CRISPR Cas-9 as a Potential Cure for Wilson's Disease By Satoshi Toya

Abstract

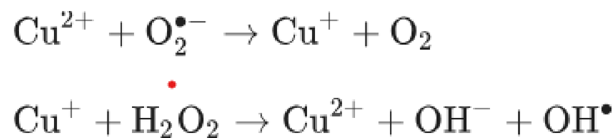
Wilson's Disease is an inherited copper metabolism disorder that is characterized by the pathological accumulation of copper throughout the body. It is caused by a mutation in the ATP7B gene – a gene that encodes a transmembrane copper-transporting protein – which inhibits the body's ability to transport copper properly. This results in the accumulation of copper in organs such as the brain and liver, which cause ophthalmological, hepatic, neurological, and psychiatric symptoms. The primary methods of treatment are chelators and zinc salts, which lower copper levels through different mechanisms; these treatments can become expensive because they need to be used continuously throughout the patient's lifetime.

However, CRISPR – an immune system used by various bacteria and archaea to eliminate invading viruses and plasmids – can be utilized as an effective gene editor, with many possible applications, including the treatment of chronic genetic diseases. The most prominent version of CRISPR used for this purpose is the CRISPR-Cas9 system. This paper aims to achieve multiple goals: to explain Wilson's Disease, mechanisms, and symptoms; to overview CRISPR and how CRISPR-Cas9 can modify DNA in genes; and to propose how CRISPR could be used to potentially permanently cure chronic genetic diseases, such as Wilson's Disease.

Introduction

Wilson's Disease is an inherited disorder of copper metabolism that causes abnormal copper accumulation in many organs, particularly the liver and brain, resulting in a wide range of symptoms[5, 121]. This copper overload can result in a myriad of negative consequences, including oxidative stress and protein toxicity, most notably in the mitochondria.

On a molecular level, Wilson's Disease arises due to oxidative stress, copper-mediated toxicity occurs when excess dissolved copper catalyzes the formation of hydroxyl radicals in the presence of peroxides via Fenton reactions[148].



$$Cu^{2+} + O_2^{\bullet -} \rightarrow Cu^{+} + O_2$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\bullet}$$

Fig 1: Fenton reaction

Hydroxyl Radicals are strong oxidants that can impede protein functionality by reacting with amino acids such as tyrosine and cysteine[2]. This substance not only directly interacts with DNA and RNA and causes oxidative DNA damage[58], but it can also interact with fatty acids, which in turn can create lipid peroxides and aldehydes that can react with DNA or amino acids to form adducts[48, 57, 82]. The adducts themselves are also detrimental to the patient's health as DNA adducts, for example, cause the DNA part to be damaged, which leads to its abnormal replication in the body and could cause mutations that potentially lead to conditions such as cancer[128].

Copper can directly target specific proteins in the body, most notably membrane proteins, by binding to thiol residues, resulting in the cross-linkage of these proteins[178]. A possible result of this phenomenon is the mitochondrial membrane permeability transition(MPT), which is caused by copper directly interacting with thiol groups in important mitochondrial proteins such as the adenine nucleotide translocase[42, 159]. Additionally, MPT can be present in organs such as the liver and brain, although the severity of MPT caused by copper varies from organ to organ[12].

Wilson's Disease Pathophysiology

Wilson's Disease is mainly caused by mutations affecting the ATP7B gene, which contains 20 introns and 21 exons, and is located on the long arm of chromosome 13 (Specifically region 13q14.3) with a genomic length of 80 kb[119, 185].

The protein is first synthesised in the endoplasmic reticulum, and then it is relocated to the Trans-Golgi-Network (TGN) within hepatocytes. The ATP7B gene itself is most highly expressed in the liver, but it is also found in other organs such as the kidneys, brain, and lungs[180].

The Genetics of Wilson's Disease

The ATP7B protein itself consists of 1465 amino acids (165 KDa) which are organized into many different parts of the protein: the phosphatase domain (A-Domain), which is identifiable by the amino acid motif TGEA alongside TGDN and GDGVND; the phosphorylation domain (P-domain), identified by the amino acid motif DKTGT; the M domain, which consists of 8 transmembrane ion channels; the nucleotide binding domain (N-Domain), identified by the amino acid motif SEHPL; and the N-Terminal metal binding domain (MBD), which contains 6 copper binding sites with the amino acid motif GMXCXXC and plays a central role in accepting copper from the copper chaperone ATOX1[20, 33, 87, 138].

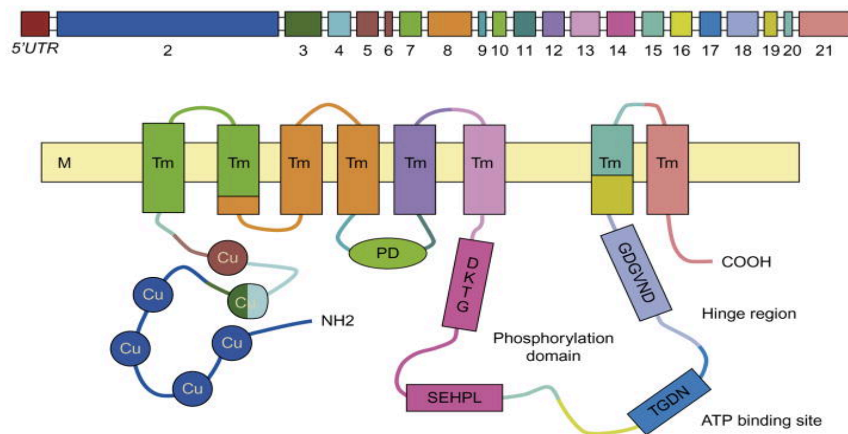


Fig 2: The general structure of the ATP7B protein
(The genetics of Wilson disease - PMC)

ATP7B is responsible for encoding a copper cation-transporting P-type ATPase that facilitates the transportation of copper between cells. These proteins are upregulated in liver

hepatocytes, and the proteins are mainly found in the Golgi Apparatus[36, 157]. This membrane-bound transporting protein facilitates the transport of copper from the cytoplasm to the Trans-Golgi Network, where copper binds to the protein apoceruloplasmin, forming ceruloplasmin, a copper transport protein. Ceruloplasmin then enters the bloodstream to distribute copper to the rest of the organs for their necessary function[36, 91, 98, 139].

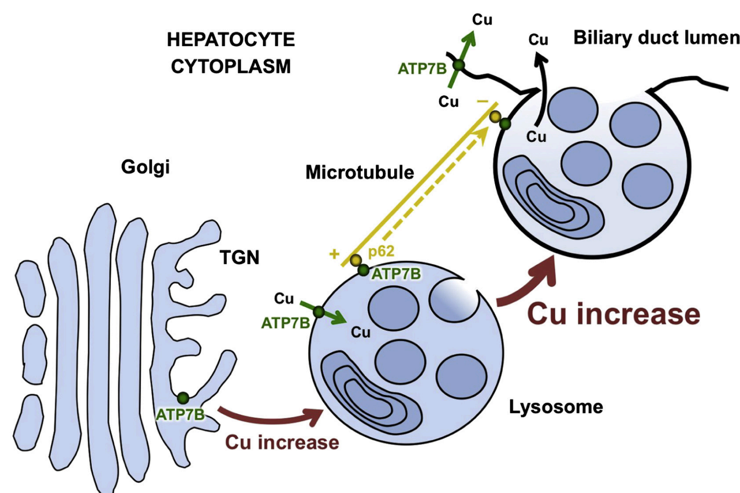


Fig 3: The general arrangement of ATP7B within the hepatocytes and its role in transporting copper

Focusing on the protein mechanism in greater detail, the active transport of copper across cell membranes begins with ATP7B binding copper at the MBD domain and transporting it across cellular membranes, driven by the hydrolysis of adenosine triphosphate (ATP). Then, the free copper binds intracellularly to GG motifs in the MBDs, followed by transport to the Cys-Pro-Cys (CPC) sequence motifs in MBD 6. Finally, the dephosphorylation of acyl-phosphate at the A-domain discharges copper across the cellular membrane[180].

