LANCEWELL TOM CAMPAGNE

HUNTINGTON'S DISEASE

A disease overview and future curative prospects through CRISPR/Cas9 genetic editing

JULY 28, 2020

RUG UNIVERSITY
GRONINGEN

Table of Contents

1. Introduction

First described as an epidemic of dancing mania in 1374, George Huntington wrote about a form of hereditary chorea in 1872. In 1993 the disorder was discovered to be a consequence of a singular mutated gene. We now refer to this disorder as Huntington's disease.

Huntington's disease (HD) is a progressive neurodegenerative disorder that initiates around the age of 40 years. The symptoms associated with HD are the loss of coordination of motor functions, cognitive decline and behavioral changes (Walker, 2007). These symptoms gradually worsen as the disease advances. There are palliative treatments to improve quality of life and some symptoms can be reduced by specific treatments but currently there is no curative treatment. Having HD results in death approximately 10 to 15 years after the initial symptoms. People of European descent are the most likely carriers of the autosomal genetic mutation with a prevalence of 5-10 per 100.000 individuals, with many more at risk of having higher amounts of repeats in successive generations (Paoli et al., 2017).

HD is caused by an autosomal dominant mutation in either one or two copies of the Huntingtin gene. This means a parent carrying this disorder on one copy has a 50 percent chance of passing it on to their offspring.

HD is a fully penetrant trinucleotide disorder caused by the IT15 gene on chromosome 4 containing a critical number of CAG codons. An excessive number of CAG repeats causes the IT15-coded protein Huntingtin (HTT) to take on a different conformation, which is called mutant Huntingtin protein (mHTT). Mutant Huntingtin is toxic to all types of neurons of the brain, but some regions are more vulnerable. Given that Huntington is a mono-genetic disease, it might be an obvious target for treatments using genetic editing, as it arises from a single gene with an excess of CAG repeats.

In the last decade the field of genetic engineering has made great strides forward. Many different genetic editing tools have been developed, with CRISPR/Cas9 being a recent addition. CRISPR/Cas9 is currently the most used genetic editing tool which can cleave DNA strands at a specific site and is able to alter DNA in all kinds of organisms by incorporating or removing genes.

Over the last years CRISPR/Cas9 has become increasingly popular, cheaper and more effective. The curative potential of clinical CRISPR/Cas9 adaptations is currently being studied in many different genetic diseases. More specifically for HD, CRISPR/Cas9 could be used to inactivate the defective gene by removing the excess of repeats to below the critical level, thereby stopping production of the toxic protein. Disease prevention could potentially be established by germline intervention, which would prevent all production of mutant protein. For late onset HD patients, somatic treatments reducing the amount of circulating mutant protein could be developed using CRISPR/Cas9. The exact functions of the huntingtin protein however, are not well understood, though it seems to be a component of many crucial cellular functions. Genetic inactivation of the Huntingtin gene has been found to be lethal in mice. (Kaemmerer & Grondin, 2019) Therefore, it is important that any treatment using genetic editing tools is very precise and only removes a specific part of the gene. This paper will explore what is currently possible regarding treatment of HD with genetic tools and what might be possible in the future as genetic tools continue to develop.

2. WHAT IS HUNTINGTON'S DISEASE

2.1 Symptoms and clinical disease development

The age at which HD becomes noticeable is different from case to case. It can begin from age 1 to 80, with symptoms usually becoming noticeable at age 35-44. This late onset means that typically, symptoms develop after HD carriers have had children. The repeat length is the major predictor of the disease severity and it influences the variability in the age of onset of HD by about 70% (Wexler et al., 2004). The remaining 30% might be attributed to other, still unknown, genetic and environmental factors. When the length of CAG repeats on exon 1 of the IT15 gene is over the critical value of 39, the disease is certain to develop. Reduced penetrance is seen in individuals with 36-39 repeats on the IT15 gene. When a parent has reduced penetrance, anticipation can occur in the offspring. In such cases the child can have a larger number of CAG repeats. Anticipation is the process where the increase of the trinucleotide expansion causes the features the disorder to become more severe with each successive generation.
This genetic anticipation in HD occurs more often when the gene is passed down from the paternal side. Both maternal and paternal transmission instability has been correlated with repeat length. In paternal lines however, this instability often results in triplet expansion, while this is not the case in maternal lines (Ridley et al., 1988). The reason why male spermatocytes generally show a larger repeat expansion during transmission than somatic cells and oocytes is still unclear.
Before the time of disease onset, HD carriers are healthy and clinically indistinguishable from non-HD carriers. In the presymptomatic phase, slight changes in behavior are often noticed by family, like increased forgetfulness, anxiety and restlessness.
Over time these symptoms worsen and psychiatric disturbances like depression and suicidal thoughts are likely to develop. Of all psychiatric disturbances, apathy is the most common, occurring in 28% of HD patients. Symptoms such as depression, irritability and obsessive-compulsive behavior manifest in 13% of patients. Many patients suffer these cognitive and behavioral problems before the onset of motor function impairments (Jacobs et al., 2016).
After an HD patient has started experiencing psychiatric problems, the early signs of motor impairments related to hyperkinesia will gradually start to develop. One of the first motor dysfunction symptoms is typically chorea. Chorea is an involuntary movement disorder characterized by randomly appearing hyperkinetic movements. In contrast to hyperkinetic movements, symptoms related to hypokinesia like bradykinesia, slowed saccadic eye movements, general loss of coordination, short-term memory loss and dysphagia, usually follow later (McColgan & Tabrizi, 2018). Early in the disease course, neuronal dysfunction seems to be the cause of the related symptoms, while later on neuronal cell death in vulnerable regions of the brain correlates with the late onset motor impairments. The length of CAG repeats has been shown to be associated with hypokinesia in later stages of HD, but does not seem attributable to chorea in the early stages (Roos, 2010).
The underlying mechanism of neuronal decay responsible for the shift from hyperkinetic to hypokinetic symptoms will be further explained in paragraph 2.2.

There is no single cause of death for Huntington patients. A recent study looking at death in HD patients in Norway found the average age of death for individuals with HD to be 56 (Solberg et al., 2018).
HD patients die around 10-25 years after disease onset with the underlying cause of death being a direct consequence of HD for 73.5% patients. The most observed cause of death was respiratory diseases, which accounts for around 44.2-53.8% of all HD related deaths (Mokdad et al., 2004; REED & CHANDLER, 1958).
A study looking into the exact kind of respiratory disease responsible for the majority of HD deaths found that the primary cause of death was Aspiration Pneumonia (Heemskerk & Roos, 2012). Aspiration pneumonia is caused by the inhalation of food or fluids into the respiratory tract which triggers an infection. This cause of death might be attributed to neurodegeneration in the central nervous system or circuitry that controls respiratory function. This might also be linked with the worsening of Dysphagia. Why respiratory failure in HD is the primary cause of death is a little studied and a not well understood part of HD.

2.2. Cellular Pathogenesis of Huntington's disease

HD is a result of mutations causing expansion of CAG repeats in exon 1 of the Huntingtin gene on the short arm of the 4th chromosome. Regions like the exon 1 of the IT15 gene containing tandem repeats are generally unstable and vulnerable to Slipped Strand Mispairing. This process involves errors in complementary base formation by DNA polymerase leading to trinucleotide expansion. This expanded number of repeats causes the translated protein to contain an expanded polyglutamine stretch near the NH2 terminus of the protein (Figure 1.). When this protein contains more than 39 glutamines, it causes neuronal degeneration through several mechanisms: disruption of axonal transport, loss of cellular proteostasis, disruption in both transcription and translation, loss of mitochondrial and synaptic functions (Ross & Tabrizi, 2011). Next to these toxic effects of the mHTT, the regular activities of the huntingtin protein which are crucial to survival and functioning of neurons are also impaired in HD patients.

