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Suppression of somatic expansion as a novel therapeutic approach for Huntington Disease and other Repeat Expansion Disorders

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Abstract

Huntington disease (HD) is one of a growing number of rare genetic diseases characterized by the inheritance of an increased number of short tandem repeats within the affected gene. Many of these repeat expansion disorders (REDs) affect the brain. While inheritance of the mutant allele is a necessary first step for disease manifestation, a second step of further expansion of the inherited expanded repeats, particularly in neurons in the brain, also appears to play a critical role toward disease manifestation. This dynamic process, called somatic expansion, is modulated by genes involved in the repair of DNA that operate upstream of the specific gene associated with each individual disease. As somatic expansion has been described in multiple REDs, genes associated with regulating somatic expansion are attractive therapeutic targets, since multiple REDs could potentially be treated with the same drug.

Introduction

Neurological, and in particular neurodegenerative diseases, have remained notoriously difficult to treat. The variable clinical presentations, the complex and intricate intertwining structure of the central nervous system (CNS), the difficulty accessing the brain, the often-lengthy time over which diseases develop, and imperfect animal models are just a few of the challenges hindering the full understanding of pathophysiologic mechanisms, and hence, of the development of targeted treatments. Huntington disease (HD) is a typically late onset neurodegenerative disorder that has been fraught with all of these characteristics and despite much effort, no rationally designed therapeutic options have been approved to date.[1–3] However, as our knowledge of HD biology has increased over the last several decades, the possibility of new, specific therapies targeting the pathophysiological underpinnings of the disease have emerged.[3]

HD neurobiology

HD is a relentlessly progressing neurodegenerative disease that impairs motor coordination, cognition and behavior, typically resulting in death 15 to 20 years after the onset of motor symptoms.[1] The first signs of the disease may be noticed in early adulthood, with full motor manifestation typically occurring around 40 to 60 years of age. Consistent with the motor manifestations, HD is associated with neurodegeneration primarily in the striatum in the early stage of the disease. However, as the disease progresses, other brain regions, particularly the cortex, are also affected.[1,4,5]

HD genetics

HD is inherited in an autosomal dominant manner and was one of the first disorders for which the emerging recombinant DNA technologies of the 1980s were applied, in an

effort to identify the causative mutation. In 1983, using DNA from two large family cohorts, one from the USA and one from Venezuela, a genetic variant linked to HD was discovered and mapped to human chromosome 4.[6] As well as setting the ball rolling for the refined genetic mapping and eventual identification of the mutation causing HD, this variant also proved useful in identifying probable mutation carriers[6–8], paving the way to modern genetic diagnosis. Through a systematic progression of cloning and linkage analysis, comparing unaffected people and those with HD, a stretch of DNA was identified on chromosome 4 that would eventually reveal the culprit.[9] The located region coded for a previously unidentified and large gene, which ultimately came to be known as huntingtin (*HTT*). A comparison of hundreds of unaffected and affected individuals revealed that exon 1 of the *HTT* gene contained a polymorphic polyglutamine-encoding trinucleotide CAG repeat that was expanded in affected individuals. The number of *HTT* CAG repeats was shown to vary from approximately 10 to 35 CAG repeats in the general population. The disorder is fully penetrant in those individuals inheriting 40 or more CAG repeats, while alleles in the range 36 to 39 CAG repeats show reduced penetrance. Age at onset is strongly driven by CAG length, with each additional inherited CAG precipitating an approximately three-year decrease in the age at onset.[10–12] In keeping with its autosomal dominant mode of inheritance, one allele is sufficient to induce disease, and people who are double mutant carriers, that are rare, do not appear to have a worse course. Rather, onset of disease is linked to the longer mutant allele.[13]