Copper is necessary for bodily function in many ways, notably acting as a cofactor for many critical cuproenzymes essential for human metabolism[156].

An example of this is in Cytochrome-c oxidase, located in the inner mitochondrial membrane, which has two copper sites in the form of CuA and CuB[37]. Cytochrome-c oxidase acts as the terminal oxidase of the mitochondrial respiratory chain in most aerobic organisms, transporting electrons from donors to a final acceptor to create ATP, simultaneously reducing molecular oxygen (O2) into water (H2O)[37].

Another example is human SOD1, a zinc-copper metalloenzyme in all cells that regulates the body's antioxidant response[41, 107]. Functionally, SOD1 catalyzes the disproportionation of superoxide anions (O2−) into molecular oxygen and hydrogen peroxide, which are further eliminated by catalase, glutathione peroxidases, and peroxiredoxins[41]. This biochemical process is important because without this type of regulation, the superoxide can react to produce more dangerous Hydroxyl radicals (•OH) that can cause further cellular damage[100, 107]. Additionally,

copper is necessary for SOD1 to catalyze the disproportionation, as reduction of superoxide occurs at the copper-zinc metal center [4, 26, 171].

Another example of a necessary copper-containing protein is ceruloplasmin, which serves as both a copper transport protein and a ferroxidase with the ability to oxidize Fe(II) to Fe(III). Oxidation of the iron substrate allows iron to bind more strongly to transferrin, which is responsible for iron transport and distribution in the body[38, 118, 156]. Ceruloplasmin also removes excess copper in hepatocytes through vesicles, where ceruloplasmin is excreted through the apical membrane to create bile[98, 139].

However, when the ATP7B gene is mutated, it causes a mutation in the copper-transporting P-type ATPase that leaves the enzyme unable to function properly[108]. As a result, this condition leads to both the impaired incorporation of copper into ceruloplasmin[33] and the excretion of copper into bile. Copper accumulates in the liver over time, in some cases increasing copper concentrations from 5 - 20 times normal copper levels in the body[27]. When the liver reaches its copper-carrying capacity, excess copper is released into the bloodstream and travels back to other organs, resulting in an array of problems[161].

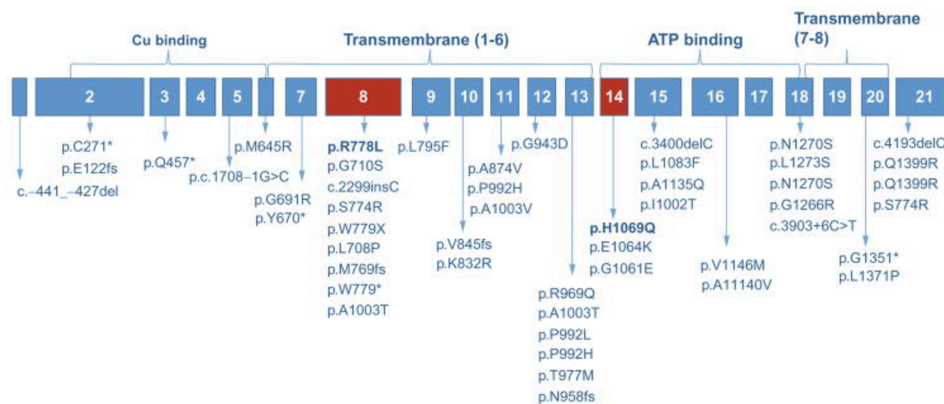


Fig 4: A schematic of the *ATP7B* gene with common mutation sites
([The genetics of Wilson disease - PMC])

One of the most common mutations that leads to the disease is the missense mutation H1069Q in exon 14. It's common in Central, Eastern, and Northern Europe, where approximately 50–80% of Wilson's Disease patients carry at least one H1069Q allele inherited from their parents[36]. In this mutation, the histidine of the conserved SEHPL motif in the N-terminal domain of the *ATP7B* protein is replaced with glutamic acid, which results in N-domain protein misfolding, abnormal phosphorylation in the P-domain, and decreased ATP binding affinity[132]. This mutation can also lead to decreased thermostability and the abnormal localization of the protein to the Trans-Golgi-Network, further disrupting transport processes[129].

Symptoms

There are multiple symptoms related to Wilson's Disease.

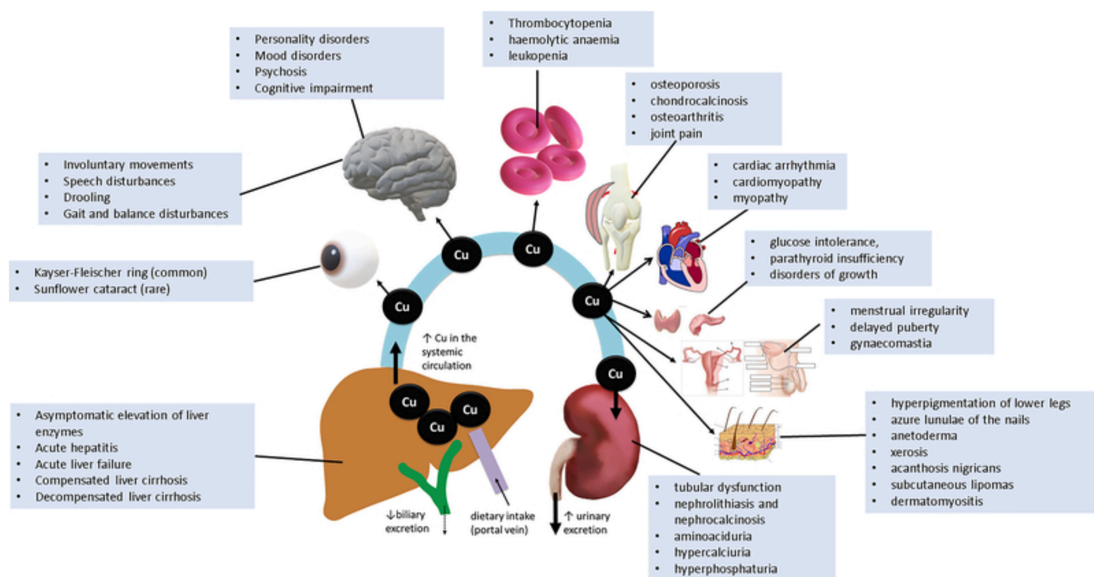


Fig 5: A listing of the organs generally affected by the excess copper caused by wilsons disease. (Nature Reviews Disease Primers article: Wilson disease - PMC)

Hepatic Symptoms

Early symptoms of Wilson's Disease usually manifest in the liver, due to excess copper buildup in hepatocytes[5, 168]. The severity of excess copper's effect on the liver can vary due to the lack of an exact genotype-phenotype correlation to Wilson's Disease. This can result in relatively small manifestations, such as a self-limiting Hepatitis-like inflammation, and in more severe cases, cirrhosis, or the scarring of the liver, and even acute liver failure[168]. Furthermore, cirrhosis leads to jaundice, which can serve as a visual symptom in patients with liver damage. Without treatment, chronic liver disease worsens alongside the appearance of conditions such as portal hypertension, hepatosplenomegaly, ascites, low serum albumin concentration and coagulopathy[27].

