one of the most observed. While CRISPR/Cas9's ability to target these mutations offers hope for a cure, the challenge lies in the complexity of editing mutations spread across multiple genes, which could possibly require multiple edits within a single therapy. Nevertheless, CRISPR/Cas9 treatment faces challenges due to potential off-target effects, where Cas9 may inadvertently cut DNA at unintended locations, leading to undesired mutations or even genetic damage (Guo et al., 2023). The specificity of the gRNA and the precision of the CRISPR/Cas9 system are critical in minimizing these risks, and to offset the risk, advancements in CRISPR technology need to be improved with either gRNA and/or enhanced Cas9 variants with greater specificity. Additionally, optimizing potential delivery systems like adeno-associated virus vectors, will be essential to ensure accurate targeting of cardiac cells and minimize immune responses that could complicate treatment.

Correcting the MYH7 p.G407C mutation in FHCM with CRISPR/Cas9 seems promising, but translating this technology from the laboratory to the clinic presents considerable obstacles in terms of delivery efficiency, precision, and long-term safety. While early studies have shown success in animal models, FHCM is influenced by a combination of genetic and environmental factors, indicating that treatment success will depend on addressing the multifaceted nature of its pathogenesis. Despite these

challenges presented by FHCM, CRISPR/Cas9 still represents an innovative approach by directly targeting and correcting genetic mutations at their source, potentially offering a cure. With continued advancements, CRISPR/Cas9 could become a powerful tool for curing FHCM, but further research, clinical trials, and refinement of the technology are necessary before it can be considered a reliable cure.

MAGE and FHCM

As MAGE has potential advantages for high-throughput genetic programming, its application in treating FHCM doesn't seem likely. The technology's ability to induce large-scale genetic diversity in organisms like *E. coli* demonstrates its potential for gene editing, but challenges in applying it to complex human diseases persist. In contrast, CRISPR/Cas9 has shown more success in targeted genome editing, particularly in human genetics (Wang et al., 2009; Guo et al., 2014). MAGE does not currently provide a better treatment for FHCM compared to CRISPR/Cas9. While MAGE can generate genetic diversity and perform large-scale modifications, its precision in correcting specific mutations, such as MYH7 p.G407C, is unproven. CRISPR/Cas9, on the other hand, has shown its ability in precise modifications at specific loci, making it the more suitable tool for addressing FHCM-related mutations. Although MAGE may complement future gene therapy efforts as its capabilities improve, CRISPR/Cas9 remains the more promising option for FHCM treatment at this stage.

Base Editing and FHCM

Base editing offers distinct advantages over CRISPR/Cas9, particularly in its precision and reduced risk of off-target effects, making it a promising tool for treating FHCM. Base editing has enhanced safety because it does not cause double-stranded breaks in the DNA. Its ability to introduce single nucleotide changes without the need for a DNA template allows for more efficient and reliable editing, especially in non-dividing cells. Given that FHCM often results from point mutations in the MYH7 gene, base editing could theoretically offer a precise method to correct such mutations (Rees & Liu, 2018; Yeh et al., 2018).

Although, while base editing's precision presents convincing advantages, CRISPR/Cas9 remains more established in treating human genetic diseases like FHCM due to its proven ability to target specific loci with high accuracy. Base editing has shown promise in preclinical research, but its applicability to complex cardiovascular diseases remains under-tested. In contrast, CRISPR/Cas9 has a longer history of clinical research, and its therapeutic potential is better understood in genetic disorders. While base editing may eventually surpass CRISPR/Cas9 in specific scenarios, CRISPR/Cas9 still holds an edge in treating FHCM due to its demonstrated success in human applications (Zafra et al., 2018; Yi et al., 2023).

ZFN and FHCM

ZFNs have shown promising potential in treating genetic diseases by enabling precise modifications in DNA, such as gene disruption or insertion, which is crucial for

disorders like FHCM. ZFNs use engineered zinc-finger proteins to target specific DNA sequences and create double-stranded breaks at precise loci. The repair of these breaks by the cell's internal mechanisms, such as non-homologous end joining or homology-directed repair allowing for targeted gene modifications. ZFNs' ability to generate controlled mutations, including insertions and deletions, show potential that could be used to correct the point mutations in the *MYH7* gene responsible for FHCM (Carroll, 2011; Davis & Stokoe, 2010).

ZFNs do face limitations despite their precision, compared to CRISPR/Cas9 in terms of efficiency and simplicity. Their application does involve a higher failure rate due to the need for customized zinc-finger domains and challenges with sequence specificity, but the potential for off-target effects and the difficulties of designing ZFNs tailored to FHCM mutations remain ongoing challenges. In contrast, CRISPR/Cas9 offers a more flexible and widely applicable tool with a simpler design process. As a result, ZFNs may offer a targeted approach for FHCM, CRISPR/Cas9's efficiency and ease of use make it a more practical option.

TALENs and FHCM

TALENs have demonstrated significant potential in gene editing and offer advantages like high specificity, modularity, and minimal off-target effects. TALENs' ability to induce precise double-stranded breaks that can be repaired by either non-homologous end joining, or homology-directed repair makes them a strong candidate for correcting genetic mutations associated with FHCM. Studies have shown that TALENs can be used for targeted gene modifications with high efficiency and low cytotoxicity,

making them a competitive alternative to the other technologies (Sun & Zhao, 2013; Mussolino et al., 2014).

However, TALENs do face some limitations compared to CRISPR/Cas9. While TALENs exhibit fewer off-target effects, they do require protein engineering for each target sequence, and this complicates their application relative to the CRISPR/Cas9 system. CRISPR/Cas9's ability to target multiple sites simultaneously and extensive clinical validation makes it more practical for widespread use. Therefore, while TALENs offer advantages in specificity, CRISPR/Cas9's broader applicability and simpler design make it the more viable tool for treating FHCM (Pickar-Oliver & Gersbach, 2019).

Cpf1 (Cas12a) and FHCM

Cpf1 (Cas12a) shows a multitude of advantages over Cas9 for genome editing, making it a competitive tool for treating FHCM. Cas12a's ability to produce staggered double-stranded breaks with five nucleotide overhangs enhances the precision of DNA repair, particularly homology-directed repair, which is crucial for genetic modifications (Zetsche et al., 2015). Additionally, Cas12a's flexible PAM recognition site (5'-TTTN-3') gives a broader range of target sequences, making it more versatile than Cas9 (Safari et al., 2019). Its ability to auto-process its crRNA simplifies experimental procedures, and its potential for multiplex genome editing could be advantageous for treating FHCM by targeting multiple loci simultaneously (Paul & Montoya, 2020). Despite concerns about the indiscriminate degradation of ssDNA, Cas12a's specificity is generally higher due to its multi-checkpoint mechanism that reduces off-target effects, making it a safer option for therapeutic applications (Chen et al., 2018).

While Cas12a has distinct advantages, its potential for treating FHCM may still be restricted by the challenges common to all CRISPR-based systems, specifically in non-dividing cells such as cardiomyocytes. Both Cas12a and Cas9 rely on the host cell's DNA repair machinery, which can affect editing efficiency in certain contexts (Peters et al., 2017). Moreover, while Cas12a's broader PAM recognition provides more flexible targeting, Cas9 still remains the more established system. Even though Cas12a shows immense potential, the proven track record of Cas9 makes it the more suitable option for treating FHCM at present (Paul & Montoya, 2020).

Hypothesis II - Cpf1 (Cas12a) and Danon Disease

It was hypothesized that the Cpf1 (Cas12a) technique could effectively treat Danon Disease by leveraging its high precision in gene targeting. Through gene silencing, insertion, or replacement, Cpf1 (Cas12a) has the potential to fully restore the *LAMP2* gene and correct LAMP2 protein function. The analysis below will interpret the viability of this approach and explore whether Cpf1 (Cas12a) can successfully achieve this therapeutic goal or if other techniques provide a more promising treatment.

Cpf1 (Cas12a) and Danon Disease

Cpf1 (Cas12a) could potentially be effective in treating Danon disease by targeting the specific genetic mutation in the *LAMP2* gene. The mutation, c.741+1G>T, results in a premature stop codon preventing the production of the essential LAMP2 protein, which is critical for lysosomal function and autophagy (Zhou et al., 2019).

Cas12a creates staggered double-stranded breaks with five nucleotide overhangs (Zetsche

et al., 2015), which has been shown to promote homology-directed repair more efficiently than other genome-editing techniques, which could facilitate the correction of the mutation in the LAMP2 gene. This precise repair could restore normal LAMP2 protein function and potentially alleviate the symptoms of Danon disease.