The aforementioned loss of proteostasis stems from mHTT hindering ubiquitination and lysosome activity. Both pathways are crucial in protein degradation, and their absence stimulates cellular mHTT aggregation. The mitochondria are affected through a different mechanism. mHTT has a direct effect which partially or fully silences the PPARGC1A promotor, this promotor produces the PPARGC1A protein. This protein is the master regulator of mitochondrial biogenesis, mitochondrial respiration and several metabolic processes (Johri et al., 2013).

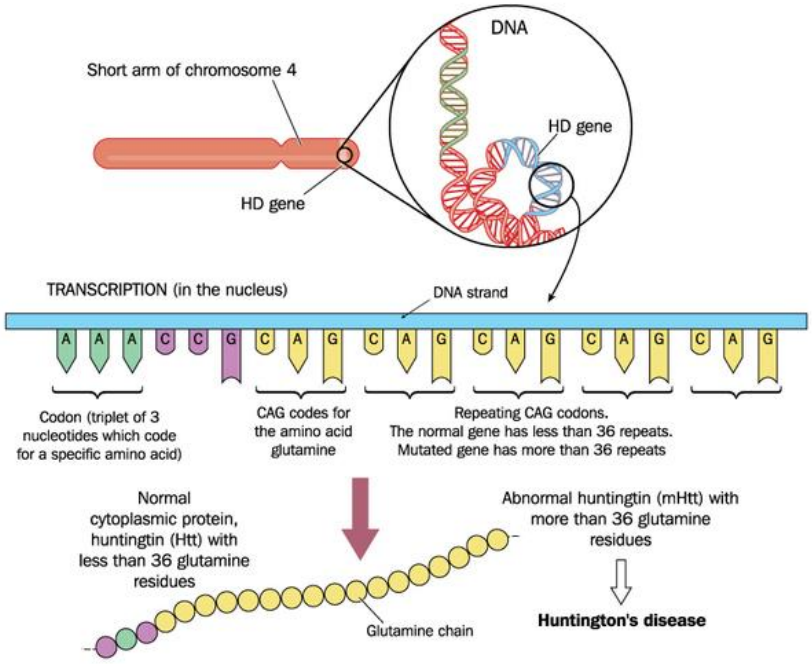

Figure 1: Location of the IT15 gene and the translated protein containing an expanded polyglutamine stretch (U.S. National Library of Medicine; 2020).

Initial Neuronal decay

Medium spiny neurons or spiny projection neurons (SPNs), which make up the majority of the striatum (caudate nucleus and putamen), are most sensitive to the toxic effect of the mHTT protein.
The characteristic change from hyperkinetic symptoms to hypokinetic symptoms can be explained by the deteriorating effects mHTT has on these GABAergic SPNs. The initial hyperkinetic symptoms are a consequence of the disruption of indirect pathway SPNs (Plotkin & Surmeier, 2015). The indirect pathway projects to the external Globus pallidus (GPe). The GPe together with the internal Globus pallidus (GPi), function to inhibit motor movement. When the indirect pathway is disrupted by mHTT, activity in the subthalamic Nucleus (STN) is suppressed by the disruption of GPe (Reiner et al., 2011). The STN has been shown to be an extrapyramidal center, which functions to inhibit muscular responses. The inhibition of movement inhibitions causes the hyperkinetic symptoms seen in early stage HD.
The hypokinetic symptoms seen in later stages of HD are attributed to the loss of the direct pathway of SPNs. The hypokinetic activity seems to the result of the subsequent loss of GPi. As mentioned previously, the GPe and GPi work together to inhibit motor movements. Direct pathway SPNs (dSPNs) that express substance P, a neuropeptide, are affected by mHTT. These dSPNs are part of the circuit of basal ganglia which is responsible for the generation of motor movements. The resulting inhibition of movement generation (hypokinesia) is the direct cause of the disruption of the circuitry of the direct pathway.
Why exactly the SPNs of the indirect pathway are more vulnerable to the mHTT, is not fully understood. Hypotheses exist which claim it is due to Dopamine D2 receptors which are only expressed by the SPNs of the indirect pathway. The increased vulnerability of indirect SPNs is the reason why in early stage HD hyperkinetic symptoms are apparent (Reedeker et al., 2010).
The SNPs are the most vulnerable neurons and deteriorate quickly in presence of mHTT, neurons which are less vulnerable are unfortunately also not immune to the toxic effects of mHTT. Studies have shown that the cerebral cortex (layers III, V, and VI), globus pallidus, thalamus, subthalamic nucleus, substantia nigra, white matter, and the cerebellum have been found deteriorated in HD patients, along with general atrophy of the hypothalamus (Reiner et al., 2011).

Aggregation formation

As mentioned before, HD is caused by a CAG tri-nucleotide repeat expansion within exon 1 of the IT15 gene. This CAG-repeat translates into a large glutamine, PolyQ, stretch of the mHTT. mHTT is proteolytically cleaved, and smaller fragments which bind together either aggregate in the nucleus or in the cytoplasm. mHTT fragments can stay in the nucleus through oligomerization and aggregation, forming inclusion bodies.
The way aggregates are formed is through a not completely understood process, where mHTT monomers arrange themselves in various intermediate oligomeric formations before forming inclusion bodies. The amino acid sequences on both sides of the polyQ stretch seem to help facilitate this accumulation process. These final formations of inclusion bodies cause disturbances in the transcriptional process (EbrahimiFakhari et al., 2012). These disruptions are the result of mHTT co-aggregating with other polyQ-containing proteins

which have crucial functions in the survival of the cell. The transcription factor CREB binding protein, for example is co-aggregated and later partially or fully depleted (Johri et al., 2013). The formation of inclusion bodies does not exclusively occur in the nucleus and also occurs when huntingtin fragments enter the cytoplasm. This creates a positive feedback loop, with cytoplasmic inclusion bodies triggering the increase of cytoplasmic aggregation formation. This eventually leads to the many cellular dysfunctions. The loss of mitochondrial and synaptic function, DNA maintenance, translational and transcriptional dysfunction.
This inclination of mHTT to aggregate in the cytoplasm and in the nucleus through the entire brain, as visualized in figure 2, is the most important cellular characteristic of HD. The mHTT protein also disrupts other proteins related with transcription and protein quality control. This means an increase of mHTT is eventually detrimental to the entire cellular function. Another factor which increases HD progression is that when a cell containing mHTT aggregates goes into apoptosis, it is possible for extracellular polyglutamine aggregates to be taken up by different cells which will further promote polyglutamine aggregation (McColgan & Tabrizi, 2018). This gives mHTT almost a prion-like quality, by being able to spread between cells and different brain regions during the development of HD.