The severity of these hepatic diseases that can cause cirrhosis is usually reflected by the Model for End-Stage Liver Disease(MELD)[69]alongside Child-Pugh scores[127]. And normally, these are determined by using abdominal ultrasound (US), CT, and MRI at diagnosis. The most common findings through these methods are events such as fatty infiltration, contour irregularity, and atrophy of the right lobe of the liver[27]. For patients with chronic liver disease, especially those with cirrhosis, screening examinations for signs of portal hypertension and hepatobiliary malignancies[120] should be performed regularly[10] and repeated depending on clinical status.

Neurological Symptoms

Neurological symptoms are the second most frequent behind hepatic symptoms and, on average, will onset around the age range of 20-30 (though outliers do exist)[32, 121]. The most predominant manifestations are tremor, dystonia, and Parkinsonism–all of which are normally associated with dysarthria, gait and posture disturbances, drooling, and dysphagia[93, 96, 168]. One of the most characteristic neurological symptoms is tremors (shaking or trembling movements),

which is a symptom described by about 55% of patients at diagnosis. Tremors can be either resting, postural, or kinetic, and movement is either unilateral or bilateral and occurs primarily in the arms. Over time, tremors can spread to the legs, head, and throughout the entire body[27].

Dystonia, or involuntary muscle contracting, is reported as one of the first Wilson's Disease symptoms in 11–65% of patients[27]. It can involve a single body part, one segment, multiple segments like a face and a leg, or even be generalized throughout the entire body. The most characteristic representation of dystonia is an abnormal facial expression, or risus sardonicus, which consists of a fixed smile due to dystonia of the risorius muscle.

Ophthalmological Symptoms

Ophthalmological symptoms, predominantly Kayser-Fleischer Rings and sunflower cataracts[28, 169], both of which are caused by pathological copper accumulation in the eyes.

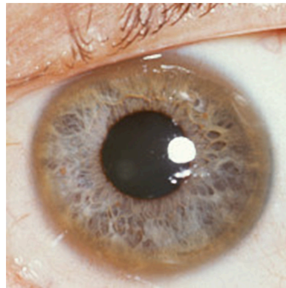


Fig 6: Kayser-Flescher Ring viewed on the outside)
(Kayser-Fleischer rings | Radiology Reference Article | Radiopaedia.org)

Kayser-Fleischer Rings (KFRs) are ring-shaped copper deposits within the eyes that develop when copper is deposited throughout the cornea; a sulfur-copper complex—found only in the Descemet's membrane—then produces visible ring-shaped copper deposits in that area[121]. Specifically, the deposit is formed in the anterior chamber angle within the internal corneal layer of the Descemet's membrane, at the Schwalbe's line[15, 74].

Normally, Kayser-Fleischer rings are present in almost all people with Wilson's Disease who have developed neurological or psychiatric symptoms. However, there are exceptions, with some patients not having KFRs despite experiencing neurological symptoms[134].

And generally, the presence of these copper deposits can alter the corneal subbasal nerve plexus(SBNP)–a monolayer of fine, amyelinated nerve fibers in the cornea. One clinical study demonstrated that Wilson's Disease patients can experience corneal neuropathy as a result of this monolayer accumulation. This was determined by an evaluation of Wilson's Disease patients' eyes using corneal confocal microscopy: factors like the number of nerve fibers, nerve fiber length density, number of nerve branchings, and number of beadings were all significantly lower when compared to those unaffected by Wilson's Disease. Additionally, in Wilson's Disease patients, the fiber tortuosity was higher compared to unaffected people, indicating that there was

a lot of nerve regeneration, an indication that nerve fibers in the eyes had died off as a result of neuropathy[68, 149].

Kayser-Flescher Rings become visible in the superior aspect of the cornea, followed by the inferior aspect, with subsequent filling in of the medial and lateral aspects. As a result, one would have to expose the entire cornea by lifting the eyelid to properly look for KFRs[150].

Furthermore, KFRs are detected and diagnosed using methods such as slit lamp examination(SLE), which makes KFRs appear as a golden-brown, golden-green, green-yellow, golden-yellow, bronze or reddish-brown coloring ring in the limbic area of the cornea[34] .

Sunflower cataracts are characterized by copper deposition in the lens capsule, distinct from the lens cortex or nucleus, forming a central disk with radiating petal-like spokes that give it its name[125]. Additionally, Sunflower cataracts seem to be the result of the accumulation of various compounds in the third posterior of the lens' anterior capsule, which includes copper, sulfur, and binding-copper proteins[181].

Sunflower cataracts typically do not impair vision and are usually detected through slit lamp examination, as they are not visible to the naked eye or an ophthalmoscope[96].

Psychiatric Symptoms

Psychiatric Symptoms appear frequently in Wilson's Disease patients[32]. A 2014 study suggested that psychiatric symptoms can occur before, in tandem with, or after the diagnosis and treatment of Wilson's Disease[177]. It can appear in younger patients as declining school performance, impulsiveness, and inappropriate behavior[142, 152]; It can often appear in older patients as paranoia, schizophrenia, or depression-but it can also commonly appear as behavioral changes[32].

The most common psychiatric symptom of Wilson's Disease is mood disturbances, with about 20–60% of Wilson's Disease patients developing depression, with a high rate of suicidal attempts ranging between 4–16% of WD patients[146, 151, 152, 177]. Adding on to this, behaviors such as emotional lability, irritability and aggression, shallow cheerfulness, euphoria, social disinhibition, hypersexuality, lack of criticism, and deficits in planning and anticipating social consequences can be due to the lesions of the frontal lobe or its pathways[19].

Additionally, Behavioural and personality disorders are also a common psychiatric aspect of Wilson's Disease, with the most common manifestations being irritability, aggression, and antisocial behaviour[122]. Other psychiatric conditions, such as catatonia, anorexia nervosa, bulimia, obsessive-compulsive disorder, and attention-deficit hyperactivity disorder, have also been reported in Wilson's Disease patients[53, 83].

Additional Symptoms

There are still more different manifestations of Wilson's Disease. However, the effect of Wilson's Disease on other parts of the human body has been less investigated compared to organs such as the liver and brain[27].

One manifestation is the negative effects that the excess copper from Wilson's Disease can cause to the skeletal system, which includes osteomalacia(softening of bones) and osteoporosis(weakening of bones), with increased incidence of spontaneous fractures being observed[165]. Additionally, the copper can negatively affect the joints, particularly larger ones, with its accumulation in the synovial membrane and cartilage being suggested as the major cause of osteoarthritis(erosion of cartilage) and accelerated degenerative changes with deformities[109].