Cas12a offers the advantage of simpler RNA processing because it only requires a single crRNA for targeting the gene, in comparison to Cas9, which needs two RNA molecules (Deltcheva et al., 2011). Additionally, its ability to induce accurate DNA cleavage and its potential for multiplex gene editing (Paul & Montoya, 2020) offers therapeutic avenues for patients with Danon disease who may have other co-occurring mutations. On the other hand, Cas12a still relies on the host cell's DNA repair machinery and can be less efficient in non-dividing cells, such as cardiomyocytes and skeletal muscle cells, which are primarily affected by Danon disease (Peters et al., 2017). There are also risks associated with Cas12a's off-target effects and potential genomic instability, particularly due to its ability to degrade ssDNA indiscriminately (Chen et al., 2018).

While Cas12a shows promise for gene editing, further research is needed to optimize its application for treating Danon disease, particularly in the context of efficient delivery and repair in non-dividing cells. In its current state, Cas12a may not yet be a definitive cure but could contribute to a potential therapeutic approach if these technical challenges can be overcome.

MAGE and Danon Disease

MAGE has an advantage in its ability to make simultaneous genetic modifications at multiple chromosomal locations, a feature critical for diseases like Danon. MAGE operates with synthetic oligonucleotides to introduce precise mutations, deletions, or insertions, generating large-scale genetic diversity, which could be beneficial for addressing the *LAMP2* gene mutation present in Danon disease (Wang et al., 2009). This high-throughput capacity and scalability make MAGE ideal for rapid genomic alterations and could potentially enable more comprehensive treatment strategies for genetic disorders like Danon disease.

MAGE's applicability to mammalian systems remains uncertain, especially those with non-dividing cells like heart and skeletal muscle. While the technique has been successful in bacteria, its utilization in more complex organisms faces significant challenges (DiCarlo et al., 2013). MAGE's reliance on oligonucleotide-mediated homologous recombination has shown limited success in mammalian cells, making its efficiency and precision in treating Danon disease uncertain. While MAGE has high potential, further research is needed before it can be considered a viable treatment option.

TALENs and Danon Disease

TALENs offer distinct advantages in treating Danon disease due to their ability for high specificity and low cytotoxicity. TALENs have shown better efficiency in modifying complex genomes with fewer off-target effects compared to CRISPR systems, making them a promising alternative for gene therapy (Sun & Zhao, 2013; Mussolino et

al., 2014). A study by Mussolino et al. demonstrated TALENs' superior specificity, with fewer off-target mutations and lower cytotoxicity than ZFNs and CRISPR/Cas9, which is imperative for a genetic disorder like Danon disease (Mussolino et al., 2014).

TALENs application in treating Danon disease may be limited by the need for protein engineering for each target sequence, complicating flexibility (Becker & Boch, 2021). However, their demonstrated precision and minimal off-target effects still make them a competitive choice for targeted genetic interventions (Mussolino et al., 2014; Sun & Zhao, 2013).

CRISPR/Cas9 and Danon Disease

CRISPR/Cas9 technology demonstrates promise for treating Danon disease due to its precision, affordability, and easy utilization. The potential for CRISPR to correct mutations in the *LAMP2* gene is compelling, especially with recent improvements to delivery systems, such as adeno-associated virus vectors to reduce immunogenicity (Redman et al., 2016). However, the main challenge lies in CRISPR's off-target effects, which again can result in unintended genetic modifications, posing risks for clinical applications (Guo et al., 2023).

While Cas12a (Cpf1) offers advantages like lower off-target effects, CRISPR/Cas9's broader application and existing therapeutic success suggest it may offer a more viable treatment option for Danon disease, particularly as research continues to improve its targeting precision (Redman et al., 2016; Guo et al., 2023).

Base Editing and Danon Disease

Base editing is a promising tool in treating Danon disease, especially due to its ability to make precise, single-nucleotide changes without the induction of double-stranded breaks, thus minimizing the risk of off-target mutations. This makes base editing highly suitable for correcting point mutations, like those in the *LAMP2* gene that cause Danon disease (Rees & Liu, 2018). As base editing can operate in both dividing and non-dividing cells, its versatility is noted where precise genetic corrections are needed (Yeh et al., 2018).

Compared to Cas12a (Cpf1), which induces staggered cuts, base editing provides better precision and efficiency for fixing SNVs without requiring template-based repair mechanisms, thus offering a safer and more reliable alternative to traditional CRISPR systems (Schatoff et al., 2019). Also, base editing has demonstrated success in mitochondrial DNA editing, a challenge for other genome editing techniques, which further expands its potential for treating genetic diseases like Danon disease (Yi et al., 2023). Therefore, base editing represents a promising tool for treating Danon disease with high precision and minimal risk.

ZFNs and Danon Disease

ZFNs have potential advantages in gene editing, including precise and targeted modifications of the genome. ZFN precision suggests that it could be effective for treating genetic disorders like Danon disease, where targeted gene modifications are required. However, ZFNs have limitations, such as the need for precise design of zinc-finger proteins, which can result in high failure rates due to sequence specificity issues

(Ramirez et al., 2008). In contrast, Cpf1 (Cas12a) is simpler to program, with fewer design constraints and a lower chance of off-target effects, making it easier to use for editing (Zafra et al., 2018). Additionally, Cpf1's ability to generate staggered cuts and its versatility with PAM sequences may make it a more robust tool for treating Danon disease. While ZFNs have shown efficacy in clinical applications like sickle cell disease (Lessard et al., 2024), their complexity and potential for lower editing efficiency may make Cpf1 a more favorable option for Danon disease.

Hypothesis III - MAGE and Pompe Disease

The potential of the MAGE technique to treat Pompe Disease lies in its ability to both edit the mutated *GAA* gene and enhance gene expression. By increasing the production of the acid alpha-glucosidase enzyme, MAGE could compensate for the genetic defect and improve glycogen breakdown in affected cells. This discussion will evaluate whether MAGE is a feasible approach for treating Pompe disease and how it compares to other gene editing strategies.

MAGE and Pompe Disease

MAGE holds promise for treating Pompe disease by enabling the simultaneous introduction of genetic modifications across multiple chromosomal locations. Pompe disease is caused by mutations in the *GAA* gene, leading to the deficiency of acid alpha-glucosidase, which is crucial for glycogen breakdown in lysosomes. MAGE's ability to generate rapid genetic diversity and target specific genomic regions could theoretically be applied to delete or correct mutations in the *GAA* gene, potentially restoring enzyme

function and halting glycogen accumulation in cells. This could be particularly useful for addressing common mutations in acid alpha-glucosidase, such as c.-32-13T>G splicing mutation or p.R854X mutation (Chien et al., 2013).

Although MAGE's ability to treat Pompe disease depends on various factors. MAGE has shown high efficacy in microorganisms, where it can introduce multiple mutations simultaneously, but its application in higher eukaryotic cells, such as human muscle cells, has been shown to be less effective (Wang et al., 2009). Challenges such as low efficiency in non-bacterial cells, difficulties in delivering oligonucleotides into human tissues, and potential off-target effects complicate its use for Pompe disease (DiCarlo et al., 2013). Pompe disease is also influenced by other pathological mechanisms, including autophagic dysfunction and mitochondrial abnormalities (Kohler et al., 2018). While MAGE could address genetic mutations, it may not be sufficient in addressing downstream cellular issues like glycogen accumulation and oxidative stress. Given these limitations, MAGE may have a role in early-stage gene therapies or research, but it absolutely requires further optimization for use in treating Pompe disease.

CRISPR/Cas9 and Pompe Disease

CRISPR/Cas9 has significant potential in treating Pompe disease. Studies have shown success in using CRISPR to correct gene mutations and restore enzyme function in animal models of genetic diseases (Redman et al., 2016). This precise ability offers advantages over techniques like MAGE, particularly for conditions like Pompe disease, where targeted correction of a single gene mutation is required. However, off-target effects still remain a challenge (Guo et al., 2023).

While MAGE allows for genetic modifications in multiple regions of the genome, its application to complex diseases like Pompe disease may be less effective than CRISPR. MAGEs reliance on oligonucleotides and homology-directed repair may not be as efficient in mammalian cells for precise single-gene corrections compared to bacterial cells. In contrast, CRISPR/Cas9 can provide a more direct approach for editing the *GAA* gene in human cells, making it a more promising therapeutic option for Pompe disease despite challenges of delivery limitations and off-target effects (Naso et al., 2017).