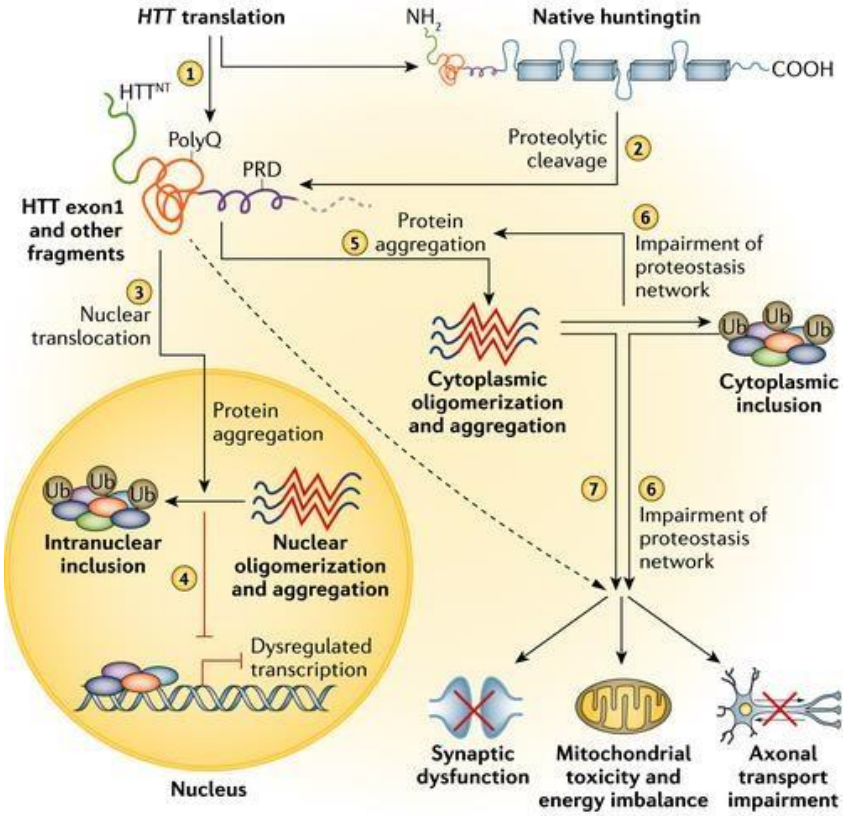

Figure 2: Pathogenetic cellular mechanisms in Huntington disease, mHTT aggregation formation in cytoplasm and nucleus (Source P. McColgan et al. 2008)

Similar pathological mechanisms in different neurodegenerative disorders

MHTT monomers, oligomers, and large inclusions can all be present at the same time and cooperate in the disruption of many different cellular mechanisms.
These mechanisms of cellular toxicity caused by aggregate formation through expanded CAG regions are not unique to HD.
Nuclear inclusion bodies containing the polyglutamine proteins have been detected in brains of Spinocerebellar ataxia type 1, 3, 7, Dentatorubral-pallidoluysian atrophy patients and in the motor neurons of Spinal-bulbar muscular atrophy patients.
All the above-mentioned diseases and other diseases like amyotrophic lateral sclerosis, Parkinson disease and Alzheimer's all involve a very similar cellular mechanism of progressive accumulation of mutant proteins in either cytoplasm, extracellular spaces and in nuclei (Figiel et al., 2012). With different conformations of mutant proteins misfolded because of a mutation. All disease-causing proteins have toxic interactions with other proteins and are processed in an irregular fashion. There is also evidence that in many of these diseases, protein degradation machinery as described in paragraph 2.2, is disrupted in a similar fashion as seen in HD. The similarities in disease causation of CAG repeat diseases makes a common treatment a viable option in the future.

2.3 Current treatments and Diagnostic tools

DIAGNOSTIC TOOLS:

Biomarkers:

To detect HD and map the stage of progression different biomarkers have been used. Biomarkers are an essential part of HD treatment, allowing insight into the progression of pathogenic processes and help assign appropriate therapeutic intervention. H2AFY is a particular histone that is elevated in individuals with HD and is used as a therapeutic marker. In early stages of HD, H2AFY levels responded to treatment with Sodium Phenylbutyrate, a histone deacetylase inhibitor, which prevents neurodegeneration (Silajdzic & Bjorkqvist, 2018). H2AFY levels do not track HD progression and therefore different biomarkers are continually being investigated.
The neurofilament light (NFL) protein is recently (2017) discovered biomarker for HD. The baseline blood plasma levels of NFL protein show a correlation with the stages of neuronal atrophy. In the pre-symptomatic phase, at which no motor dysfunctions are visible but deteriorating processes have already started. The baseline blood plasma levels of NFL were found to be correlated with disease progression over a 3-year period (Byrne et al., 2017). A correlation between blood plasma NFL and cerebrospinal fluid (CSF) NFL was also found. This is important, as usually biomarkers are invasively collected via lumbar puncture of the lumbar spine. The possibility of blood sampling being sufficient for the measurement of NFL to predict progression makes it a very viable biomarker.

MRI:

Magnetic resonance imaging (MRI) is the most commonly used tool to diagnose the progression of HD. The study TRACK-HD looked at changes in volume of grey and white matter of HD patients three times in one year. They found grey matter volume loss in the striatum. While around the striatum a loss of white matter volume was detected. Figure 3 shows the differences of the striata between a healthy individual and an HD patient. Atrophy rates in early HD were found to be highest in the caudate and in white matter (Tabrizi et al., 2012). The reason why cognitive and motor function in presymptomatic stages is limited, seems to point to compensatory mechanisms which try to maintain normal neuronal functions.

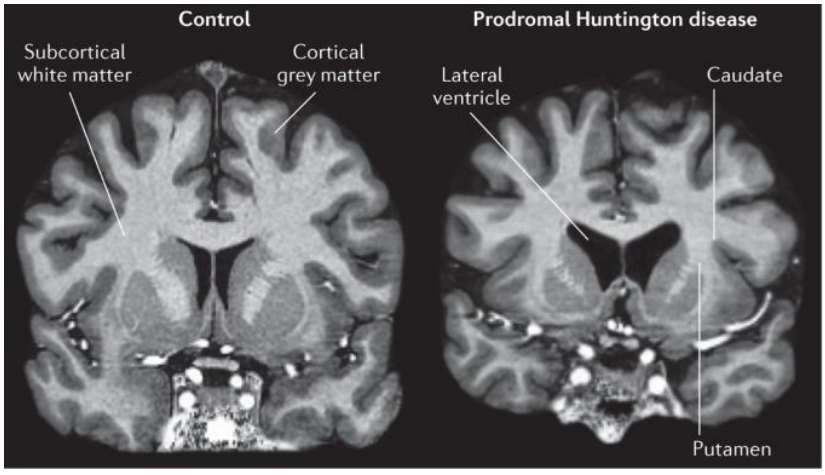

Figure 3: MRI imaging comparison of control (left) and presymptomatic HD patient brain (right). partial atrophy of head of caudate nucleus, general atrophy of the putamen visible. (Bates et al., 2015)

Medicinal treatment

There are currently no curative treatments for HD, but there are palliative treatments to improve symptoms and overall quality of life. One of the earliest symptoms in HD is Chorea. The synaptic vesicular amine transport inhibitor Tetrabenazine is the first HD drug and the only one used to reduce hyperkinetic symptoms associated with chorea (Marshall, 2006). Tetrabenazine is a monoamine storage inhibitor which inhibits vesicular monoamine transporter 2, resulting in reduced monoamine uptake in synaptic vesicles which makes the monoamines exposed to premature degradation. The reduction of monoamines has decreased locomotor activity in animals, but the exact underlying mechanisms are poorly understood. This observational evidence has led to it being a staple treatment for chorea in HD.
Tetrabenazine has been found to have side effects which induce several psychiatric symptoms. Treatment with Deutetrabenazine, which has 6 hydrogens replaced with deuterium atoms, seems to have less psychiatric side effects, but this remain unproven (Rodrigues et al., 2017). The treatment of motor symptoms along with the development of HD leads to psychiatric disturbances. The overall treatment of these psychiatric symptoms in HD is based on current clinical understanding of depression, anxiety, obsessive compulsive disorder and irritability. These symptoms can be treated with cognitive behavioural therapy or psychodynamic therapy. Success of these treatments seem to rely heavily of the state of cognitive dysfunction within the HD patient. Antidepressants, typically SSRI's, are pharmacologically used to treat these symptoms as well (Pidgeon & Rickards, 2013).