Another type of manifestation is cardiovascular symptoms, with myocardial copper accumulation being able to cause cardiomyopathy(decrease of heart muscle function) and arrhythmias(irregular heartbeat)[27]. Additionally, pathological cardiac examination done on Wilson's Disease patients has shown symptoms such as interstitial and replacement fibrosis, intramyocardial small vessel sclerosis, and focal inflammatory cell infiltration[16].

Some manifestations can affect the red blood cells in Wilson's Disease patients with the excess copper in the bloodstream being able to cause symptoms such as Coombs-negative haemolytic anaemia; this alongside other symptoms may be caused through the reaction of copper with membrane lipids, or copper inhibiting sulfhydryl groups of glucose-6-phosphate dehydrogenase and glutathione reductase which all can reduce cellular antioxidant capacity which leads to oxidative damage of the haemoglobin and cell membrane in red blood cells[40, 160].

Last but not least, there are also renal manifestations that are caused by the renal tubular epithelium filtrating the non-ceruloplasmin-bound copper in the blood that can cause excess copper buildup in the renal parenchyma, leading to renal tubular dysfunction[176].

Wilson's Disease Incidence / Prevalence

Initially, it was believed that the universal prevalence of Wilson's Disease ,except for a select few small isolated populations, was 1:30,000, based on a study done by Scheinberg and Sternlieb in 1984[136].

More recent data from the U.S, Europe, and Asia suggest a prevalence range of about 1:30,000-1:50,000, clarifying the 1984 study results[136]. Prevalence is relatively greater in Pakistan and India, due to factors like genetic consanguinity.

Wilson's Disease Treatment Available

Once a patient has been definitively diagnosed with Wilson's Disease, treatment is provided for a patient's entire lifetime[35, 131].

Chelating Agents

One type of treatment for Wilson's Disease is pharmaceutical treatment that can induce cupriuresis, or the urinary excretion of copper. This is done primarily with copper chelating agents, such as D-penicillamine and Trientine, or using zinc salts that decrease the absorption of copper in the digestive tract.

D-Penicillamine functions as a chelator that binds divalent metal ions like copper with its sulfhydryl functional group (−SH) to form a water-soluble complex that is excreted out of the body through urine[47].

Trientine is another copper chelating agent and can act as an alternative to D-penicillamine, particularly for those who are unable to handle D-penicillamine. Trientine binds to copper in a 1:1 ratio to form a strong, stable complex that is excreted in the urine.

D-penicillamine and trientine result in similar patient responses; However, trientine was more likely to exacerbate the neurological effects of Wilson's Disease compared to D-penicillamine[47]. Conversely, D-penicillamine treatment is more likely to be discontinued due to side effects when compared with trientine[166].

When considering overall performance, trientine and D-penicillamine are not inferior to one another, as evidenced by a recent randomized open-label non-inferiority trial[141].

However, these types of treatments can be expensive. An example of this is the price for the 2 drugs around 2015, where trientine(Syprine) was $200 per 250-mg capsule and D-penicillamine(Cuprimine) costing $55-$60 per 250-mg capsule[182]. Around 2022, the annual cost for treatment was about $78,000 for trientine, and from $50,000 to $200,000 for penicillamin[3].

Zinc Supplements

There are also Zinc supplements, which can have multiple versions such as zinc acetate, zinc gluconate, and zinc sulfate. However, there is no statistically significant difference between these variants in terms of the improvement of liver function[18]. Zinc preparations work by inducing the synthesis/creation of metallothioneins while promoting the binding of copper to these metallothioneins in the enterocyte of the small and large intestines. The copper-containing enterocyte is then disposed of through it shedding naturally into the gut lumen, then it accompanies the rest of the waste out of the body. This overall process hinders the body's absorption of copper, and prevents the further exacerbation of Wilson's Disease from increasing[47]. Its effects are generally slow as a de-copperating agent, with its effects happening over time rather than manifesting as a sudden, noticeable event. As a result, it's not suitable for treating more florid symptoms such as cirrhosis[140]. Wilson's Disease still shows progression in the first few months of treatment[14]. When compared to the other types of treatment for Wilson's Disease, this is the cheapest one out of all of them, with the annual cost from 2022, with $100 worth for nutriment types, and from $550 to $3650 for pharmaceutical-grade Zinc[3].

Liver Transplant

As a more complex and surgical option, liver transplantation also counts as a treatment for Wilson's Disease[1, 30, 167]. This approach is based on the concept that Wilson's Disease results from gene defects, such as those in ATP7B, which impair the liver's ability to manage excess copper. Consequently, a liver transplant can be viewed as a phenotypical correction of the gene, thereby restoring copper homeostasis. Normally, liver transplantation in Wilson's Disease

patients is suggested in cases with acute liver failure or decompensated liver cirrhosis with a lack of pharmacological treatment effect. The need for a liver transplant in cases of acute liver failure should be based on a specific WD clinical scale, such as the revised King's prognostic Wilson Index[30]. On another note, the idea of whether uncontrolled neurological disease constitutes a need for liver transplantation as the "last chance" treatment option is highly controversial[1, 30, 167]. The problems with this treatment, however, are that it can give rise to a myriad of long-term medical complications in the patient as a result of the transplant. This can include various conditions: cardiovascular complications that account for 20% of the deaths of the patients surviving at least 3 years from a liver transplant[126]; Kidney disease where up to 80% of patients 5 years after a liver transplant will develop chronic kidney disease[50]; Hyperuricemia which has been reported in 14–47% of post Liver transplant patients[116], and may be related to deteriorating renal function due to acid uric having a high renal excretion[95, 113]; Bone complications where 65% of Liver transplant recipients were affected by Atraumatic bone fractures because of cholestatic disease[9]; and many other possible side effects.

Additionally, liver transplants themselves can cost a lot of money, with the operation itself costing about $600,000, not including the mandatory preoperative evaluations, which can cost from $168,000 to $308,000, and the postoperative medications that cost from $671,000 to $949,000 in the 10 years that follow the procedure[55].

When considering overall treatment, the average healthcare cost for a Wilson's Disease patient, based on a 2021 study on the cost burden for Wilson's Disease patients, was about $3,887 per month after a Wilson's Disease diagnosis[135]. And when looking at a larger scale, the total cost for Wilson's Disease hospitalizations was $20.90, $27.23, $24.20, and $27.25 million US dollars for the years 2016, 2017, 2018, and 2019, respectively[86].

CRISPR History

CRISPR (or clustered regularly-interspaced short palindromic repeats) describes a family of DNA sequences found in bacteria and archaea that facilitate an adaptive immunity against mobile and exogenous genetic elements such as viruses. Since their characterization by Emmanuelle Charpentier and Jennifer Doudna, CRISPR systems are now utilized as a precise gene-editing tool that has revolutionized the field of molecular biology[6, 23].