TALENs and Pompe Disease

TALENs have several advantages over MAGE for treating genetic diseases such as Pompe disease. TALENs have proven to be a precise and efficient genome-editing tool with high specificity and minimal off-target effects, making them ideal for therapeutic applications where accuracy is crucial (Sun & Zhao, 2013; Mussolino et al., 2014). In comparison to MAGE, which relies on oligonucleotide-based editing, TALENs can induce targeted DNA double-strand breaks that are repaired through homology-directed repair, leading to precise sequence modifications. This method has shown to be particularly advantageous in editing complex genomic regions (Becker & Boch, 2021).

While MAGE offers the potential to edit multiple genes simultaneously, it can result in less accurate genome modifications. TALENs are highly specific and can be engineered to target specific genes with less unintended effects, addressing a major concern with MAGE (Becker & Boch, 2021). Even though CRISPR/Cas9 has become more widely utilized, TALENs remain valuable in cases where precision is key and a requirement for Pompe disease treatment, where even minor errors can have significant

consequences (Pickar-Oliver & Gersbach, 2019). Therefore, TALENs present a potentially better alternative to MAGE in the context of therapeutic gene editing for Pompe disease.

ZFNs and Pompe Disease

ZFNs have demonstrated potential in the treatment of genetic disorders like sickle cell disease, with high efficiency in editing HSCs with minimal adverse effects (Lessard et al., 2024). ZFNs work by introducing targeted DNA double-stranded breaks that are repaired through either non-homologous end joining or homology-directed repair, allowing for precise genetic modifications. This precision, coupled with their ability to generate biallelic edits, makes ZFNs a promising for treatment Pompe disease because they offer targeted modifications with minimal unintended mutations.

However, their application for diseases like Pompe disease may face limitations in terms of adaptability. In contrast to MAGE which offers multiplexing capabilities, allowing for the simultaneous editing of multiple genes, posing as beneficial for complex diseases like Pompe disease that may involve multiple genetic mutations. While ZFNs provide high precision, their application in treating diseases requiring editing several genetic loci may not be as efficient as MAGE. Therefore, while ZFNs hold significant potential, MAGE may offer broader applicability for diseases with complex genetic backgrounds (Davis & Stokoe, 2010; Carroll, 2011).

Base Editing and Pompe Disease

Base editing can convert single nucleotides without causing DBs which lessens the risk of unintended mutations (Rees & Liu, 2018). This ability to target specific point mutations makes base editing particularly useful for correcting pathogenic alleles associated with SNVs, a common cause of genetic disorders (Schatoff et al., 2019). Base editing's efficiency in both dividing and non-dividing cells and its ability to simultaneously introduce multiple mutations using multiple sgRNAs gives it a distinct advantage over technologies like CRISPR knockout, which rely on less efficient homology-directed repair for precise edits (Yeh et al., 2018). Although, base editing's application for Pompe disease seems limited compared to MAGE as it has the ability for a more robust method for editing multiple loci simultaneously, which is essential for diseases like Pompe disease, where multiple genes may need to be targeted for therapeutic efficacy. While base editing is effective for single-nucleotide corrections, MAGE's ability to perform multiplexed edits across larger genomic regions shows to be a more comprehensive treatment approach for Pompe disease (Schatoff et al., 2019).

Cpf1 (Cas12a) and Pompe Disease

Cpf1 (Cas12a) has leverage over Cas9, including its ability to perform multiplex genome editing and production of staggered double-stranded breaks with its nucleotide overhangs. This feature could be particularly beneficial for Pompe disease, where precise modifications across multiple loci may be required. Cas12a also uses a simpler crRNA system and can auto-process its crRNA, making it more versatile and reducing experimental complexity compared to Cas9 (Paul & Montoya, 2020). Moreover, the

indiscriminate degradation of ssDNA by Cas12a raises concerns about possible genomic instability, which could complicate its therapeutic use if not carefully controlled (Chen et al., 2018).

Cas12a faces challenges when considering treatment for Pompe disease, mainly in balancing its efficiency with the potential risks of genomic instability. MAGE, on the other hand, is specifically designed for precise, efficient, and scalable genome editing across multiple loci without these concerns. It could be better suited for treating Pompe disease, where precise and safe manipulation of multiple genes is required (Paul & Montoya, 2020; Jasin & Rothstein, 2013).

Hypothesis IV - ZFNs and ARVD/C

Due to its high specificity and precision in gene targeting, the ZFN technique has been proposed as a potential treatment for Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy (ARVD/C). This approach could effectively modify not just a single desmosomal protein but multiple genes implicated in ARVD/C, including desmoglein (DSG), desmoplakin (DSP), and plakoglobin (JUP). Below is an assessment of the feasibility of using zinc finger nucleases for ARVD/C treatment and a discussion of its advantages and limitations in comparison to other gene editing methods.

ZFNs and ARVD/C

ZFNs show considerable potential for treating ARVD/C, particularly due to their ability to target specific genetic mutations and facilitate precise genetic modifications. In ARVD/C, the most common mutations are found in the desmosomal genes, particularly

PKP2, which is responsible for stabilizing cell to cell adhesion in cardiac tissue (Groeneweg et al., 2015). ZFNs could be employed to edit the mutated *PKP2* gene directly, disrupting the pathogenic mutations or replacing the faulty gene with a functional copy. By introducing a double-stranded break at the targeted site and relying on the cell's repair mechanisms including non-homologous end joining or HR, ZFNs could correct mutations in the *PKP2* gene, restoring normal desmosomal function and preventing the fibrous and fatty tissue replacement characteristic of ARVD/C (Carroll, 2011; Davis & Stokoe, 2010). Furthermore, the ability of ZFNs to introduce small indels or precisely integrate a corrected gene provides a viable strategy for addressing the genetic root causes of the disease.

ZFNs have been successfully utilized in other genetic diseases, such as sickle cell disease, where gene editing in hematopoietic stem cells led to the reactivation of fetal hemoglobin, alleviating disease symptoms (Lessard et al., 2024). This approach showcases the potential of ZFNs to modify genes in a way that restores normal function and prevents disease progression. On the other hand, the application of ZFNs to ARVD/C would require careful consideration of delivery methods to ensure precise editing within cardiac tissue. As the technology does hold promise, challenges in achieving targeted gene repair in vivo and ensuring long term stability of the edited cells remain areas of active research.

While ZFNs have significant potential for treating ARVD/C, the question of whether they can fully cure the disease remains complex. One primary concern is the delivery of ZFNs to the affected cardiac tissue, particularly the right ventricle, where the mutations primarily manifest (Riasat et al., 2022). Efficient and targeted delivery of the

ZFN system to heart cells remains a major hurdle. Therefore, while ZFNs hold great optimism for treating ARVD/C, their ability to cure the disease would depend on overcoming these delivery and precision challenges, making the application of ZFNs a potential but not yet fully realized treatment for ARVD/C.

MAGE and ARVD/C

MAGE offers advantages over ZFNs in treating ARVD/C, due to its ability to introduce simultaneous modifications at multiple chromosomal locations. MAGE enables precise genetic alterations, generating substantial genomic diversity in a controlled manner. This capability could be highly beneficial for diseases like ARVD/C, where multiple gene mutations contribute to the pathology. By targeting several genomic regions simultaneously, MAGE could address the complexity of ARVD/C, such as mutations in the PKP2 gene, with greater efficiency than ZFNs, which typically target one locus at a time (Wang et al., 2009). Additionally, MAGE's scalability allows for large-scale modifications, potentially enabling more comprehensive treatments compared to ZFNs, which require more labor-intensive, individual targeting of mutations.

MAGE's application faces significant limitations in non-model organisms like humans. While MAGE has demonstrated success in *E. coli* and yeast, its efficiency and effectiveness in human cells remain underdeveloped (DiCarlo et al., 2013). ZFNs, on the other hand, have a proven track record in clinical trials and gene therapy, with more established methodologies for delivering targeted edits to human tissues. While MAGE holds promise for genetic modification, it is not yet on par with ZFNs for treating

complex human diseases like ARVD/C, requiring further validation before it can rival ZFNs as a clinical tool (Singh & Braddick, 2015).

CRISPR/Cas9 and ARVD/C

CRISPR/Cas9 also has the potential to surpass ZFNs in treating ARVD/C due to its precision, and ability to edit multiple genes simultaneously. The CRISPR system enables targeted gene edits with minimal complexity (Redman et al., 2016). Its homology-directed repair mechanism allows for concise precision, giving the ability to correct genetic errors associated with ARVD/C, such as mutations in the PKP2 gene. Unlike ZFNs, which require the construction of specific zinc finger proteins for each target site, CRISPR/Cas9's RNA based guidance system allows for rapid adaptation, making it a more versatile and efficient tool for gene therapy (Hsu et al., 2014). CRISPR also has shown success in treating genetic disorders such as Duchenne muscular dystrophy, demonstrating its potential for therapeutic applications in human diseases (Tabebordbar et al., 2016).