HD pathology

The *HTT* CAG expansion is assumed to mediate its pathogenic effect primarily through a toxic gain of function of the aggregation-prone expanded polyglutamine tract in the resulting mutant HTT protein.[2] However, there is also evidence that the mutant *HTT* transcript may have direct toxic effects at the RNA level.[14–18] In addition, it has been shown that the expanding CAG length increases defective splicing of exon 1 to exon 2, thereby creating a highly toxic mutant fragment (translated from the exon 1/intron 1 ~~fragment~~transcript, commonly referred to as the exon 1 fragment). This resultant fragment appears to confer greater toxicity than full length HTT and has been detected in the brain of people with HD.[19,20] The complexities of the downstream pathways raise questions as to which aspects of downstream pathology, mediated by which toxic entities (RNA/protein), from which transcripts (truncated/full length), would make the most efficacious therapeutic targets. Nevertheless, all data point to greater toxicity as repeat length increases.[21,22]

HD anticipation

Once the *HTT* CAG repeats have expanded into the disease associated range the repeats become highly genetically unstable and frequently change in length from one generation to the next. Notably, these intergenerational changes are biased toward

further expansion, particularly during male transmission, such that HD displays the phenomenon of anticipation (decreasing age at onset in successive generations). [23–27]

Somatic Expansion in HD

In addition to being intergenerationally unstable, it was noted shortly after the CAG expansion mutation was identified that the CAG repeats were also somatically unstable in a process that appeared to be biased toward additional repeat length gains. Notably, these somatic expansions were most prominent in the brain, and in particular in the striatum and the cortex, which are affected early on and most profoundly in HD.[28–31] Of particular note, it was shown that the earliest gains in repeats were observed in the striatum ~~at a time when striatal neuropathology was absent and thus~~ pr~~ior to~~ ~~eceding~~ the first overt neuropathological abnormalities[32] and coinciding with the first biomarker changes (*e.g.*, caudate volume loss, neurofilament light protein (NfL) increase in cerebrospinal fluid (CSF).[5] Moreover, these analyses revealed that some cells in the striatum could acquire somatic expansions of many hundreds of CAG repeats. Similar gains in repeat length, occurring first and being most pronounced in striatum, followed by cortex, were also seen in HD mouse models.[30,33] Subsequent analyses revealed that the striatal specific expansions occurred predominantly in the medium spiny neurons that are the primary target of neurodegeneration in HD.[34,35]

Given that longer inherited alleles cause an earlier age at onset, and that in nearly all the model systems greater pathologic effects are observed with longer CAGs, it seems logical to assume that somatic expansion of the CAG repeat in HD may contribute toward the progressive nature and tissue specificity of the symptoms. Consistent with this hypothesis, it was shown that in HD individuals with the same inherited repeat length, those with greater somatic expansion in the cortex showed earlier disease onset.[36] More recently, similar effects have been observed linking the degree of somatic expansion of the *HTT* CAG repeat in blood DNA with the severity of the symptoms.[37–39]

Repeat structures in HD

Further support for a role for somatic expansion in HD was revealed by the observation that age at onset in HD is best predicted by the number of pure CAG repeats inherited, rather than by the number of glutamines encoded.[39–41] In the majority of HD patients, the CAG repeat is proceeded by a CAA CAG two codon cassette. Since CAA also encodes glutamine, in typical alleles the length of the polyglutamine tract is thus equal to the number of CAG repeats, plus the two encoded by the CAA CAG cassette. However, some rare individuals have inherited *HTT* alleles which have either lost or duplicated the CAA CAG cassette. Despite the fact that for the same number of CAGs, the mutant transcripts from such alleles encode respectively either two fewer, or two more glutamines than a typical allele, disease onset is best predicted by the number of pure CAG repeats and not the number of glutamines encoded.[39–41] Likewise, the

propensity to somatic expansion is best predicted by the number of pure CAG repeats, rather than the number of glutamines encoded.[39–41] These data suggest that the propensity to expand somatically trumps the glutamine encoding potential of the inherited allele.