DNA/RNA targeting therapies:

DNA/RNA targeting therapies are approaches which can be used to target the DNA or RNA of the Huntingtin gene to try to inhibit gene expression. Antisense oligonucleotides (ASOs), splicing inhibitors, RNA interference (RNAi) can be used to disrupt processes in translation (Figure 4.).
RNA interference (RNAi) uses small cytoplasmic dsRNAs 20–30 bp in length to disrupt posttranscriptional translation of the target gene. The interference is caused by dsRNAs binding to target mRNAs with a specific sequence. In HD treatment only the mutant allele containing the critical number of 39 repeats or higher should be targeted. An endoribonuclease called a dicer is used to cut dsRNA into siRNA molecules. These siRNAs then become part of the RNAinduced silencing complex (RISC). This complex of multiple proteins then cleaves the mRNA encoding for the mHTT protein. The usage of this method to reduce mHTT protein in mice has given positive results in ameliorating the symptoms of HD (Kordasiewicz et al., 2012). However, using RNAi is not curative and has to be a life-long treatment with repeated lentiviral administration. This might pose a problem for HD patients in later stages of disease progression. The lack of specificity of RNAi is a cause of concern regarding HD therapy. It is crucial that only mHTT and not normal HTT is targeted when disrupting translation. As previously mentioned HTT has many crucial functions regarding cellular survival and accidentally silencing the healthy IT15 gene can have life threatening consequences.

Using ASOs is a different method to disrupt RNA. ASOs are chemically synthesized oligonucleotides which can be used as catalysts in the degradation of mHTT RNA by using RNAse H. ASOs are typically 12–30 nucleotides long and bind to RNA which eventually leads to RNA degradation. This degradation works either by promoting RNA cleavage or through steric blocking in which access to mRNA is blocked. These two pathways also lead to the reduction of mHTT protein production. A study using mouse models found using ASOs not only delays the progression of HD, but was also successful in reversing the disease phenotype in late onset HD (Southwell et al., 2018). The authors conclude that this therapy could be clinically feasible. It should however be noted that also with ASOs the downside of repeated treatment and lack of specificity come into play. The current research regarding ASOs and HD is based only on mouse models, which might not translate fully to human models.

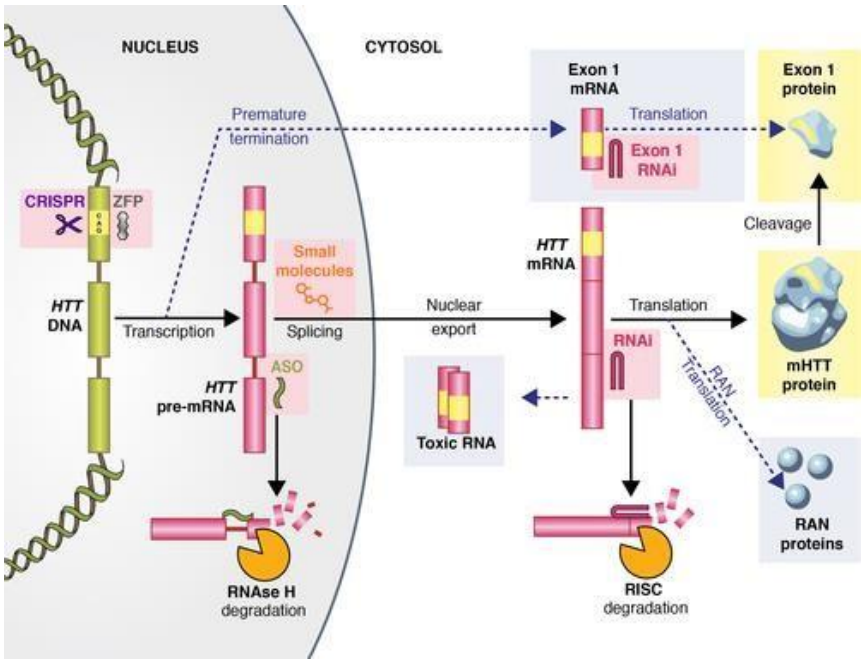

Figure 4. different translational disruption pathways of mHTT (Kordasiewicz et al., 2012; McColgan & Tabrizi, 2018)

3. GENETIC ENGINEERING

3.1 Different genetic editing tools

Targeted genetic editing is a popular tool for studying gene function or for modifying genomes by adding or removing specific genes. Many different strategies have been developed to create targeted double stranded breaks (DSBs) in DNA. These DSBs are repaired by the cell's own repair mechanisms which helps facilitate the required edit. This repair system relies on either on homology-directed repair (HDR) or on the error prone nonhomologous end joining (NHEJ). To find the appropriate DNA target, all genetic editing tools need mechanisms to recognize a specific target and require a mechanism to create the DSB. Creating a DSB without donor template is used to silence the gene by either insertions or deletions via NHEJ. Creating two DSBs at the same time can be used to delete or invert the sequence in between (Figure 5.). When using a donor template, it is possible to introduce a gene at your specified target using a DSB. The most important different genetic editing tools are:
Transcription activator-like effector nucleases (TALENs), Meganucleases (MNs), Zinc Finger Nucleases (ZFNs) and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/ CAS9 (CRISPR-associated protein 9). TALENs, MNs and ZFNs all achieve sequence-specific DNA-binding via protein-DNA interactions (Guha et al., 2017).

ZFNs

ZFNs are one of the first genetic editing tools, discovered in 1985 and first used as such in 1994. ZFNs are fusions of a zinc-finger protein and a cleavage domain of a restriction endonuclease. The mechanisms for DNA recognition are located within the α-helical domain and could interact with a maximum of three DNA bps. A major problem with ZFNs was the number of off-target mutations and cellular toxicity. ZFNs have been heavily engineered to enhance cleavage efficiency. Creating DNA templates for ZFNs to recognize, proved very difficult and some DNA sequences were not effectively recognized, which hindered ZFN's popularity.

TALENS

The novel TALEN gene editing technology appeared in 2011, 15 years after the usage of ZFNs. TALE proteins originate from gram-negative bacteria. These proteins aid bacteria to infect plants, by entering the nucleus of the plant cell. TALE protein will then bind to the plant promoter sequences to activate transcription genes aiding further infection of the bacteria. This code to bind to DNA sequences was first discovered quite recently (2009) and it used the same restriction endonuclease as used in ZFNs (Gaj et al., 2016). Each TALE repeat could interact with just a single nucleotide and thus offered an advantage over the ZFNs. Furthermore, the time needed to create a functional nuclease was significantly shorter to ZFNs. TALENS are also more specific and cause less off-target mutations and are less toxic to cells than ZFNs. A downside to TALENS was their size, requiring lentiviral delivery into cells. Nonetheless, TALENS were a step up from previous genetic editing tools ZFNs and are still used in scientific research today.