CRISPRs were first detected in the E. coli bacteria's genome in 1987, where they were characterised as unusual insertion elements consisting of a series of 29 nucleotide repeats separated by 32 nucleotide "spacer" sequences, all of which had appeared whenever bacteria came into contact with invading phage DNA[60] . At the time, scientists were unable to determine the function of these sequences due to the lack of sufficient DNA sequence data, especially for mobile genetic elements[61]. In 1993, CRISPRs were first observed in the archaea *Haloferax mediterranei*[111], and they were subsequently detected in an increasing number of bacterial and archaeal genomes as gene sequencing became cheaper and more widely available in the life sciences. All of these example sequences were conserved, which helped to further the understanding of their function and importance.

Research related to CRISPR came to a head in the early 2000s when two parallel breakthroughs greatly accelerated genetic research efforts.

One breakthrough was an increased understanding of the function of CRISPR as an immune defense after the discovery of the sequence similarities between the spacer regions of CRISPRs and the sequences of bacteriophages, archaeal viruses, and plasmids were reported on by three different research groups[11, 112, 124].

The other major discovery was that several genes previously thought to encode DNA repair proteins for hyperthermophilic archaea were actually strictly associated with CRISPR and were designated as Cas (CRISPR-associated) genes as a result[62, 102]. Comparative genomic analysis had suggested that CRISPR and the Cas proteins both work together to constitute an immune system to protect prokaryotic cells against invading viruses and plasmids (DNA-based threats), comparable to the eukaryotic RNA interference (RNAi) system[103].

A 2007 experiment proved this hypothesis using the lactic acid bacteria *Streptococcus thermophilus,* where the molecular mechanism of the adaptive immune response to a phage infection was explained[6].
Additionally, it was demonstrated that CRISPR sequences are transcribed into RNA that is then cleaved and loaded into CRISPR–Cas proteins, resulting in an RNA–protein complex sufficient for RNA-guided double-stranded DNA endonuclease activity[44, 66].

CRISPR Mechanism

Specifically, CRISPR consists of two major parts. First, a CRISPR system must contain a genome with an entire CRISPR locus that consists of an array of short direct repeats interspersed with spacer sequences where the spacers and repeats are both uniform in length, but the repeats are identical in sequence content, and the spacers are highly variable in sequence content[73].
Second, it also consists of Cas enzyme-producing genes organized in operon(s) to allow transcription by the cell[7, 158, 170].

Regarding the CRISPR locus in different species of bacteria and archaea, the repeats are about 32 base pairs (bp) long on average, and the spacers are generally of a similar size in the range of 20-72bp[49, 52, 110]. Related species may have similar repeat sequences, but there is great diversity in the sequences of both spacers and repeats in bacteria and archaea[84].

In the original immune defense system, the CRISPR–Cas mechanism of action consists of 3 primary stages: adaptation, expression, and interference. In the adaptation step, a complex of Cas proteins encounters a protospacer-adjacent motif (PAM) and causes 2 double-strand breaks in the PAM's invading DNA molecule. The resulting fragment of foreign DNA (termed protospacer, is the target) is then integrated into the CRISPR locus between 2 repeats of the CRISPR array and becomes a spacer around the proximal end of the CRISPR array[31].

In the expression stage, the Cas gene's expression, which produces Cas proteins, and the transcription of the repeat-spacer element from the protospacer, which produces a precursor CRISPR RNA (pre-crRNA), in which proteins and accessory factors process the pre-crRNA into short mature crRNA, all occurs[31].

Finally, in the interference step, the crRNA and Cas proteins work together to recognize and mediate the cleavage of the foreign DNA to protect the host cells from the infection[6, 103].

Classification of CRISPR/Cas9 Systems

CRISPR/Cas systems are divided into 2 main classes, 6 main types, and 33 subtypes. Class 1 contains multiprotein effector complexes, while class 2 is defined by a single, multidomain, multifunctional effector protein[67].

All types are distinguished by the different structures of the effector modules, which contain unique signature Cas proteins. Additionally, each type is also classified into numerous subtypes that are characterized by the subtle differences in CRISPR locus organization and encode subtype-specific Cas proteins[56, 81, 145].

Overall, the primary features that define the type and subtype of the CRISPR/Cas systems are the Cas genes and the proteins they encode, which functionally and genetically differ across the different variants, as shown in the various steps of CRISPR-mediated immunity.

For example, the type I and III systems(both in Class 1) both produce specialized Cas endonucleases, which process the pre-crRNAs, which mature into crRNA and assemble into a large multi-Cas protein complex capable of recognizing and cleaving nucleic acids complementary to the crRNA[31].

Type II(which is Class 2) systems, by contrast, process pre-crRNAs with a different mechanism where a trans-activating crRNA (tracrRNA) complementary to the repeat sequences in pre-crRNA triggers pre-crRNA maturation in the presence of the double-stranded RNA-specific ribonuclease RNase III and the Cas9 protein[29, 51]. Additionally, Cas9 is thought to be the sole protein responsible for the crRNA-guided silencing of foreign DNA[6, 43, 137].

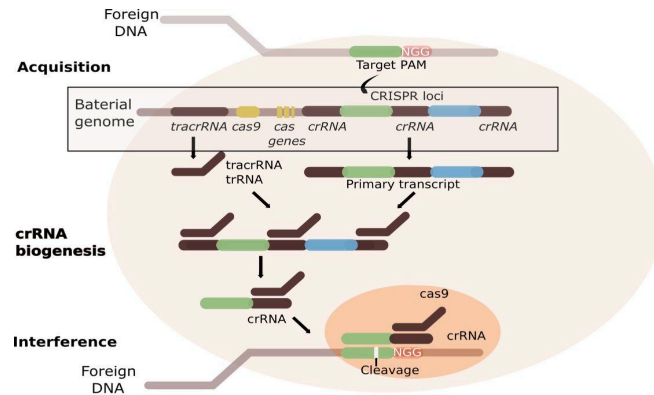
The type II CRISPR-Cas, specifically, with the Cas9 protein, is an overall easier, cheaper, and more efficient gene editing tool compared to the pre-existing editing technology, such as ZFNs and TALENs[31].

Mechanism for CRISPR-Cas 9

The CRISPR-Cas9 system-one of the most prominent and widely used gene editing tools-has the same three main stages of a CRISPR-Cas system: adaptation, expression, and interference.

Adaptation

For the adaptation stage, a complex of Cas proteins may encounter a PAM in the foreign/invading DNA, where the complex will induce 2 double strand breaks close to it; The resulting fragment of foreign DNA (Termed protospacer, is the target) is then integrated into the CRISPR locus between 2 repeats and becomes a spacer around the proximal end of the CRISPR array[31, 158, 170].