Mechanism of expansion

While expansion can probably occur during any vulnerable state of DNA, the presence of somatic expansions in non-replicating neurons[30,42] led to the hypothesis that somatic expansion was not linked directly to cell proliferation and DNA replication. While the exact mechanisms are still being elucidated, evidence suggests that once a threshold length of CAG repeats is reached, the DNA tends to form secondary structures such as slipped strand DNA. These structures appear to provide the substrate for components of the DNA repair machinery, primarily DNA mismatch repair, to assemble and mediate an inappropriate DNA repair reaction that actually increases the number of repeats. As longer repeats are more likely to form aberrant secondary structures, and are more genetically unstable, this establishes a positive feedback loop in which the rate of somatic expansion increases with age. The hypothesis that expansion is mediated by inappropriate DNA mismatch repair is well supported by data from HD mouse models in which key components of the DNA mismatch repair pathway have, counterintuitively, been shown to actually be required to generate somatic expansions.[31,43–46] In particular, both MSH2 and MSH3 have been shown to be essential to mediate expansions in HD animal models. MSH2 and MSH3 form the MutSβ heterodimer, which is known to have the role of recognizing small insertion/deletion loops in canonical DNA mismatch repair, and is assumed to similarly recognize slipped strand structures as part of the expansion process.[47] Notably, the absence of *Msh2* was shown not only to suppress somatic expansion in HD mouse models, but also to improve disease course.[31,45] In addition to the MutSβ, downstream components of the mismatch repair complex such as MLH1, MLH3 and PMS2 have also been shown to be involved in the processing of expansions, suggesting that expansion is not simply mediated by the stabilization of atypical secondary structures, but likely involves an inappropriate DNA mismatch repair reaction.[44,48]

Members of the DNA Damage Response (DDR) Gene Family are modifiers of HD onset and progression by Modulating Somatic Expansion~~SE~~

While the inherited number of CAG repeats clearly represents the primary determinant of age at onset in HD, considerable variation is nonetheless observed in age at onset for individuals inheriting the same number of CAG repeats. These individual-specific differences are almost certainly contributed to by environmental factors, but heritability studies indicated that genetic modifiers also play a critical role.[49] The identification of genetic modifiers of a disease process is important because their nature may provide an unbiased insight into the key pathogenic pathways and reveal novel, potentially more

druggable, genetically validated therapeutic targets. The identification of common naturally occurring polymorphisms that modify the disease process can also provide insights into plausible therapeutic effect sizes and safety windows. Indeed, it has been determined that therapeutic targets that are supported by human genetic data are more likely to proceed successfully through the drug development pipeline than targets identified via other more traditional routes.[50] To this end, several unbiased genome wide association studies (GWAS) for modifiers of HD severity have been conducted.[37,41,44,51–54] Somewhat surprisingly, these GWAS have not revealed genes or pathways known to be involved in aggregate biology, RNA processing or neuronal survival as might reasonably have been expected. Rather, most of the genes that have reached genome-wide significance levels are DNA repair genes.[37,41,44,51–54] This specifically includes genes with known roles in the DNA mismatch repair pathway such *MSH3*, *MLH1*, *PMS2* and *LIG1* – the pathway that mouse model data has directly implicated in the somatic expansion process. Interestingly, one of the genes in which variants are highly significantly associated with variation in HD severity, is *FAN1*.[37,41,44,51–54] This was a known DNA repair gene, but there was no prior indication that this gene was involved in the somatic expansion process. Subsequent studies have demonstrated that the *FAN1* gene is involved in somatic expansion, but in contrast to key components of the DNA mismatch repair pathway such as *MSH3*, *MLH1*, *MSH2* and *MLH3*, *FAN1* actually protects against somatic expansion.[55–57] These data further highlight the utility of an unbiased human genetics approach in identifying key molecular players in what otherwise may appear to be relatively well understood pathways. Most critically in this case however, combined with prior knowledge on the tissue specific dynamics of somatic expansion in HD patients and its association with disease severity, and the known role of the mismatch repair proteins in the expansion process, these data strongly suggest that the genetic polymorphisms in the DNA genes revealed by the GWAS are exerting their effects by modulating the rate of somatic expansion in patients. Consistent with this, candidate gene analyses have revealed that some of the same DNA repair polymorphisms that modulate HD severity, also modify the degree of somatic expansion of the *HTT* repeat in blood DNA.[39] Additional studies have indicated that DNA repair gene variants modulate the pathogenesis of HD prior to full motor manifestation, via a genetic interaction with the *HTT* gene.[58] Specifically, striatal and putamen volume, and cognitive capacity were affected by genetic modifiers in premanifest people with HD, while no effect of these variants was noted in the control group. These data are further corroborated by a recent GWAS analysis demonstrating that *MSH3* variants had a particularly strong association with disease progression landmarks that included cognitive deterioration or a combination of cognitive, functional and motor deterioration.[59]