MNs

Meganucleases were first discovered in the late 1980's after the discovery of ZFNs. Meganucleases or homing endonucleases are naturally occurring DNA-cleaving enzymes which interact and cleave long (20-40 bps) DNA sequences. These enzymes are very specific, but not easy to repurpose for genetic editing. Therefore, their applicability for genetic editing has been limited. MNs are minimal in their toxicity but perhaps less user-friendly. The reason for this is the need for large recognition sites which might not be large enough or not present at all in the target organism's genome (Stoddard, 2011). This would require the researcher to a priori add a target sequence before using Meganucleases to cleave the target DNA. Recently, studies have tried to combine MNs with TALE proteins creating megaTALs (Boissel et al., 2014). This fuses the specific homing endonuclease to a TALE binding domain. These hybrids have been found highly specific for genetic editing. Meganucleases were generally overshadowed by different genetic editing tools and are currently the least used genetic editing tool in this list.

CRISPR/Cas9

CRISPR/Cas9 appeared as a genetic editing tool in 2013, only 2 years after the introduction of TALENS and 26 years after the first publication about CRISPR in 1987. As mentioned previously all abovementioned gene editing techniques used sequence-specific DNA-binding via protein-DNA interactions. CRISPR/Cas9 however, is an RNA-guided system. This means site recognition is facilitated completely by using only one gRNA. gRNAs are short RNA sequences which bind to specific complementary DNA sequences. These gRNAs consist of a trans-activating CRISPR RNA (tracrRNA) composed of a scaffold sequence for Cas binding along with a CRISPR RNA (crRNA) which defines the genomic target. These two components together provide sequence specificity, and means no recombinant protein is needed for every DNA-sequence (Wang et al., 2016). This has made CRISPR/Cas9 into the most popular, flexible tool for genetic editing. No time had to be used to engineer proteins to recognize each target site. One downside is the need for a protospacer adjacent motif (PAM) to be downstream of the gRNA target site (Gaj et al., 2016). The PAM is recognized by Cas9 and essential for DNA cleavage. As the name Cas9 implies, there are multiple Cas variants with different PAMs. The exact function and origin of the PAM will be discussed in the following paragraph. Despite the requirement of a PAM, CRISPR/Cas9 has gained immense popularity and is the current gold standard when it comes to genetic editing.

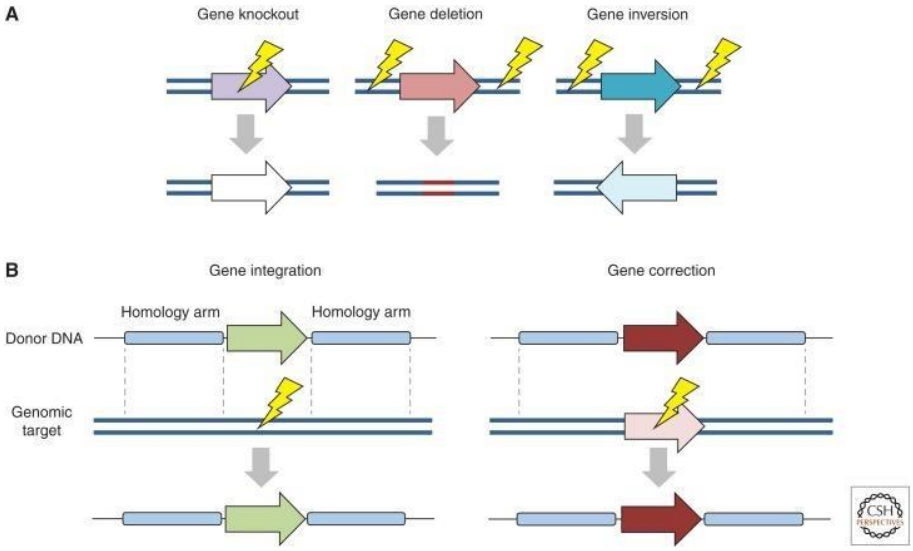

Figure 5. The different options of genetic editing, (Gaj et al., 2016)

3.2 CRISPR/Cas9

CRISPR is a defense mechanism found in bacteria and archaea, formed to prevent bacteriophages infecting them by degrading foreign nucleic acids entering the cell. When bacteria or archaea are attacked by bacteriophages, they will incorporate bacteriophage DNA into a CRISPR locus as spacers. The area excised from the bacteriophage is not random but always adjacent to a short DNA sequence called the PAM. This PAM prevents the CRISPR locus from being targeted itself by CRISPR/Cas. The PAM in bacteria and archaea allows them to distinguish self from foreign DNA.
Scientists have used this bacterial mechanism and have been able to transfer it for use in mammalian cells. The bacterial system is able target specific sites in an organism' genome and Cas9 a nuclease, is also able to cleave mammalian DNA. The transfer to mammalian cells is conducted via viral, physical or chemical factors. Cas9 is a nuclease which is able to cleave both DNA strands at the specified site and create DSBs. These DSBs are repaired by DNA repair mechanisms via NHEJ or HDR (Gaj et al., 2016).
CRISPR uses a short sequence of guide RNA (gRNA) which pairs with a complementary DNA strand. The specificity comes from both this gRNA and a short PAM. The PAM is typically 2-6 base pairs and follows the Cas9 DNA sequence. Just by changing the gRNA sequence CRISPR/Cas9 can be used to target any site of interest which makes it a perfect tool for sequence specific genetic editing. The bacterial CRISPR immune system contains many different Cas proteins, Cas9 was specifically chosen for genetic editing purposes because only one RNA-guided endonuclease is required for cleavage.

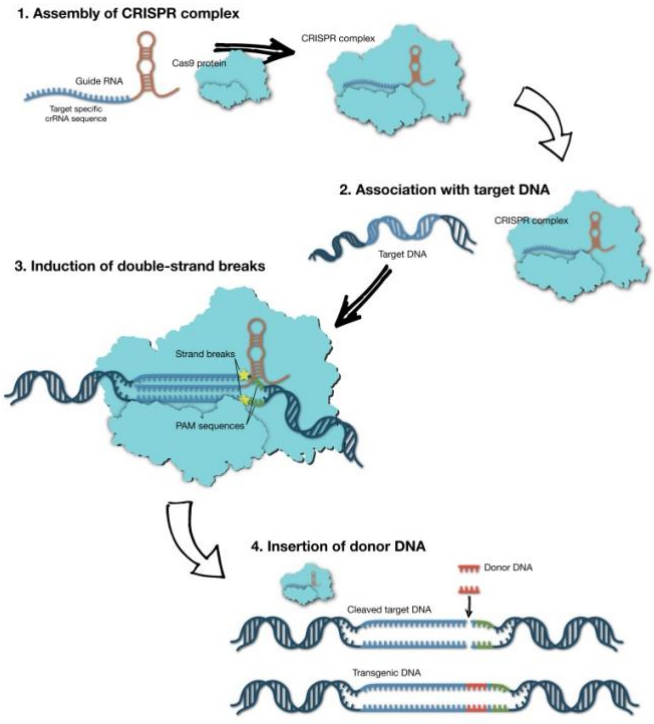

Figure 6. The CRISPR/Cas9 complex (Sittampalam et al., 2016)

With Cas9 it is also possible to induce a genomic deletion via the NHEJ pathway by introducing two DSBs by using one guide RNAs in conjunction with Cas9. A deletion at the DSB site can cause a reading frame shift, or a mutation in a critical region, which both lead

to the inactivation of a gene. HDR can be used to replace a sequence at the DSB site by homologous recombination guided by a donor DNA template. This allows to induce targeted deletions or insertions and even corrections of a mutated gene (Guha et al., 2017). It is even possible for CRISPR/Cas9 to edit multiple genes at the same time by using multiple gRNAs at the same time. This has been used to create large chromosomal rearrangements. These can be used to recreate specific genetic disorders in humans. These Cas9-mediated, targeted rearrangements may be useful for creating disease models by mimicking rearrangements that occur in human disease states. The versatility, specificity and simplicity of CRISPR/Cas9 make it a wildly popular genetic tool for many different types of research.