The major concern with CRISPR/Cas9 is the risk of off-target effects, where the Cas9 enzyme may unintentionally edit non-target genomic sites, leading to undesirable mutations (Guo et al., 2023). While efforts to minimize off-target effects, such as optimizing gRNA design and improving Cas9 variants are ongoing, these risks still need to be addressed before CRISPR can be considered superior to ZFNs for treating ARVD/C. ZFNs are already known for their precision in targeting specific DNA sequences, with fewer off-target effects compared to CRISPR/Cas9 in certain contexts.

Cpf1 (Cas12a) and ARVD/C

Cpf1 (Cas12a) shows the potential to be superior to ZFNs in treating ARVD/C due to its simplicity, and ability to perform multiplex genome editing. Unlike Cas9, which requires two RNA molecules for processing, Cpf1 simplifies the process by using only a single crRNA, eliminating the need for additional components like tracrRNA (Deltcheva et al., 2011). Cpf1 also recognizes a more flexible PAM sequence, allowing for wider genomic targeting, and produces staggered DNA breaks with nucleotide overhangs, enhancing homology-directed repair (Zetsche et al., 2015). This feature makes Cas12a particularly advantageous for precise genome modifications and potentially for correcting genetic mutations associated with ARVD/C. Additionally, its ability to process its own crRNA and perform multiplex gene regulation adds versatility to gene-editing tasks (Paul & Montoya, 2020).

Cpf1 still faces challenges, particularly with the uncontrolled degradation of ssDNA, which could disrupt genomic stability (Chen et al., 2018). Moreover, like Cas9, Cpf1 is dependent on host cell DNA repair machinery for efficient gene editing, which can be less effective in non-dividing cells. While these limitations persist, Cpf1's improved precision, simplified process, and reduced off-target effects give it significant potential to outperform ZFNs in treating ARVD/C.

Base Editing and ARVD/C

Base editing does show potential in treating ARVD/C because of its ability to facilitate single nucleotide conversions without inducing double-stranded breaks (Rees & Liu, 2018). Unlike traditional CRISPR methods that rely on double-stranded breaks and

the cell's repair mechanisms, base editing avoids unwanted mutations by directly converting one DNA base pair into another, minimizing the risk of genomic instability. This precision allows base editing to correct point mutations seen in ARVD/C (Schatoff et al., 2019). Base editing also has proven particularly effective in generating mutations such as missense and nonsense making it an ideal candidate for altering genes that contribute to ARVD/C (Zafra et al., 2018).

TALENs and ARVD/C

TALENs do show potential as an alternative to ZFNs in treating ARVD/C as they do have high specificity and low off-target effects. Compared to ZFNs, TALENs have demonstrated lower cytotoxicity, with minimal apoptosis and cell cycle arrest, making them a safer option for gene editing in human cells (Mussolino et al., 2014). TALENs use a modular design which contributes to better versatility, especially when editing complex genomic regions. This makes them suitable for therapeutic applications, as evidenced by their successful use in the first genome-editing treatment that cured cancer (Qasim et al., 2017). Also, TALENs have been shown to achieve high efficiency in gene disruption, with mutation efficiencies of up to 100% in certain human loci, offering a more controlled approach to genome editing than ZFNs, which can suffer from higher cytotoxicity and off-target mutations (Mussolino et al., 2014).

Hypothesis V - Base Editing and Barth Syndrome

The base editing technique has been hypothesized as a potential treatment for Barth Syndrome due to its ability to precisely edit individual nucleotides, which could directly target the mutation in the *TAZ* gene. This section will examine the feasibility of

base editing as a viable approach for treating Barth Syndrome, while also considering other gene editing techniques and their potential effectiveness in addressing the condition.

Base Editing and Barth Syndrome

Base editing demonstrates great promise as a treatment for Barth Syndrome due to its precision in correcting specific point mutations in the *TAZ* gene (G4.5), which is responsible for the disorder. Since SNVs cause Barth Syndrome in *TAZ*, base editing is particularly well-suited for making these corrections without introducing double-stranded breaks in DNA (Rees & Liu, 2018). Mutations in *TAZ* lead to dysfunction in the tafazzin protein, which disrupts cardiolipin (CL) remodeling in mitochondria and compromises mitochondrial function (Jefferies, 2013). By directly fixing these point mutations, base editing has the potential to restore tafazzin function and improve mitochondrial health. Through precise base conversions (C>T or A>G transitions), base editing could repair mutations that interfere with cardiolipin synthesis which is an essential component of mitochondrial energy metabolism and structural stability.

One of the biggest advantages of base editing is that it works effectively in both dividing and non-dividing cells. This is crucial for targeting tissues affected by Barth Syndrome, like the heart and muscles, which aren't always in active division. Additionally, the development of mitoBEs opens new possibilities for directly targeting mitochondrial mutations (Yi et al., 2023). Given tafazzin's essential role in mitochondrial function, base editing's ability to modify mitochondrial DNA makes it a compelling option for addressing the root genetic defects in Barth Syndrome.

Whether base editing can fully "cure" Barth Syndrome depends on several factors, including the specific nature of the mutations and how effectively the editing process works in affected tissues. While base editing excels at fixing single nucleotide mutations, Barth Syndrome mutations vary widely and can include deletions or truncations that may not be as easily corrected. If base editing can accurately target and repair the relevant TAZ mutations, it could help restore cardiolipin synthesis and improve mitochondrial function. However, its overall success will depend on factors like the efficiency of the editing process, the extent of mitochondrial damage that has already occurred, and how well the edited cells restore function in critical organs such as the heart, muscles, and immune system.

MAGE and Barth Syndrome

MAGE has an edge over base editing in certain situations, especially when large-scale, simultaneous genetic modifications are needed. It's particularly effective at generating combinatorial genetic diversity which allows for multiple chromosomal sites to be modified across a population in a high-throughput, scalable way (Wang et al., 2009). This capability to introduce precise mutations, deletions, and insertions could be valuable for treating Barth Syndrome because of its range of mutation types. By creating genetic variants and helping researchers better understand gene functions, MAGE could contribute to new therapeutic strategies, including those targeting mitochondrial genes like TAZ in Barth Syndrome (DiCarlo et al., 2013; Bao et al., 2015).

Despite its potential, MAGE faces significant challenges in higher organisms, primarily due to inefficiencies in introducing oligonucleotides for homologous

recombination outside of bacteria and yeast (DiCarlo et al., 2013). In contrast, base editing is a more precise and targeted gene-editing approach, making it a stronger candidate for treating disorders like Barth Syndrome, particularly when caused by point mutations in the TAZ gene. Since base editing can correct specific mutations without creating double-stranded breaks, it offers a safer and more reliable option for clinical use. While MAGE may hold promise for future applications, base editing is currently the more practical and effective tool for directly correcting the mutations responsible for Barth Syndrome, making it the better choice for therapeutic development (Jefferies, 2013).

CRISPR/Cas9 and Barth Syndrome

CRISPR/Cas9 has the potential to be a powerful tool for treating genetic diseases like Barth Syndrome by making precise genetic modifications, such as correcting point mutations in the TAZ gene. Its ability to target specific DNA sequences with high accuracy could make it an effective approach for directly fixing the mutations that cause Barth Syndrome, particularly if homology-directed repair is utilized to restore the faulty gene (Redman et al., 2016). CRISPR/Cas9 has already shown promise in other genetic disorders, such as DMD, where it was successfully used to delete a mutated exon, leading to restored dystrophin expression and improved muscle function (Tabebordbar et al., 2016).

CRISPR/Cas9 does come with challenges which includes risk of off-target effects and difficulties with efficient delivery, especially when using viral vectors like adeno-associated virus, which have limited payload capacity (Guo et al., 2023; Naso et al.,

2017). In comparison, base editing provides a more precise and controlled method for gene correction with a lower risk of unintended mutations, making it a safer and more reliable option for fixing single-point mutations in diseases like Barth Syndrome. While CRISPR/Cas9 has enormous potential, base editing currently appears to be the better choice for the precise treatment of Barth Syndrome.