Overall, these data suggest that, while inheriting a pathologically expanded allele predisposes to the disease and can be seen as step one, a second step, *i.e.*, somatic expansion, contributes toward triggering neuropathology and the first clinical signs and symptoms that then progress relentlessly towards full manifestation. Considering the delay in onset of disease in HD, which often takes thirty to forty years, this type of a dynamic process makes perfect sense. Nonetheless, while somatic expansion is clearly making things worse, it remains unclear if somatic expansion is actually required to precipitate symptoms. It is possible that the threshold number of repeats required to

mediate pathology in neurons in the adult brain is actually much greater than the number of repeats typically inherited by adult-onset patients and that somatic expansion is necessary. Such a model would also be consistent with the observation that HD is a true dominant disorder[13] with onset being driven by the time taken for the larger CAG allele to somatically expand beyond the pathological threshold.[60]

~~Somatic Expansion as a therapeutic target~~

~~Several lines of evidence have now converged to reveal that somatic expansion is a key part of the pathogenic pathway in HD. Therefore, a logical therapeutic goal is to suppress somatic expansion. If such therapy could be started when the first clinical signs and symptoms are measurable, it may be possible to inhibit further disease progression. Ideally however, if somatic expansion was suppressed early, before the critical threshold of affected cells has been reached, several years prior to motor manifestation, then it may be possible to delay, or possibly even prevent, the onset of symptoms altogether, assuming that somatic expansion is required to mediate pathology. Another major advantage of targeting the underlying mutation itself at the DNA level of the mutant *HTT* gene, is that such an approach would be expected to be efficacious without an understanding, and independent of, the key toxic events downstream, whether they occur at the RNA or protein level.~~

Somatic Expansion is seen in multiple Repeat Expansion Disorders (REDs)

HD is one of a large number of diseases collectively termed “repeat expansion disorders” or REDs, caused by the expansion of a simple sequence within the associated gene (annotated recently in[61]). These expansions are located at different positions within the different genes in different disorders, and may cause different effects on gene function including loss of function, or toxic gain of function, at both the DNA and RNA levels.[61] In many of these disorders, the repeat expansion is genetically unstable in the germline, and many of these disorders also present with genetic anticipation, and/or other unusual inheritance patterns. More critically however, many of these disorders such as spinal and bulbar muscular atrophy,[62–66] spinocerebellar ataxia type 1 (SCA1),[4,67–72] dentatorubral-pallidoluysian atrophy (DRPLA),[63,64,70,73–78] SCA3,[62,63,68,69,79] SCA7,[80,81] and myotonic dystrophy type 1 (DM1),[82–85] all exhibit somatic expansion. Given that in the majority of these disorders, longer inherited repeats also lead to earlier onset, it seems logical that somatic expansion is also contributing toward the progressive nature of the pathology. Indeed, there is direct data that in myotonic dystrophy type 1, as observed in HD, individual-specific somatic expansion rates are inversely associated with individual-specific measures of disease severity (*i.e.*, individuals in whom the repeat expands faster have earlier and/or more severe disease)[86–88]. An observation across many REDs that primarily affect the brain is that somatic expansion of the inherited expanded gene is typically greatest in the striatum, followed by the cortex, while occurring at much lower levels in the cerebellum (**Table 1**).

These observations point to factors intrinsic to the striatum, as well as the cortex, which particularly facilitate somatic expansion. These factors may also be driving the tissue-specificity of the symptoms in HD. Similarly, in multiple SCAs, such as SCA1, 2, 3, and 7, early clinical presentation and neuropathology implicate the basal ganglia, including the striatum, as well as the cortex[89,90] and somatic expansion in these brain regions has been described.[4,63,80,91] In other SCAs, where cerebellar neurodegeneration may be a predominant driver of disease manifestation, somatic expansion may not play a central role since the cerebellum is a tissue in which the repeat appears to remain relatively stable. However, this may be a detection problem associated with the very low proportion of vulnerable Purkinje cells in the cerebellum, and does not preclude a role for somatic expansion in driving pathology within the cerebellum, or mitigate against the likely negative effects of somatic expansion in the striatum in the disease course.[22]

Overall, DM1, and probably many of the other REDs that display somatic expansion, may also be subject to a two-step genetic process that opens an entirely new approach to a therapy not only for HD, but for multiple other REDs as well. Instead of targeting each disease gene, or the downstream pathologies in each disorder, the pathologic process upstream of the individual disease gene product presents a more universal target. Theoretically, if somatic expansion could be suppressed, it may be possible to slow further progression in any RED for which somatic expansion contributes toward disease pathology.