3.3 How has CRISPR/Cas9 been used and how can it be used in HD

CRISPR/Cas9 gene editing has been used for many different purposes. It has been an easy and effective method to silence genes or engineer point mutations, which can be helpful in discovering the function of different genes.

CRISPR/Cas9 has also been used in HD research in rodents to remove promotor regions, transcription start sites and even the CAG stretches of the IT15 gene (Yang et al., 2017). Removal of promotor regions and start sites can completely silence the IT15 gene, this is not deleterious as having one copy of the IT15 gene is sufficient for normal development and neuron maintenance. Removing parts of the CAG stretches would only alter the translated protein, resulting in production of functioning HTT protein without toxic cellular effects. RNAi and antisense oligonucleotide methods have been used to lower mRNA levels of HD, but often were found not sufficiently allele-specific. In some instances, it caused inactivation of the normal allele, a result of the inability of these methods to readily distinguish mHTT from HTT (Harper, 2009). As previously stated, absence of HTT is highly deleterious to healthy individuals. Another issue with RNAi is the requirement of repeated treatment, which can be a risk factor for individuals with a chronic neurogenerative disease like HD. The aforementioned downsides are not present in the CRISPR/Cas9 method. Treatment would permanently change the DNA of the patient and therefore not require repeated treatment. The removal of the mutant allele would halt the neuronal toxicity at the source. It might be possible to apply this strategy to similar genetic gain of function disorders, whereof there are many. Inactivation of mHTT in elderly HD patients might also be effective in removing neurological symptoms. One study has shown that neuropathological phenotypes in mice at 9 months old were reversable after CRISPR/Cas9 removal of CAG repeats (Yang et al., 2017). This indicates that neuronal cells affected by the mutant protein still have the ability to remove the protein aggregates and repair damages after mHTT expression was halted. Further research will be needed to find if this effect translates to humans. CRISPR/Cas9 therapy can also be used prenatally, after genetic testing, to prevent HD altogether. This preventive intervention on germline cells would use CRISPR/Cas9 to reduce the CAG repeats on the IT15 gene to a number below 39. This would remove the underlying cause of HD and prevent its onset. The changes made in this way will be passed on to future generations, unlike the previously mentioned somatic gene treatment strategy in HD patients. The exact treatment for HD patients via CRISPR/Cas9 will include either the elimination of the promoter region, the transcription start codon or the critical part CAG region to a prevent the production of mHTT from the mutant allele.

When creating a CRISPR/Cas9 mediated gene inactivation therapy it will be crucial to establish DNA targeting strategy catered to the individual with flawless mutant allele specificity to avoid any inactivation or mutations of the healthy allele.

3.4 What are the downsides of CRISPR/Cas9 mediated genetic editing?

As eluded to above, HD therapy critically relies on perfect specificity to the mutant allele. A major concern for such a treatment right now would be off-target mutations or inactivation of either the healthy allele or any other part of the genome. Recently, algorithms have been used to predict the locations of off-target mutations which researchers may use to check for unwanted genetic changes after CRISPR/Cas9 intervention (Lin & Wong, 2018). This software is unfortunately not without its flaws and only provides a statistical analysis.

Another big issue is the current method of delivery. The CRISPR/Cas9 machinery typically is introduced into the body via viral vector. Cells effected are then engineered to produce the Cas9 protein indefinitely. This continuous production can have deleterious effects on healthy cells. Alternate delivery systems are being developed, with researchers looking at a using viral nanoparticles (VNPs) to introduce the machinery without adverse effects (Steinmetz, 2010). VNPs themselves can be designed either genetically or chemically. This delivery system could provide a solution to the delivery problem and is currently under development.
Another alternative is the accurately named KamiCas9. This method includes an additional guide RNA for Cas9. This would mean that after initial Cas9 activity, Cas9 is inactivated permanently (Merienne et al., 2017).

The main issue of off target mutations still remains however, with additional concerns about poor translatability of research from mouse models to humans.
Off-target effects are currently a big concern when it comes to germline editing. An off-target mutation while germline editing critical CAG repeats could induce even more life-threatening issues because it affects all cells including germ cell, which affects not only the child but its offspring as well (“Human Genome Editing: Science, Ethics, and Governance,” 2017). In contrast, somatic gene editing affects only some of the cells of the patient being treated.

Besides off-target effects, the concern of poor translatability of research from mouse models to humans is another issue worth looking into. The way HD is recreated and develops in mouse models is not exactly the same as in humans. A cause of this discrepancy might be the lifespan of a mouse. A maximum lifespan of one to two years is just not enough to map a disease which causes neuronal degeneration over the span of decades. The structure of a mouse brain might be similar to a humans’ but also not identical. A possible solution to this issue could be working with larger mammals with longer life spans and more comparable brain structures. Another distinct difference is the rate of CNS development in rodents. Rodents’ CNS develop relatively quickly compared to humans and might play a part in increased toxicity resistance to mHTT found in rodents’ neuronal cells (Chang et al., 2015).

A study from 2018 used HD knockin (KI) pig models modified with CRISPR/Cas9 to more accurately recreate the neurodegeneration seen in humans (Yan et al., 2018). The downsides to using pigs are the high costs of the animals and the required facilities. However, no animal model will completely replicate the development of HD in humans, the loss of SPNs in KI pigs was found not to be as prevalent as those found postmortem in human HD patients. The pig model however, comes close to reflect human neurodegeneration and seems to be the best model to use so far.

3.5 The many ethical aspects of CRISPR/Cas9

The question of viability for the CRISPR/Cas9 genetic editing mechanism is not only a technical one, but an enormous ethical question too.
There are many ethical concerns attached to this method of genomic intervention, not all of which concern humans, but also different species and the environment. In this paragraph we will only focus on the ethical side concerning human genome editing.