Cpf1 (Cas12a) and Barth Syndrome

Cpf1 (Cas12a) brings several advantages over traditional CRISPR/Cas9 for gene editing, particularly its ability to create double-stranded breaks with 5 nucleotide overhangs enhancing homology-directed repair and allowing for a more precise genome modification (Zetsche et al., 2015). Another key benefit is its simpler crRNA system, which doesn't require tracrRNA or RNase III, making it easier to work with and reducing experimental complexity (Deltcheva et al., 2011). Combined with its ability to edit multiple genes at once and its lower risk of off-target effects, Cas12a stands out as a promising tool for correcting mutations in Barth Syndrome (Paul & Montoya, 2020). With that being said, Cas12a still does have its limitations. One concern is its tendency to degrade ssDNA, which could threaten genomic stability (Chen et al., 2018). Additionally, it depends on the host cell's DNA repair mechanisms, which are often less efficient in non-dividing cells which is something that could limit its effectiveness in certain therapeutic applications (Peters et al., 2017). Because of these challenges, base editing still offers a more precise and reliable approach for treating Barth Syndrome.

ZFNs and Barth Syndrome

ZFNs are a powerful gene editing tool that creates precise double-stranded breaks at specific genome locations, which are then repaired by either non-homologous end joining or homology-directed repair (Carroll, 2011). ZFNs have already shown promise in clinical settings where they were used to edit hematopoietic stem cells and reactivate fetal hemoglobin, demonstrating their therapeutic benefits (Lessard et al., 2024). This success highlights their potential for treating Barth Syndrome. ZFNs do come with challenges, particularly in designing highly functional ZFPs, which can be complex and sometimes inefficient. While advancements in selection-guided approaches have helped refine the technology (Davis & Stokoe, 2010), ZFNs are still more complicated and prone to errors compared to base editing. Base editing offers a more precise method for making single base pair changes with fewer off-target effects, making it a more reliable option for correcting the key mutations that cause Barth Syndrome.

TALENs and Barth Syndrome

TALENs allow for accurate genetic modifications by creating site-specific double-stranded breaks. Known for their high specificity, minimal off-target effects, and modular design, TALENs offer a promising alternative to CRISPR/Cas9 and ZFNs (Sun & Zhao, 2013; Mussolino et al., 2014). Their ability to generate targeted edits has made them a strong candidate for therapeutic applications (Qasim et al., 2017). When it comes to treating Barth Syndrome, TALENs may not be as efficient as base editing. Base editing is better suited for making precise single-base pair changes that often are necessary for correcting the mutations associated with Barth Syndrome. In contrast, TALENs are more effective for broader genetic modifications and may not offer the same

level of precision for point mutations. Additionally, designing TALENs for specific targets requires extensive protein engineering, which adds complexity to their use. This makes base editing a simpler and more practical option to treat Barth Syndrome.

Results and Conclusions

In this section, a summary of the analysis conducted on the original five hypotheses, evaluating the feasibility of each proposed gene editing technique for treating inherited cardiovascular diseases. The hypotheses explored the potential of CRISPR/Cas9 for FHCM, Cpf1 (Cas12a) for Danon Disease, MAGE for Pompe disease, ZFNs for ARVD/C, and base editing for Barth Syndrome. Through a comprehensive review of the efficacy, precision, and safety profiles of these techniques, whether the proposed techniques represent the most effective treatment options or if alternative strategies may offer superior outcomes. Below are the key findings and conclusions from this evaluation.

H1: CRISPR/Cas9 for FHCM

CRISPR/Cas9 stands out as the most viable gene editing tool for addressing FHCM. Its well-established clinical success and broad applicability in genetic modification make it a strong candidate for therapeutic use. The system's ability to create precise double-stranded breaks with customizable gRNAs allows for targeted gene correction or disruption. Additionally, its compatibility with both non-homologous end joining and homology-directed repair enables precise genome modifications necessary for treating FHCM. Another key advantage of Cas9 is its functionality in various cell

types, including dividing cardiomyocytes, further supporting its potential for FHCM treatment.

H2: Cpf1 (Cas12a) for Danon Disease

Cpf1 (Cas12a) emerges as the most promising genetic editing approach for treating Danon Disease because of its ability to introduce staggered double-stranded breaks, which enhance the efficiency of homology-directed repair (Zafra et al., 2018). Compared to CRISPR/Cas9, Cpf1 offers several advantages, including a simpler targeting mechanism, greater flexibility in recognizing PAM sequences, and a lower likelihood of off-target mutations. These characteristics make Cpf1 particularly suited for precise genetic modifications needed to restore proper gene function in Danon Disease.

H3: ZFNs for Pompe Disease

Among the gene-editing techniques evaluated, ZFNs show the most promise for addressing Pompe disease. ZFNs have been extensively studied for their ability to induce precise genetic modifications, with notable clinical success in conditions like sickle cell disease (Lessard et al., 2024). Their capacity to generate targeted double-stranded breaks and facilitate repair through homology-directed repair or non-homologous end joining makes them particularly effective for complex genomic alterations required for Pompe disease. In addition, studies have demonstrated ZFNs' potential in human disease models, highlighting their ability to facilitate gene replacement and mutation correction (Carroll, 2011; Davis & Stokoe, 2010). This strong track record positions ZFNs as the most suited candidate for treating Pompe disease.

H4: Base Editing for ARVD/C

Base editing presents itself as the most effective technique for treating ARVD/C, mostly due to its ability to correct point mutations with exceptional precision. Unlike methods that rely on inducing double-stranded breaks, base editing modifies single nucleotides directly, minimizing the risk of unintended genomic alterations (Rees & Liu, 2018). This makes base editing a safer and more reliable approach for therapeutic applications. Additionally, because base editing does not depend on the cell's DNA repair pathways, it is effective in both dividing and non-dividing cells, which is particularly relevant for treating ARVD/C (Yeh et al., 2018). The technique's accuracy in correcting single-nucleotide variants makes it the best option in addressing the specific mutations that contribute to ARVD/C.

H5: Base Editing for Barth Syndrome

For Barth Syndrome, base editing stands out as the most convincing gene-editing strategy. Since Barth Syndrome is often caused by point mutations in the *TAZ* gene, base editing's ability to precisely modify single base pairs makes it an ideal solution (Anzalone et al., 2020). Unlike other gene-editing approaches that may introduce insertions or deletions, base editing allows for targeted correction without disrupting the surrounding DNA, ultimately reducing the risk of off-target effects and maintaining genomic stability. Another key advantage of base editing is its efficiency and ease of design seen in other techniques such as TALENs, which require custom protein engineering for each new target, base editing offers a streamlined and scalable approach for addressing genetic mutations in Barth Syndrome.

Appendix.

Disease Profiles and Gene Editing Methods

Table 1. Clinical and molecular genetic analysis of FHCM

<i>Family member</i>	<i>Sex/age(years)</i>	<i>IVS, cm</i>	<i>LVOT, m/s</i>	<i>LVEF, %</i>	<i>Symptoms</i>
Patient					
4	M/51	3.84	1.4	66	Fatigue, palpitations, angina, dyspnea, swelling of limbs
7	M/44	3.82	1.0	60	Fatigue, palpitations, angina, dyspnea
9	F/25	1.62	1.4	60	Fatigue, dyspnea
13	M/37	3.85	1.0	66	Fatigue, dyspnea
22	M/14	4.19	4.05	68	Fatigue, dyspnea
Normal					
6	F/46	1.26	0.8	64	—
8	F/43	1.18	0.8	66	—
17	F/25	1.0	0.8	58	—
18	F/23	1.2	1.0	62	—
21	F/16	0.8	0.7	64	—
23	F/7	0.5	1.0	60	—
24	M/6	0.4	1.0	59	—

M, male; F, female; IVS, interventricular septum diameter; LVOT, left ventricle out tract; LVEF, left ventricle ejection fraction (Guo et al., 2014).