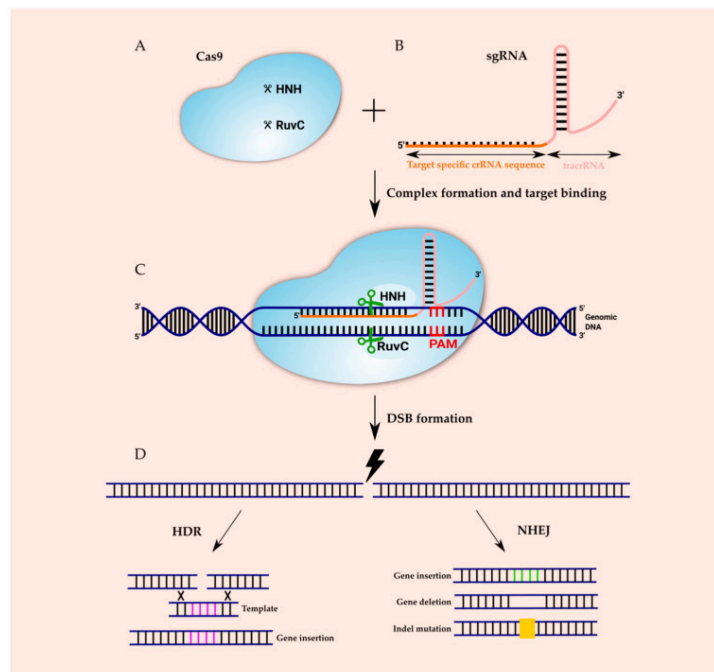


Fig 7-8: The process of CRISPR-Cas9 DNA editing

Expression

For the expression stage, two main events occur in the genome.

One event that occurs is the expression of the Cas9 gene that results in the Cas9 enzyme, a DNA endonuclease that consists of two nuclease domains–the RuvC domain, which cleaves non-complementary DNA strands, and the HNH domain, which cleaves complementary DNA strands–that are responsible for inducing a double-strand break in the target DNA sequence[31].

The other event—which occurs within the CRISPR Locus—is the transcription of the repeat-spacer element derived from the protospacer, which yields pre-crRNA (**pre**cursor-**CRI**SPR **R**NA). Additionally, transactivating crRNAs (tracrRNA) are also produced from another section in the CRISPR locus.

The tracrRNA anneals with the pre-crRNA and directs the maturation of the pre-crRNA into mature crRNA in the presence of the endoribonuclease RNase III and Cas9(also called Csn1) protein[17, 29, 64]. The resulting crRNA is also reduced to a length of 20 nucleotides(nt) through trimming of the 5′ end by other nucleases[64].

This results in a single-guide RNA (sgRNA) composed of two parts: the crRNA, whose 20 nt 5’ end sequence is complementary to the protospacer in the target DNA, and the tracrRNA that’s connected to the crRNA which will help bind the entire sgRNA to the Cas9 protein[31].

Overall, the result of these 2 parallel processes is that a CRISPR–Cas9/sgRNA genome editing complex capable of searching for its target is formed, with the sgRNA binding the complex to the target DNA, and the Cas9 protein actually editing the DNA[63].

Interference

Ultimately, the interference stage consists of the Cas9/sgRNA searching for its target, with its search and recognition process requiring both a complementary nt base pairing between the 20-nt crRNA and a protospacer in the target DNA, as well as the presence of the Cas9’s corresponding PAM sequence that's close to the target site[44, 66].

The PAM sequence is crucial for the complex’s ability to differentiate between the CRISPR locus itself and foreign sequences, preventing autoimmunity[105], and even single mutations in the PAM can disable Cas9’s DNA cleaving ability in vitro while allowing foreign DNA to avoid the complex[8, 65, 66]. The PAM sequence that the most common Cas9 type SpCas9(from *Streptococcus pyogenes*) corresponds with is 5′-NGG-3′, with N being any 1 of the 4 DNA nucleotides[24, 59].

Cas9 initiates the target DNA search for the complex by first probing for its corresponding PAM sequence before interrogating the DNA adjacent to the PAM to check for potential guide-RNA complementarity[147]. The target recognition itself occurs through 3-dimensional collisions where Cas9 rapidly splits from the DNA that doesn’t contain the appropriate PAM sequence, and the dwell time depends on the complementarity between guide RNA and adjacent DNA when the proper PAM sequence is present[77, 101, 147].

Once Cas9 has found the target site with the appropriate PAM sequence, it triggers local DNA denaturing, which causes the DNA strand to split in 2 at the PAM-adjacent target site; this is followed by the crRNA binding to the complementary target strand to form a RNA-DNA hybrid alongside the displaced noncomplementary DNA strand–which is all termed as the R-loop–from the PAM-proximal to PAM-distal ends[147, 153].

After the R-loop is formed, the Cas9 is now activated for DNA cleavage[147]. The Cas9’s two nuclease domains each cut one strand of the target three bp from the PAM sequence to produce a mainly blunt double strand break(DSB)[44, 66].

After this, two possible DNA repair mechanisms can occur as a result of the DSB from the Cas9 enzyme: non-homologous end joining (NHEJ) and homology-directed repair (HDR)[54, 94].

NHEJ consists of the repair of the DSB by joining DNA fragments through an enzymatic process in the absence of exogenous homologous DNA. This process is active in all phases of the

cell cycle. It is a predominant and efficient cellular repair mechanism that is most active in cells, but it is error-prone and may result in the random insertion/deletion (indels) of base pairs at the cleavage site, which can lead to the generation of frameshift mutation or premature stop codon[92, 115, 133, 172].

In contrast, HDR is a highly precise repair mechanism that requires the use of a homologous DNA template and is the most active in the late S and G2 phases of the cell cycle. In CRISPR gene editing, HDR requires a large amount of homologous donor (exogenous) DNA templates from either sister chromatids or foreign DNA, which is used in precise insertions and base substitutions at the DSB site[13, 90, 94].

Applications for CRISPR-Cas9

In general, the CRISPR-Cas9 system could be used for many applications.

Functional Genome Screening

One application of CRISPR-Cas9 is in genome functional screening to identify key genes involved in various biological processes across different biological models. It's being used in genome-wide, targeted loss-of-function screens as an alternative screening system to RNA interference (RNAi)[39]. An instance of this is when a genome-scale CRISPR–Cas9 knockout (GeCKO) library was used to identify genes essential for cell viability in cancer and pluripotent stem cells and was then used to screen for genes whose loss is involved in resistance to vemurafenib (treatment for late-stage melanoma) in a melanoma model[143]. Additionally, other studies have identified host genes important for the intoxication of cells by diphtheria and anthrax toxins using the GeCKO library, which was supported by functional validation[175].

Overall, the application of CRISPR screening in functional genomics is helping researchers to discover new gene functions and, in the future, may help explore the molecular mechanisms of a variety of cellular functions[31].

Virus Treatment

Another application is related to its potential use as a therapy for chronic viral infections[85]. Most antivirus therapies for conditions such as human immunodeficiency virus(HIV) and hepatitis B virus (HBV) aren't a clinical cure because of the inability to remove the viral genome from the infected host cell due to a latent state where viruses minimize their activity inside the host cell to avoid host immune surveillance. As a result, the latency-related life cycles of these viruses play a key role in the incurability of chronic infections, and patients with the virus will have to take antiviral drugs for the rest of their lives.

But CRISPR itself has shown great promise for the treatment of different viral infections in experiments: one instance is in a study where CRISPR–Cas9 has been shown to inhibit HBV replication and gene expression with the inhibition sustained for 3 days after the CRISPR–Cas9 administration, and the results suggest that the Cas system may provide a simple, inexpensive and short-term process for mammalian genome modification[174]; CRISPR–Cas9 delivered by

lentivirus significantly decreasing HIV-1 replication in infected primary CD4+ T cell cultures and reducing the viral load in ex vivo CD4+ T cell culture obtained from HIV-1 infected patients suggests that CRISPR-Cas9 can act as a new type of therapy for HIV, and CRISPR–Cas9 may be used as a novel and efficient platform for the cure of AIDS itself[70]; CRISPR-cas9 when used on a Burkitt's lymphoma cell line with latent Epstein–Barr virus (EBV) infection was able to get rid of EBV completely for 25% of the cells, and caused significant EBV load decrease for another 50% of the treated cells when compared to the untreated ones[162].