Somatic Expansion as a therapeutic target

Several lines of evidence have now converged to reveal that somatic expansion is a key part of the pathogenic pathway in HD, and other REDs. Therefore, a logical therapeutic goal is to suppress somatic expansion. If such therapy could be started when the first clinical signs and symptoms are measurable, it may be possible to inhibit further disease progression. Ideally however, if somatic expansion was suppressed early, before the critical threshold of affected cells has been reached, several years prior to motor manifestation, then it may be possible to delay, or possibly even prevent, the onset of symptoms altogether, assuming that somatic expansion is required to mediate pathology. Another major advantage of targeting the underlying mutation itself at the DNA level of the mutant *HTT* gene, is that such an approach would be expected to be efficacious without an understanding, and independent of, the key toxic events downstream, whether they occur at the RNA or protein level.

The *MSH3* gene as a therapeutic target

Development of a therapy to target the process of somatic expansion relies on the rational design of an agent that will selectively and safely slow or prevent somatic expansion. There are a variety of DNA repair genes that have been shown to modify somatic expansion and disease phenotypes in HD and other REDs in both humans and

animal models. However, from both safety and efficacy data, *MSH3* repeatedly emerges as a particularly appealing candidate.

MSH3 expression levels show a clear and dose-dependent link to impact on somatic expansion and disease symptoms,[37,41] while this clear link has not yet been demonstrated for some other repair pathway genes that have emerged as significant genetic modifiers of age of onset in HD. Specifically, a transcriptome-wide association study (TWAS) that tested the association between gene expression and residual age at onset in HD identified four genes and three loci that were significant after correction for multiple comparisons.[41] One of these was *MSH3*, for which decreased expression was linked to later HD onset, while for *FAN1*, another DNA repair gene, increased expression was associated with later onset,[92] consistent with the effects of loss of function knock-out alleles of *Msh3* and *Fan1* on somatic expansion in cell and animal models. A comparison of the heritability estimation predicted that for *FAN1 vs. MSH3*, 40% *vs.* 87%, respectively, of their contribution to the age at onset in HD could be explained by *cis*-expression effects.[41] Similarly, *MSH3* has a particularly useful therapeutic aspect in that it was shown to be the limiting resource in MutSβ activity, as deletion of only one allele lowered expansion in a myotonic dystrophy type 1 CTG repeat expansion model.[93] Human genetics studies provide further compelling evidence for the role of *MSH3*. For example, in one study of a cohort of DM1 patients, a polymorphism in *MSH3* was associated with a variation in somatic expansion,[94] indicating a direct relationship. Candidate gene studies in people with HD and DM1 also identified polymorphisms in the *MSH3* gene that were associated with disease progression.[37,51] More recently, *MSH3*, and *PMS2*, were revealed as genetic modifiers of X-linked dystonia Parkinsonism which is caused by the expansion of a genetically unstable CCCTCT repeat.[95]

Given the strong association between expression and impact on disease for *MSH3*, lowering *MSH3* expression represents a rational approach to treat HD and other REDs. ASOs can be rationally designed to specifically lower expression of a target gene and have shown to permit a rapid development path.[96,97] While *FAN1* is also a strong genetic modifier of HD, its activity suppresses somatic expansion, which makes it unsuitable for ASO mediated transcript degradation.[55,56]