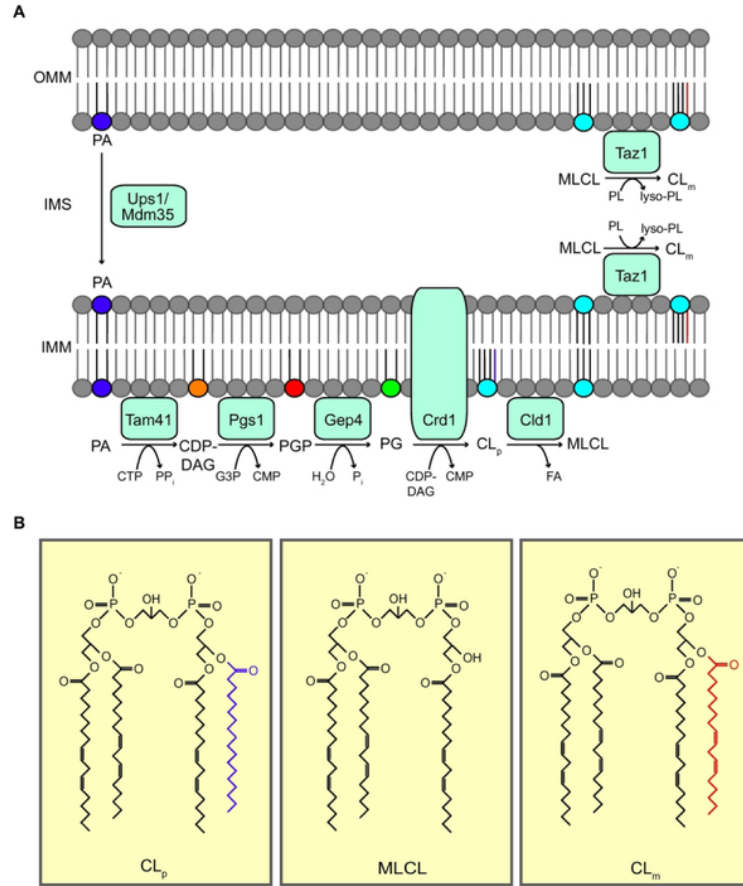


Figure 1. Overview of the Biosynthetic Pathway of CL

Figure 1a. Cardiolipin (CL) synthesis takes place exclusively within the mitochondria, beginning with the transfer of phosphatidic acid (PA) from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) through the action of the Ups1/Mdm35 complex. Sequential enzymatic reactions mediated by Tam41, Pgs1, and Gep4 transform PA into phosphatidylglycerol (PG). Crd1 then produces nascent CL (CL_p) from PG. This precursor CL undergoes remodeling into mature CL (CL_m) via deacylation and reacylation processes facilitated by Cld1 and Taz1. Adapted from (Ghosh et al., 2019).

Figure 2b. Depicted are the molecular structures of premature cardiolipin (CL_p), monolysocardiolipin (MLCL), and mature cardiolipin (CL_m). Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; CTP, cytidine triphosphate; PPi, pyrophosphate; CDP-DAG, cytidine diphosphate-diacylglycerol; CMP, cytidine monophosphate; PA, phosphatidic acid; PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate; G3P, glycerol-3-phosphate; PL, phospholipid; FA, fatty acid; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; IMS, intermembrane space. Adapted from (Ghosh et al., 2019).

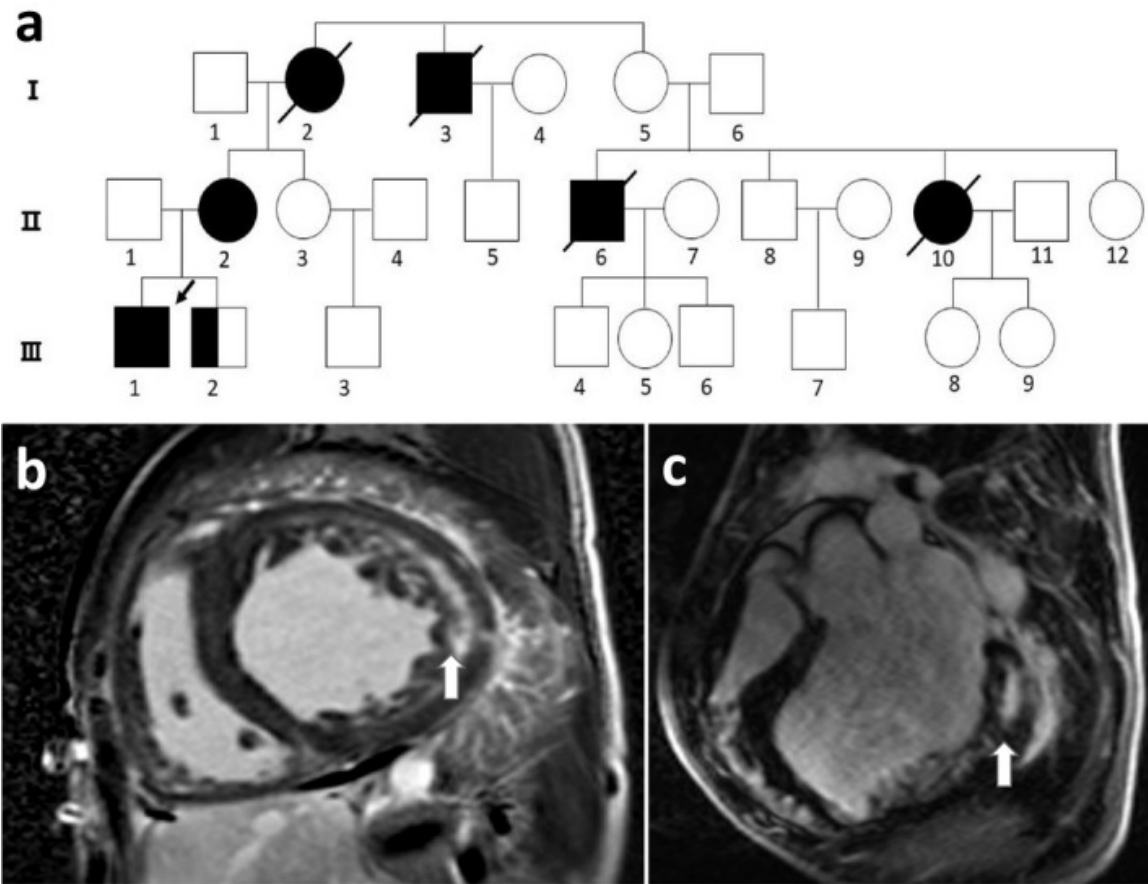


Figure 2. Pedigree of Family with Danon Disease and Cardiac MRI

Figure 2a. Pedigree of the family included in this report. Squares, male individuals; circles, female individuals; slashes, deceased individuals; filled black shapes, affected patients; arrow, proband.

Figure 2b, 2c. Cardiac MRI showing left and right ventricular wall hypertrophy, with reduced overall systolic activity, left atrioventricular enlargement, decreased myocardial T1 value, and delayed enhancement (Zhou et al., 2019).

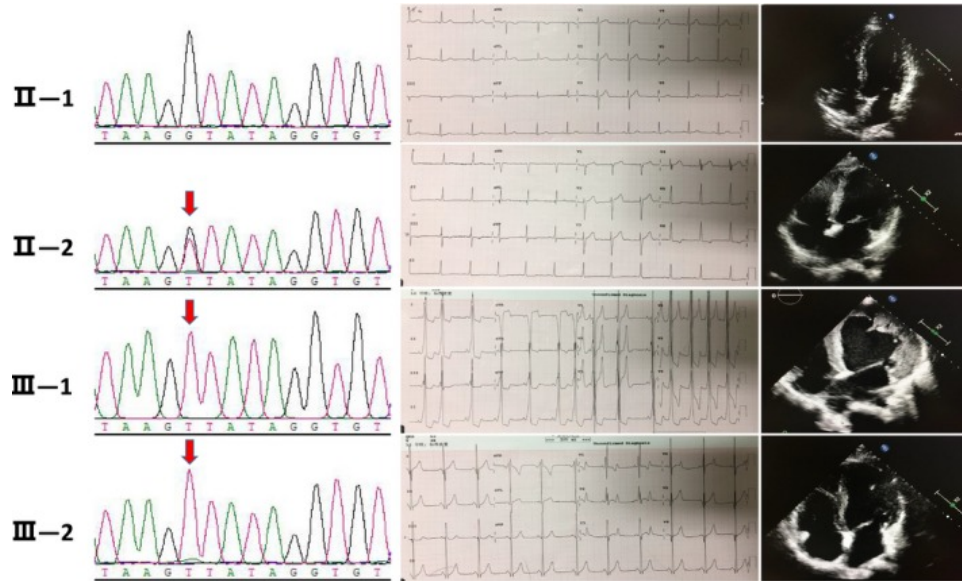


Figure 3. Sanger sequencing, Electrocardiogram, and Echocardiogram of family with Danon Disease

II-1: Father of affected, II-2: mother of affected, III-1: affected patient, III-2: brother of affected patient. Probands of II-2, III-1, and III-2 carry the mutation in LAMP2, c.741 + 1G>T. II-1 is not a carrier of mutation (Zhou et al., 2019).



Figure 4. Pedigree of Family with FHCM

I, II, and III are referring to the first, second, and third generations of the family. 22 is the proband analyzed while 4, 6, 7, 8, 9, 13, 17, 18, 21, 23, 24 are members screened for mutations (Guo et al., 2014).