Overall, the use of CRISPR–Cas9 as an antiviral strategy is a promising prospect, with various in vitro and in vivo studies, giving rise to potential clinical applications for humans that could result in treatment or prevention methods for pathogenic viral infections in humans.

Combating Antibiotic-Resistant Bacteria

CRISPRs can also be used to control and combat antibiotic-resistant bacteria.

One experiment where 2 CRISPR–dCas9 systems were used to repress antibiotic resistance in MRSA(Methicillin-resistant *Staphylococcus aureus*-a human pathogen which is resistant to β-lactam antibiotics such as penicillin) by targeting the *mecA* methicillin resistance gene resulted in a 77% decrease in gene expression in the CRISPR-treated samples, but it did not kill the bacteria, which makes it a viable option for treatment against antibiotic-resistant bacteria[163].

Another experiment with MRSA introduced a nonviral delivery method(Known as Cr-Nanocomplex) for CRISPR targeting *mecA*(major antibiotic resistance gene involved in MRSA,) which consisted of SpCas9 covalently modified with a cationic polymer called polyethyleneimine (bPEI), as the carrier for packaging sgRNA and boosting their delivery to bacteria. Furthermore, the cultured MRSA strains which were treated with the Cr-Nanocomplex were not able to grow in agar media, including oxacillin at the dose 6 µg/mL, while the untreated samples could grow[71, 164].

Overall, the use of CRISPR–Cas9 to specifically remove resistance genes in bacteria can be a powerful tool to counteract antibiotic resistance and could be a part of the solution to keep antibiotics working; CRISPR–Cas9 would also have to be used as a conventional broad-spectrum antibiotic, which can potentially target only antibiotic-resistant bacteria while leaving the rest alone[31].

Cancer Therapy

Another application is CRISPR as a potential cancer treatment[31]. This is shown through multiple studies and experiments. One is in an experiment where epidermal growth factor receptor (EGFR) mutations in EGFR mutation-bearing tumors in vivo were targeted by CRISPR–Cas9 through adenovirus vector delivery which had disrupted the EGFR mutation(L858R) and resulted in cancer cell death and significant reduction of tumor size[80].

Another is in a study where CRISPR–Cas9 was used to generate chimeric antigen receptor T(CAR-T)cells, which can recognize specific antigens on cancer cells. These CAR-T

cells were used in therapy for patients with acute lymphoblastic leukemia which targeted CD19 and resulted in an effective treatment for the lymphoblastic leukemia while being related to a high remission rate for patients where Stem-cell transplantation was ineffective[106]. Some problems face CRISPR cancer therapy, however. One of these problems is the lack of a safe and efficient delivery mechanism that can be applied in clinical trials, alongside the fact that tumor heterogeneity caused by tumors normally having different subclones with different DNA mutations within can make creating the treatment difficult with CRISPR[21, 46].

Another problem is editing efficiency, as the effectiveness of DSB repair with HDR and NHEJ can vary in different cell types and states, and the potentially low editing efficiency caused by that factor in certain cells can cause a lack of effectiveness from the treatment[25, 173].

Additionally, Cas9–due to being a protein of bacterial origin–can activate the body's immune system, again lowering the effectiveness of the treatment[22].

But despite all the problems, CRISPR is still crucial in cancer research[31]; If its safety, specificity, and efficacy is optimized enough for clinical use, then it could be a widespread cancer treatment.

CRISPR Base Editing

Base editing is a form of genome editing that enables the direct and irreversible conversion of one basepair to another at a target genomic locus without requiring DSBs, or HDR processes with their necessary donor DNA templates[78, 79, 117]. Compared to the standard genome editing methods to introduce point mutations, base editing can do so more efficiently[79], with far less problems such as stochastic insertions/deletions (indels) or translocations[76, 78, 79, 117, 130, 184].

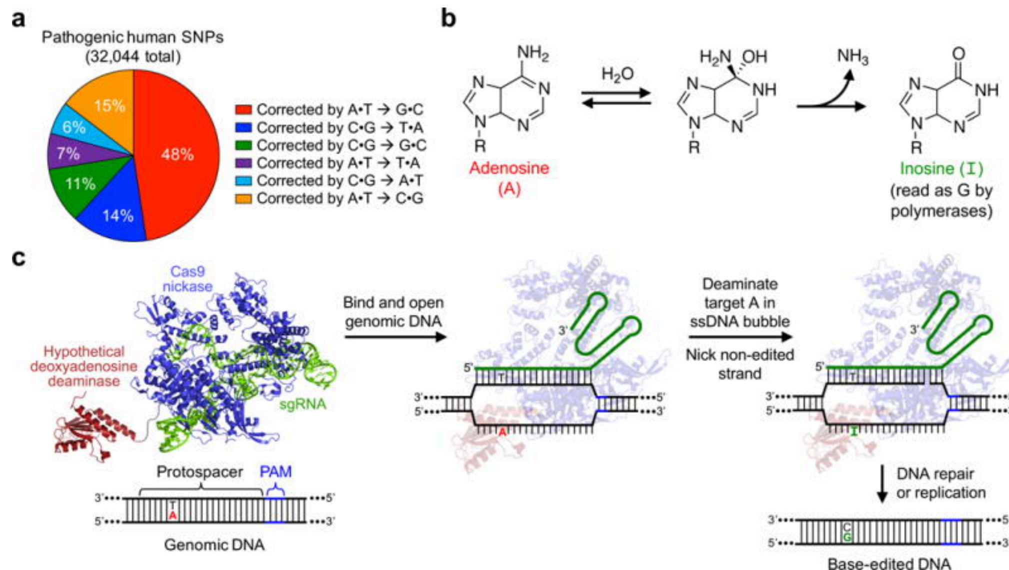


Fig 9: In b, the deamination of Adenosine to Inosine, which is read as G by polymerases. In c, the general structure of how an ABE would work with a deoxyadenosine deaminase working alongside an enzymically impaired Cas9, alongside sgRNA to find a spot in the target DNA and catalyze the deamination of Adenosine to Inosine[45]

There are two main categories of Base editors(BE): cytosine base editors (CBEs) and adenine base editors (ABEs), which are all used with sgRNA for guidance[31].

Regarding CBEs, one of the most commonly used versions are 3rd generation designs (CBE3), which consist of four main parts:

The first piece is a catalytically impaired CRISPR-Cas9 mutant that is unable to create DSBs (known as nuclease-dead Cas9 or dCas9 for short), the second is a single-strand-specific enzyme capable of converting one base to another with an example in this case being a cytidine deaminase that converts Cytosine(C) to Uracil(U) within a 5-nt window in a single-stranded DNA containing bubble created by the Cas9, 3rd is a uracil glycosylase inhibitor (UGI) that impedes uracil removal and the downstream processes that decrease the base editing efficiency and product purity[78], and the 4th is an enzymatic nickase that nicks the other non-edited DNA strand complimentary to the first strand, directing cellular DNA repair processes to replace the G-containing DNA strand[78, 79].