Therapeutic Targeting of DNA Repair Genes to Halt Somatic Expansion

With advances in DNA technology, new genetically-based therapeutic approaches have been devised including inserting a wild-type version of a gene in loss of function disorders, and degrading the target gene mRNA by way of introduction of short nucleic acid molecules in the form of small interfering RNAs (siRNA) or antisense oligonucleotides (ASOs) in gain of function disorders.[98–101] For a dominant gain of function disease such as HD, ASOs may be a logical choice since: i) degradation of the mutant gene can be specifically targeted with an ASO; and, ii) a key cellular target are neurons that readily take up ASOs - as long as the ASO reaches the neuronal tissue at sufficient concentration. ASOs can be injected into the CSF for targeted delivery to the

brain, while limiting distribution to peripheral tissues.[102,103] In the case of *MSH3* lowering in HD, the target cells are primarily neurons in the striatum and cortex in the brain. To address the neuropathology of HD, *MSH3* lowering in peripheral tissues is not needed, and a brain focused ASO delivery may even be desirable.

ASOs can be designed to block expression (by engaging RNA degradation via an RNAse H-dependent mechanism), or induce alternative splicing, to skip exons and facilitate expression of a functional, albeit modified, protein product.[104–106] Multiple clinical trials are either ongoing or in preparation that are investigating safety and potential benefits of rationally designed molecules, including ASOs, siRNAs, and gene therapies, aiming to slow, delay or altogether prevent disease progression in HD and other REDs. Detailed recently in:[96,107]

To investigate possible therapeutic approaches targeting somatic expansion, mouse models that recapitulate somatic expansion in brain regions similar to people with HD have been employed. One of the first rodent models used for *in vivo* studies, the R6/2 mice, were generated by introducing the promoter and the exon 1 fragment of the human *HTT* gene, which includes the expanded CAG repeat, into the mouse genome. With ~ 120 repeats, the animals exhibit a very aggressive disease course, with a median survival of 15 weeks, and motor coordination deficits that were considered similar to the motor symptoms of the human disease[108,109] and also somatic expansion in the striatum as well as the cortex. Other often used HD models are “knock-in” (KI) models, in which the human HD mutation is inserted into the mouse *Htt* locus.[110–112] The KI models have been generated with various CAG lengths, with the Hdh^{Q175} model exhibiting arguably the most translationally relevant behavioral phenotype, while mice with fewer starting CAGs show very mild behavioral abnormalities late in their lives.[113–115] As the KI mouse models show somatic expansion in striatum and cortex, they are useful for investigating therapeutics that modulate somatic expansion. An allelic series of different CAG repeats lengths established repeat-length and age dependent transcriptional alterations in the striatum, with very pronounced alterations detected in mice with 140 CAG repeats by 6-months of age, but almost no alterations at 2-months of age.[114]

Corroborating the importance of *MSH3* for somatic expansion were studies in Hdh^{Q111} KI mice crossed onto backgrounds deficient in the *Msh3* gene. Homozygotic deletion of *Msh3* led to a complete ablation of somatic expansion[31] In heterozygote *Msh3* +/- mice, pronounced reduction in somatic expansion in the striatum was observed, while heterozygote *Msh2* +/- mice did not detectably reduce somatic expansion in the striatum, indicating two important points: first, lowering of *Msh3* by 50% was sufficient to achieve a marked therapeutic-type effect; and second, the level of *Msh3* was likely to be rate limiting.[31]

Safety Considerations of *MSH3* as a Target

The clear gene dose effect for *MSH3*, in both rodent models and humans, and the growing evidence that 50% lowering suffices to substantially reduce or halt somatic expansion bode well for therapeutic developments targeting *MSH3*. Lowering rather than abrogating *MSH3* reduces potential safety concerns and permits at least partially maintained function of the MutSβ complex for canonical DNA repair. These safety aspects are of particular importance for initiating treatment in people with HD prior to full motor manifestation. Importantly, two publications in 2019 showed that heterozygous loss of function alleles of *MSH3* do not lead to an overt cancer predisposition phenotype in humans.[116,117]

Further, in 2019, Triplet has interrogated the gnomAD database of humans with no known pediatric disorders. This analysis confirmed less constraint and greater tolerance of partial loss of function for *MSH3* than for *MSH2*, *PMS1*, *MLH1*, and *MLH3* which are all implicated in HD and other REDs (**Figure 2**). In particular, for some of these latter genes heterozygous loss of function has been linked to cancer predisposition syndromes in humans ~~(**Figure 2**)~~. In addition, genetic knockout models and targeted gene suppression in mouse studies have demonstrated safety of partial as well as full knockout of *Msh3*, while knockout of *Msh2*, *Msh6* and *Mlh1* shortened life span and increased tumor burden compared to wildtype animals.[118,119]