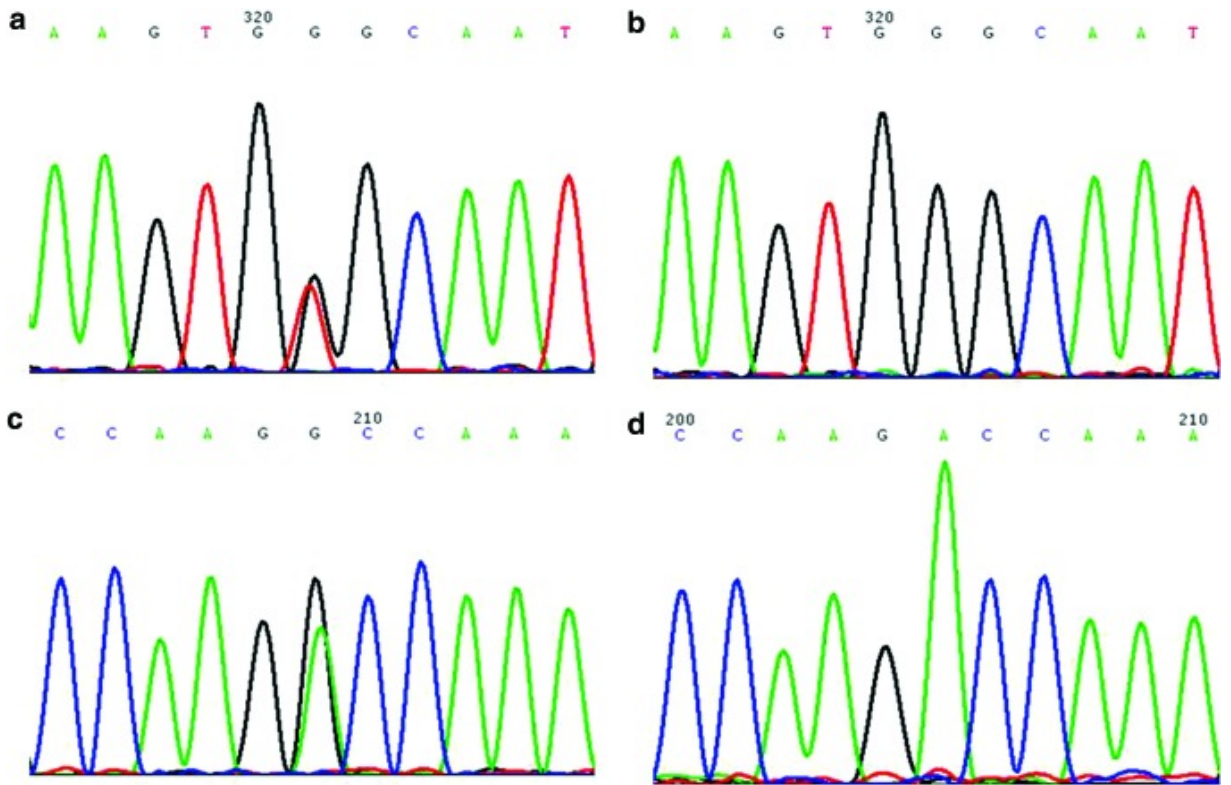


Figure 5. Exome analysis and mutation detection of Family with FHCM

Figure 5(a) Shows mutation of MYH7 p.G407C of the proband; **(b)** shows the wild type of MYH7. **(c)** Shows mutation of JPH2 p.A396T of the proband; **(d)** shows the wild type of JPH2 (Guo et al., 2014).

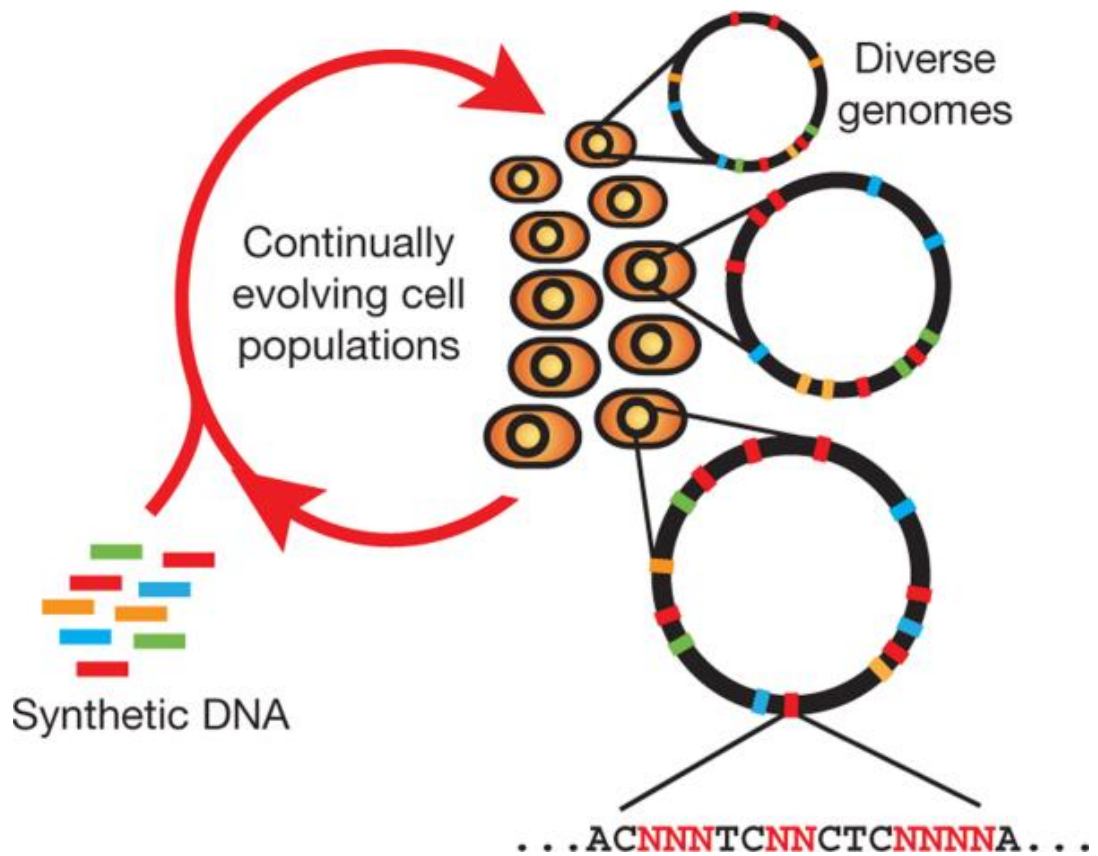


Figure 6. MAGE Overview

MAGE rapidly creates sequence diversity at multiple targeted chromosomal sites in a large cell population by repeatedly introducing synthetic DNA. Each cell carries a unique set of mutations, creating a diverse population. Degenerate oligo pools target specific genome sites, generating varied sequences at each location (Wang et al., 2009).

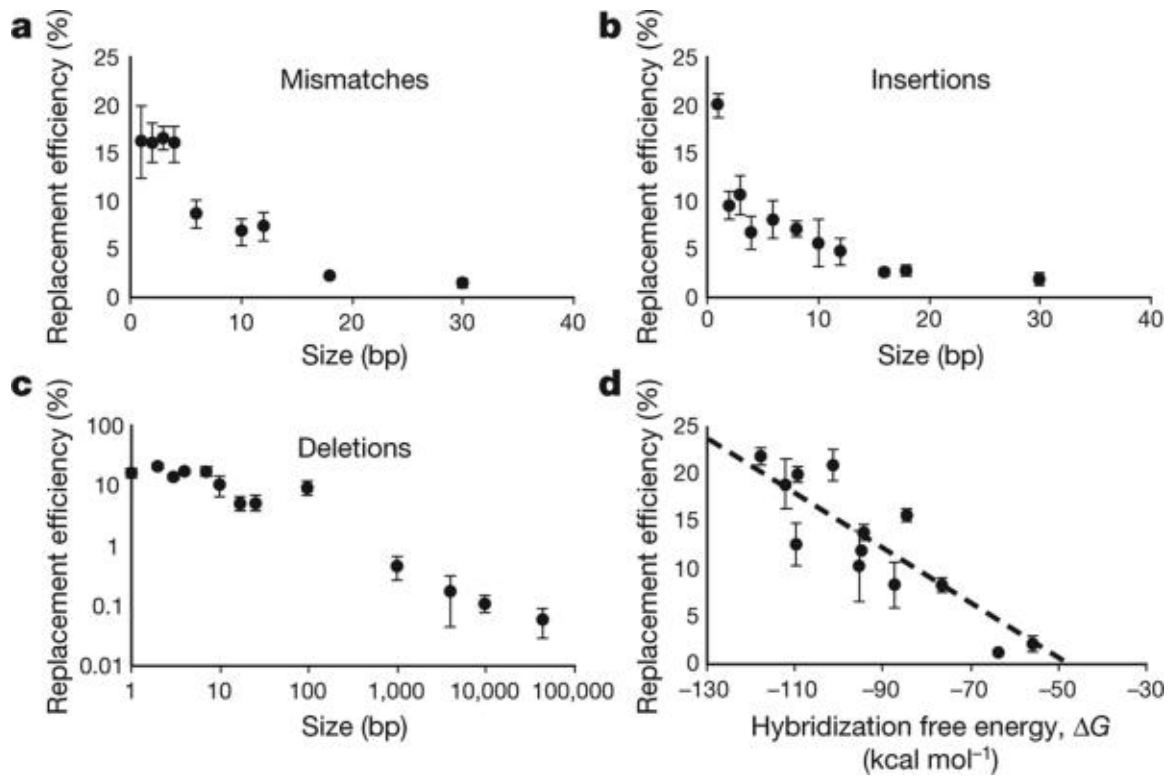


Figure 7. Evaluating allelic replacement efficiency by modification type and size