With all these components, BE3 can carry out the conversion of C•G to T•A efficiently and permanently in many different organisms, from bacteria and plants[97, 179], to things such as mice[75, 130, 183] and human embryos[88, 89].

Base editors have expanded in capability through the development of base editors with different properties: different PAM compatibilities[76], narrower editing windows[76], enhanced DNA specificity[130], and small-molecule dependence[154].

Regarding ABEs, these are a relatively recent development, but they share similarities with CBEs in terms of their mechanical design, except for some major differences.
One big difference is that CBEs and ABEs have a different single-strand-specific enzyme capable of converting one base to another, with ABEs having a deoxyadenosine deaminase that can catalyze the deamination of Adenine to Inosine in DNA where the Inosine is interpreted as Guanine[45, 72]. A note about this is that there were no naturally occurring single-stranded DNA Adenine deaminases; only RNA adenosine deaminases were found, which were all unable to edit DNA[45].

But it was engineered through the directed evolution of *Escherichia coli's* tRNA adenosine deaminase(TadA) which was chosen since it shares homology with the APOBEC enzyme (cytidine deaminase) used in the previous CBEs, and some APOBECs bind single-stranded DNA in a conformation that resembles tRNA bound to the TadA[144].

The experiment first consisted of introducing a C•G to T•A mutation in the CamR gene of *Escherichia coli*–a gene that codes for chloramphenicol resistance in bacteria–in the form of an H193Y substitution that would weaken the bacteria's chloramphenicol resistance[45]. Then, the researchers had created unbiased libraries of *Escherichia coli(*ec*)*TadA-dCas9 fusions that contained mutations only in the adenine deaminase portion of the construct to avoid altering the favorable properties of the Cas9 portion of the editor, and were then transferred into the *E. coli* harboring the CamR H193Y mutation with the idea that A•T to G•C conversion at the H193Y mutation should restore chloramphenicol resistance, linking the TadA's Adenine base editing activity to bacterial survival[45].

The surviving bacterial colonies that were able to catalyze the A•T to G•C in the CamR gene had the TadA mutations, A106V and D108N[45]. These mutated TadAs were then incorporated into a mammalian codon-optimized TadA–Cas9 nickase fusion construct, where the dCas9 was replaced with the Cas9 D10A nickase used in BE3 to manipulate cellular DNA repair to favor the desired base editing outcomes[79] alongside a C-terminal nuclear localization signal (NLS)[45]. The resulting TadA*–XTEN–nCas9–NLS construct–where TadA* is the new TadA variant and XTEN is a 16-amino acid linker used in BE33– was designated as ABE1.2, which was capable of carrying out very low efficiency, but observable A•T to G•C editing[45].

From here on out, efforts would be made to optimize ABEs through engineering a single-chain heterodimer, consisting of a wild-type non-catalytic TadA monomer and an evolved TadA monomer(TadA-TadA*). And further work would result in prominent versions such as ABE7.10 and ABE8e that would convert A•T to G•C base pairs efficiently with very high product purity and very low rates of indels[45, 72].

Additionally, ABEs compared with CBEs yield a far cleaner product with fewer mistakes and reports of significant off-target (A-to-non-G) edits to date[72]. Adding on to this, unlike CBEs with their UGI mediated inhibition of Uracil-DNA glycosylase(UDG-enzyme that removes Uracil), ABE editing in cells that lacked alkyl adenine DNA glycosylase (AAG)–the enzyme known to recognize and remove inosine in DNA which would in theory inhibit ABE ability–failed to increase the editing efficiency or product purity compared with cells containing AAG, which shows the sheer converting power of ABE compared to CBE[72].

CRISPR’s Use for Curing Chronic Disease

CRISPR-Cas9 base editing could be used to cure chronic genetic diseases, such as Wilson’s Disease, that would otherwise be incurable and require treatment for the rest of the patient's lifetime. This would be done by first identifying the specific mutation in the target gene (ATP7B in this case) that results in the disease. Mutations can occur in multiple different places on a gene, such as H1069Q and R778L in patients with Wilson’s Disease, making genomic screening a valuable and necessary precursor to a newly designed treatment. The mutation itself can be of types that are in different places within the gene for each person which would require the use of genomic screening, with Wilson's Disease having many different missense mutations, such as H1069Q and R778L. Once the mutation is identified, the sequence of the mutation area would be inserted into a CRISPR locus, where the proper sgRNA would be generated alongside Cas9 with an engineered base editor; this would then form the basis for a Cas9/sgRNA genome editing complex. The complex would then be injected into the patient in a specific bodily region most affected by the disease. For Wilson's Disease, a medical team would most likely target the liver for gene editing complex delivery. Finally, the patient would be monitored to ensure there are no negative effects from treatment.

The most apparent benefit is that CRISPR-Cas9 base editing would possibly be able to cure the genetic disease for good; moreover, another benefit is that with a one-time treatment, the

patient receiving it would never have to spend additional money on temporary treatments, saving thousands of dollars a year.

The idea of CRISPR curing a genetic disease has been made a reality quite recently. A real-life example of CRISPR-based editing used to treat disease is the case of an infant named "KJ" who received treatment for a rare inherited metabolic disease in 2025, detailed in a study by The New England Journal of Medicine. Specifically, KJ experienced a severe carbamoyl phosphate synthetase 1 (CPS1) deficiency. This is a rare genetic disease that causes the lack of a liver enzyme in the liver needed to convert ammonia (generated from the normal breakdown of proteins in the body to urea), which causes ammonia to build up to toxic levels in the subject and can damage organs such as the brain and liver. Patients with CPS1 deficiency, like KJ would normally be treated with a liver transplant; however, to undergo a liver transplant, patients would need to be medically stable and old enough to handle such a major procedure-neither of which was true for KJ.

Ahrens-Nicklas and Kiran Musunuru MD, PhD (Barry J. Gertz Professor for Translational Research in Penn's Perelman School of Medicine) are co-corresponding authors on the published report and began collaborating in 2023 to study the possibility of creating customized gene editing therapies for rare metabolic disorders. They had mainly focused on urea cycle disorders. Soon after KJ's birth, they were able to identify KJ's specific variant of CPS1 through genetic sequencing. The team, including Nicklas and Musunuru, was able to design and manufacture a CRISPR-based editing therapy in 6 months. In late February 2025, the treatment was delivered to KJ's liver via nanoparticles to correct KJ's lack of functional CPS1 enzyme, and then he received follow-up doses in March and April of 2025. As of the time of reporting, KJ has experienced no serious side effects from treatment; additionally, KJ has been able to increase consumption of protein and has been weaned off of nitrogen scavenger medication.
KJ has also demonstrated recovery from certain typical childhood illnesses like rhinovirus(virus that's predominant cause of common cold) without ammonia building up in his body. Longer follow-up is needed to fully evaluate the benefits of the therapy, but so far the treatment seems promising[114].

Although KJ will need to be monitored carefully for the rest of his life due to possible immunogenic or genetic side effects, his case demonstrates the possibility for CRISPR to be able to treat genomic diseases that would otherwise put great strain and pressure on thousands of people.

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