Designer babies

The possibilities of CRISPR/Cas9 are far reaching. Not only curative interventions are possible, but also enhancive interventions. Through germline editing It will be possible to alter or select specific traits like: increased immunity, heightened IQ, gender, improved athleticism, height and many other traits carrying a genetic component. Parents using IVF can currently use preimplantation genetic diagnosis to screen an embryo for some preferred traits. IVF was met with a lot of backlash before its inception, but has since been fully integrated into our society with little to no ongoing social debate. If germline editing will integrate as seamlessly into our society is doubtful. A possible social outcome could reaffirm a statement of 1994 by the Council on Ethical and Judicial Affairs which stated genetic selection as a means to prevent or cure specific diseases, but that selection based on benign characteristics was not ethical. In this scenario using genetic editing to cure HD and other genetic diseases would be possible. There is however, currently no scientific of societal consensus on genetic intervention treatment. But the first likely candidates of genetic treatment will be monogenic diseases like HD. The abovementioned ethical aspect of the CRISPR/Cas9 debate is only a very small tip of a much larger iceberg. The range of this debate is as big as you can imagine it and it deserves a paper on its own. It is however inevitable that science will at some point be able to offer such treatments and social debates will have to be had to decide how and if we will incorporate it.

4. Conclusion & Discussion

Huntington's disease is a progressive and disastrous disease. Since the moment the gene causing HD was found in 1993, a lot has improved in our understanding of HD on a cellular and genetic level. Current treatments for HD are very limited and many clinical trials for new treatments have proven fruitless.
Despite this, researchers are constantly thinking of new innovative pathways to finally cure this deadly disorder, and they continue building the scientific understanding to realize this goal. It is uncertain which of these pathways will eventually lead to a therapeutic target.
Much of the current scientific fundaments of HD is based on either cellular or in vivo animal models. The insight that neurotoxicity was ameliorated and symptoms reduced in mice after CRISPR/Cas9 was used to delete the CAG stretch of the IT15 gene, is very promising. Replicating such findings in Pigs or primates are necessary steps to translate this information into clinical trials involving humans.
To translate all relevant scientific understanding of HD into suitable patient specific models, will take a large amount of time and research. Removing a number of critical CAG repeats from the IT15 gene, or completely deactivating the IT15 gene using CRISPR/Cas9 are feasible therapeutic options for the future. Before human trials with CRISPR/Cas9 are possible, this too will need to be improved upon. There must be a reduction of off-target edits along with the prevention of deleterious CRISPR/Cas9-induced mutations. The current way of administering CRISPR/Cas9 involving viral delivery has shown to pose problems as well. Improving animal models and creating systems providing maximum safety are essential in supporting clinical trials in the future.
Recent in vivo research using CRISPR/Cas9 has shown it might already be experimentally possible to remove the critical amount of CAG repeats in HD patients and ameliorate neurodegeneration (Stadtmauer et al., 2020). But for a therapeutic intervention to become widespread, the method has to be completely safe and all current technical obstacles have to be overcome.
Besides the technical issues, there are a vast amount of ethical concerns surrounding CRISPR/Cas9 treatment. Society will have to make the final decision; social debates will have to be had in which the risks and rewards are weighed.
There is currently no societal or scientific consensus regarding this topic and figuring out the details will take a lot of time.
Despite so many obstacles, the promise of CRISPR/Cas9 to redefine the curation of a plethora of genetic diseases including HD is an exciting future perspective.

5. References

Bates, G. P., Dorsey, R., Gusella, J. F., Hayden, M. R., Kay, C., Leavitt, B. R., Nance, M., Ross, C.A., Scahill, R. I., Wetzel, R., Wild, E. J., & Tabrizi, S. J. (2015). Huntington disease. In *Nature Reviews Disease Primers*. https://doi.org/10.1038/nrdp.2015.5

Boissel, S., Jarjour, J., Astrakhan, A., Adey, A., Gouble, A., Duchateau, P., Shendure, J., Stoddard, B. L., Certo, M. T., Baker, D., & Scharenberg, A. M. (2014). MegaTALs: A rarecleaving nuclease architecture for therapeutic genome engineering. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkt1224

Byrne, L. M., Rodrigues, F. B., Blennow, K., Durr, A., Leavitt, B. R., Roos, R. A. C., Scahill, R. I., Tabrizi, S. J., Zetterberg, H., Langbehn, D., & Wild, E. J. (2017). Neurofilament light protein in blood as a potential biomarker of neurodegeneration in Huntington's disease: a retrospective cohort analysis. *The Lancet Neurology*. https://doi.org/10.1016/S14744422(17)30124-2

Chang, R., Liu, X., Li, S., & Li, X. J. (2015). Transgenic animal models for study of the pathogenesis of huntington's disease and therapy. In *Drug Design, Development and Therapy*. https://doi.org/10.2147/DDDT.S58470

Ebrahimi-Fakhari, D., Wahlster, L., & McLean, P. J. (2012). Protein degradation pathways in Parkinson's disease: Curse or blessing. In *Acta Neuropathologica*. https://doi.org/10.1007/s00401-012-1004-6

Figiel, M., Szlachcic, W. J., Switonski, P. M., Gabka, A., & Krzyzosiak, W. J. (2012). Mouse models of polyglutamine diseases: Review and data table. Part I. In *Molecular Neurobiology*. https://doi.org/10.1007/s12035-012-8315-4

Gaj, T., Sirk, S. J., Shui, S. L., & Liu, J. (2016). Genome-editing technologies: Principles and applications. *Cold Spring Harbor Perspectives in Biology*. https://doi.org/10.1101/cshperspect.a023754

Guha, T. K., Wai, A., & Hausner, G. (2017). Programmable Genome Editing Tools and their Regulation for Efficient Genome Engineering. In *Computational and Structural Biotechnology Journal*. https://doi.org/10.1016/j.csbj.2016.12.006

Harper, S. Q. (2009). Progress and challenges in RNA interference therapy for Huntington disease. In *Archives of Neurology*. https://doi.org/10.1001/archneurol.2009.180

Heemskerk, A. W., & Roos, R. A. C. (2012). Aspiration pneumonia and death in Huntington's disease. *PLoS Currents*. https://doi.org/10.1371/currents.RRN1293

Human genome editing: Science, ethics, and governance. (2017). In *Human Genome Editing: Science, Ethics, and Governance*. https://doi.org/10.17226/24623

Jacobs, M., Hart, E. P., van Zwet, E. W., Bentivoglio, A. R., Burgunder, J. M., Craufurd, D., Reilmann, R., Saft, C., Roos, R. A. C., & The REGISTRY investigators of the European Huntington's Disease Network. (2016). Progression of motor subtypes in Huntington's disease: a 6-year follow-up study. *Journal of Neurology*. https://doi.org/10.1007/s00415016-8233-x

Johri, A., Chandra, A., & Beal, M. F. (2013). PGC-1α, mitochondrial dysfunction, and Huntington's disease. In *Free Radical Biology and Medicine*. https://doi.org/10.1016/j.freeradbiomed.2013.04.016

Kaemmerer, W. F., & Grondin, R. (2019). The effects of huntingtin-lowering: what do we know so far? . *Degenerative Neurological and Neuromuscular Disease*. https://doi.org/10.2147/dnnd.s163808

Kordasiewicz, H. B., Stanek, L. M., Wancewicz, E. V., Mazur, C., McAlonis, M. M., Pytel, K. A., Artates, J. W., Weiss, A., Cheng, S. H., Shihabuddin, L. S., Hung, G., Bennett, C. F., & Cleveland, D. W. (2012). Sustained Therapeutic Reversal of Huntington's Disease by Transient Repression of Huntingtin Synthesis. *Neuron*. https://doi.org/10.1016/j.neuron.2012.05.009

Lin, J., & Wong, K. C. (2018). Off-target predictions in CRISPR-Cas9 gene editing using deep learning. *Bioinformatics*. https://doi.org/10.1093/bioinformatics/bty554