7a. Introduction of mismatch mutations of up to 30 bp. **7b.** Inserting exogenous sequences of up to 30 bp. **7c.** Removing up to 45 kbp of chromosomal sequence using a single oligo. **7d.** Correlation of replacement efficiency and two-state hybridization energy ΔG between the oligo and the targeted complement region in the genome. (Dashed line is the linear regression correlation ($y = -0.288x - 13.7$, $R^2 = 0.799$). All oligos used were 90 bp with two phosphorothioate bonds at the 3' and 5' ends. All error bars indicate \pm s.d.; $n = 3$.) (Wang et al., 2009).

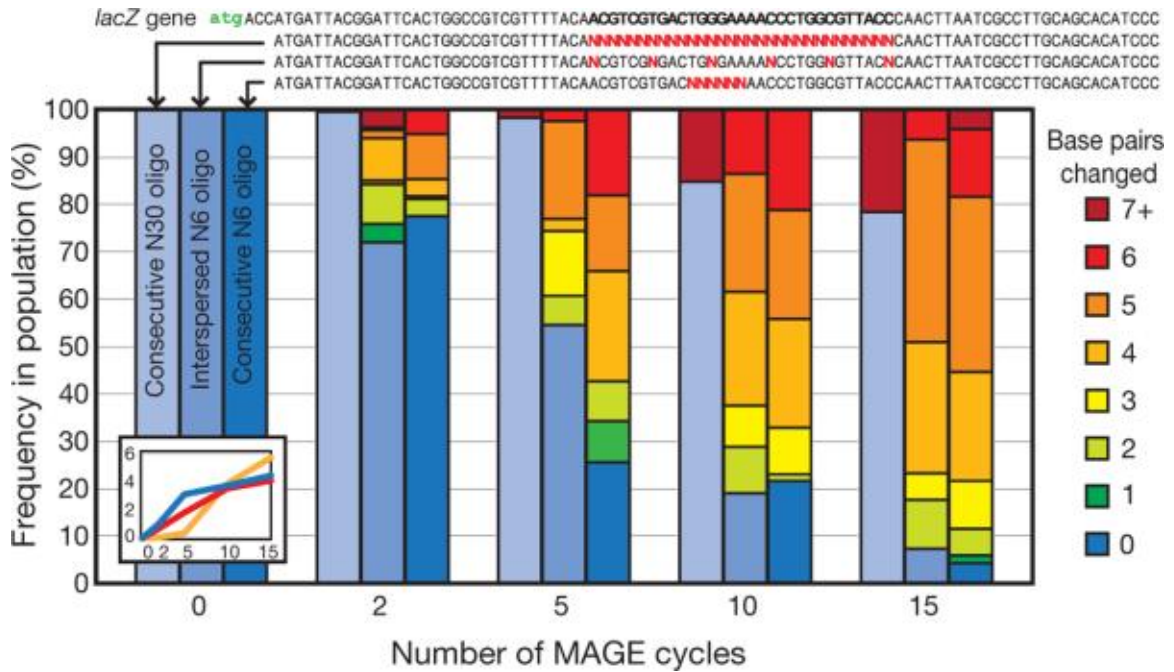


Figure 8. Sequence diversity developed in three cell populations as a function of the number of MAGE cycles

Three 90-mer oligo pools were tested: cN30 (30 consecutive degenerate bases), iN6 (6 degenerate bases spaced every 5 bases), and cN6 (6 consecutive degenerate bases). The frequency of strains with varying base differences from the wild-type lacZ sequence is color-coded. The inset shows the average number of base changes across the population after each MAGE cycle for the three pools: cN30 (orange), cN6 (blue), and iN6 (red) (Wang et al., 2009).

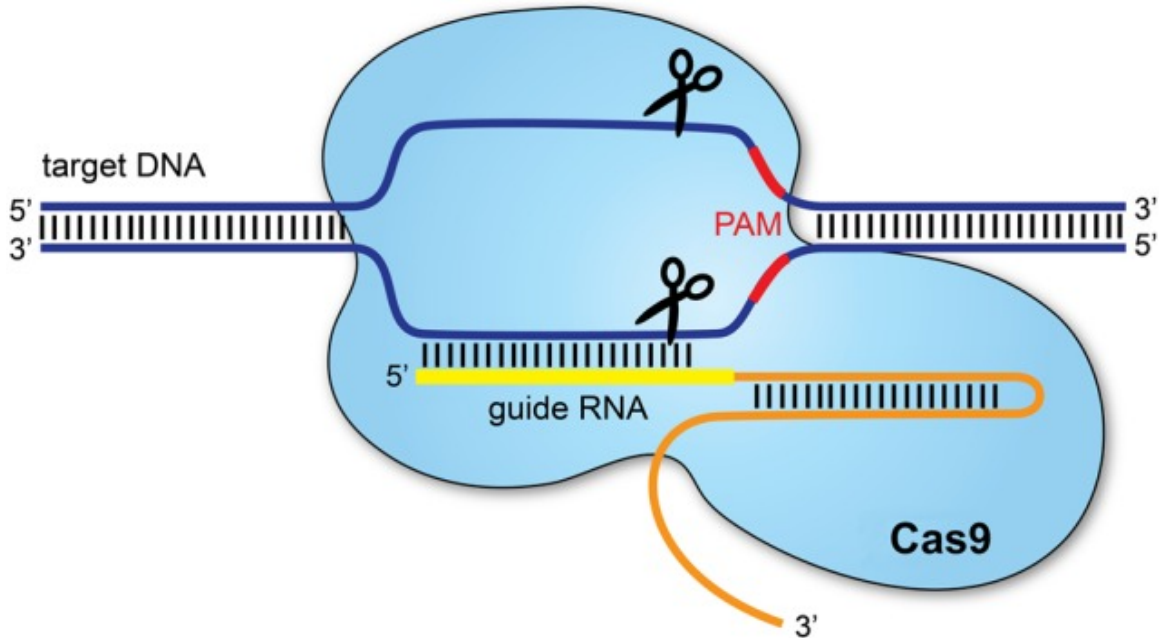


Figure 9. CRISPR/Cas9 System

CRISPR/Cas9 is a gene-editing tool where the Cas9 protein acts as molecular scissors, cutting both strands of target DNA. Cas9 is guided by a synthetic single guide RNA (sgRNA), which contains a sequence of 18–20 nucleotides complementary to the target DNA. For the cut to occur, a specific sequence called the protospacer adjacent motif (PAM) must be present near the target site. After the DNA cut, repair can occur through non-homologous end joining (causing random insertions or deletions) or homology-directed repair, which enables precise genome editing by using a provided DNA template to introduce specific changes (Redman et al., 2016).




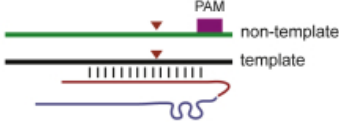
	Cas12a	Cas9
Size of protein	~1300 amino acids	~1000-1600 amino acids
RNA	 crRNA Single RNA molecule	 crRNA tracrRNA Two RNA molecules
Nuclease sites	Single nuclease site RuvC-Nuc	2 nuclease domains HNH and RuvC
Type of cut	 PAM non-template template Staggered ends	 PAM non-template template Blunt ends
PAM requirements	Recognises 5' T-rich PAM sequences of 3-4 nt	Recognises 3' G-rich PAM sequences of 3-5 nt
precrRNA processing	possesses intrinsic RNase activity to process precr-RNA	requires host RNase III and tracrRNA

Figure 10. CRISPR/Cas9 vs CRISPR/Cpf1 (Cas12a)

This chart identifies the differences and similarities between Cas9 and Cas12a (Paul & Montoya, 2020).

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