Marshall, F. J. (2006). Tetrabenazine as antichorea therapy in Huntington disease: A randomized controlled trial. *Neurology*. https://doi.org/10.1212/01.wnl.0000198586.85250.13

McColgan, P., & Tabrizi, S. J. (2018). Huntington's disease: a clinical review. In *European Journal of Neurology*. https://doi.org/10.1111/ene.13413

Merienne, N., Vachey, G., de Longprez, L., Meunier, C., Zimmer, V., Perriard, G., Canales, M., Mathias, A., Herrgott, L., Beltraminelli, T., Maulet, A., Dequesne, T., Pythoud, C., Rey, M., Pellerin, L., Brouillet, E., Perrier, A. L., du Pasquier, R., & Déglon, N. (2017). The SelfInactivating KamiCas9 System for the Editing of CNS Disease Genes. *Cell Reports*. https://doi.org/10.1016/j.celrep.2017.08.075

Mokdad, A. H., Marks, J. S., Stroup, D. F., & Gerberding, J. L. (2004). Actual Causes of Death in the United States, 2000. In *Journal of the American Medical Association*. https://doi.org/10.1001/jama.291.10.1238

Paoli, R. A., Botturi, A., Ciammola, A., Silani, V., Prunas, C., Lucchiari, C., Zugno, E., & Caletti, E.

(2017). Neuropsychiatric burden in huntington's disease. In *Brain Sciences*. https://doi.org/10.3390/brainsci7060067

Pidgeon, C., & Rickards, H. (2013). The pathophysiology and pharmacological treatment of Huntington disease. *Behavioural Neurology*. https://doi.org/10.3233/BEN-2012-120267

Plotkin, J. L., & Surmeier, D. J. (2015). Corticostriatal synaptic adaptations in Huntington's disease. In *Current Opinion in Neurobiology*. https://doi.org/10.1016/j.conb.2015.01.020

REED, T. E., & CHANDLER, J. H. (1958). Huntington's chorea in Michigan. I. Demography and genetics. *American Journal of Human Genetics*.

Reedeker, N., Van Der Mast, R. C., Giltay, E. J., Van Duijn, E., & Roos, R. A. C. (2010). Hypokinesia in Huntington's disease co-occurs with cognitive and global dysfunctioning. *Movement Disorders*. https://doi.org/10.1002/mds.23136

Reiner, A., Dragatsis, I., & Dietrich, P. (2011). Genetics and neuropathology of huntington's disease. In *International Review of Neurobiology*. https://doi.org/10.1016/B978-0-12381328-2.00014-6

Ridley, R. M., Frith, C. D., Crow, T. J., & Conneally, P. M. (1988). Anticipation in Huntington's disease is inherited through the male line but may originate in the female. *Journal of Medical Genetics*. https://doi.org/10.1136/jmg.25.9.589

Rodrigues, F. B., Duarte, G. S., Costa, J., Ferreira, J. J., & Wild, E. J. (2017). Tetrabenazine Versus Deutetrabenazine for Huntington's Disease: Twins or Distant Cousins? *Movement Disorders Clinical Practice*. https://doi.org/10.1002/mdc3.12483

Roos, R. A. C. (2010). Huntington's disease: A clinical review. In *Orphanet Journal of Rare Diseases*. https://doi.org/10.1186/1750-1172-5-40

Ross, C. A., & Tabrizi, S. J. (2011). Huntington's disease: From molecular pathogenesis to clinical treatment. In *The Lancet Neurology*. https://doi.org/10.1016/S14744422(10)70245-3

Silajdzic, E., & Bjorkqvist, M. (2018). A critical evaluation of wet biomarkers for huntington's disease: Current status and ways forward. In *Journal of Huntington's Disease*. https://doi.org/10.3233/JHD-170273

Solberg, O. K., Filkukovà, P., Frich, J. C., & Feragen, K. J. B. (2018). Age at Death and Causes of Death in Patients with Huntington Disease in Norway in 1986-2015. *Journal of Huntington's Disease*. https://doi.org/10.3233/JHD-170270

Stadtmauer, E. A., Fraietta, J. A., Davis, M. M., Cohen, A. D., Weber, K. L., Lancaster, E., Mangan, P. A., Kulikovskaya, I., Gupta, M., Chen, F., Tian, L., Gonzalez, V. E., Xu, J., Jung, I. young, Joseph Melenhorst, J., Plesa, G., Shea, J., Matlawski, T., Cervini, A., … June, C. H. (2020). CRISPR-engineered T cells in patients with refractory cancer. *Science*. https://doi.org/10.1126/science.aba7365

Steinmetz, N. F. (2010). Viral nanoparticles as platforms for next-generation therapeutics and imaging devices. In *Nanomedicine: Nanotechnology, Biology, and Medicine*. https://doi.org/10.1016/j.nano.2010.04.005

Stoddard, B. L. (2011). Homing endonucleases: From microbial genetic invaders to reagents for targeted DNA modification. In *Structure*. https://doi.org/10.1016/j.str.2010.12.003

Tabrizi, S. J., Reilmann, R., Roos, R. A. C., Durr, A., Leavitt, B., Owen, G., Jones, R., Johnson, H., Craufurd, D., Hicks, S. L., Kennard, C., Landwehrmeyer, B., Stout, J. C., Borowsky, B., Scahill, R. I., Frost, C., & Langbehn, D. R. (2012). Potential endpoints for clinical trials in premanifest and early Huntington's disease in the TRACK-HD study: Analysis of 24 month observational data. *The Lancet Neurology*. https://doi.org/10.1016/S14744422(11)70263-0

Walker, F. O. (2007). Huntington's disease. In *Lancet*. https://doi.org/10.1016/S01406736(07)60111-1

Wang, H., La Russa, M., & Qi, L. S. (2016). CRISPR/Cas9 in Genome Editing and Beyond. *Annual Review of Biochemistry*. https://doi.org/10.1146/annurev-biochem-060815014607

Wexler, N. S., Lorimer, J., Porter, J., Gomez, F., Moskowitz, C., Shackell, E., Marder, K., Penchaszadeh, G., Roberts, S. A., Gayán, J., Brocklebank, D., Cherny, S. S., Cardon, L. R., Gray, J., Dlouhy, S. R., Wiktorski, S., Hodes, M. E., Conneally, P. M., Penney, J. B., … Landwehrmeyeri, B. (2004). Venezuelan kindreds reveal that genetic and environmental factors modulate Huntington's disease age of onset. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.0308679101

Yan, S., Tu, Z., Liu, Z., Fan, N., Yang, H., Yang, S., Yang, W., Zhao, Y., Ouyang, Z., Lai, C., Yang, H., Li, L., Liu, Q., Shi, H., Xu, G., Zhao, H., Wei, H., Pei, Z., Li, S., … Li, X. J. (2018). A Huntingtin Knockin Pig Model Recapitulates Features of Selective Neurodegeneration in Huntington's Disease. *Cell*. https://doi.org/10.1016/j.cell.2018.03.005

Yang, S., Chang, R., Yang, H., Zhao, T., Hong, Y., Kong, H. E., Sun, X., Qin, Z., Jin, P., Li, S., & Li, X. J. (2017). CRISPR/Cas9-mediated gene editing ameliorates neurotoxicity in mouse model of Huntington's disease. *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI92087