Notably, *MSH3* appears to be at least partially redundant and thus partial loss of function is tolerated better than deficiency of some other DNA mismatch repair genes such as *MSH6*.[118,120] Most prominently, heterozygous *MSH2* deficiency in both humans and mice has been associated with a predisposition to early cancer, and in particular Lynch Syndrome in humans. No such association has been shown for *MSH3*,[116,121] and acquired mutations within *MSH3* are associated with less severe cancer outcomes.[122] Safety considerations in support of *MSH3* were recently summarized in:[123]

The development of TTX-3360, an antisense oligonucleotide targeting *MSH3*

While the initial GWAS data identified multiple modifier genes involved in somatic expansion and HD onset, several recent publications have strengthened the association between lower *MSH3* expression, reduced somatic expansion, later disease onset and slower progression in HD and other REDs, validating *MSH3* lowering as a rational and logic therapeutic approach.[37,39,41,59] Towards this end, Triplet Therapeutics has initiated in 2019 the development of a novel ASO, TTX-3360, which selectively binds to *MSH3* mRNA and lowers its expression. ~~TTX-3360 has been studied in multiple rodent and non-human primates (NHPs) and has shown overall good safety and tolerability, and IND enabling studies are ongoing. As TTX-3360 has full sequence homology to the NHP, but not the rodent *Msh3* sequence, its effect on *MSH3* expression could only been studied in the latter. At clinically relevant and well tolerated dose levels, TTX-3360 administered via repeat intracerebroventricular (ICV) injections has shown sustained (>12 weeks) and pronounced (≥ 50%) reduction in *MSH3* expression in the striatum as~~

~~well as cortex. (https://chdifoundation.org/2021-conference/#antonijevic) As the NHPs used do not carry a pathologically expanded HTT gene, proof of concept of halting somatic expansion by lowering *MSH3* by approximately 50% was demonstrated in induced pluripotent stem cells generated from people with HD (https://chdifoundation.org/2020-conference/#bettencourt). Further, a tool ASO with sequence homology to the mouse *Msh3* gene has been administered to two different HD model mice, *i.e.* the aggressive R6/2 model and the phenotypically mild HD$^{Q111/-}$ model. In both models, reducing Msh3 mRNA in cortex and striatum by approximately 50% effectively halted somatic expansion in these brain areas (https://chdifoundation.org/2021-conference/#antonijevic).~~

T~~aken together, t~~he data with Triplet's ASOs are in line with published data demonstrating 1) that when *MSH3* expression is reduced by approximately 50% somatic expansion is markedly reduced[31] and 2) that ASOs once distributed to brain tissues, have a long duration of effect.[97] Triplet Therapeutics is preparing a First-in-human clinical trial in people with HD to start in the 2nd half of 2022.

Overcoming the challenge to deliver an ASO to the striatum and cortex

One persistent challenge of treating CNS diseases is effective delivery of drugs into the CNS, and in the case of HD and other CNS REDs, delivery to deep brain regions such as the striatum, along with the cortex. For a therapeutic that aims to halt somatic expansion, which has been shown to start in the striatum, followed by the cortex, effective drug delivery to those areas is paramount, while distribution to other brain areas seems less critical. In HD, it has been known for decades that the striatum is affected early on and very profoundly.[124]

MSH3 lowering targets the inappropriate response of the DNA repair system to the DNA structure formed by a pathologically expanded repeat, rather than a mutant gene. Therefore, allele selectivity is not relevant when targeting *MSH3*. Further, as discussed above, multiple data indicate that a 50% lowering of *MSH3* expression is sufficient to substantially reduce or halt somatic expansion, and thus the remaining protein can continue to exert its physiological function for DNA repair such that no increased ~~predisposition to~~cancer risk ~~ensues~~would be expected. Because of their specificity and spontaneous uptake by neurons, ASOs provide an appealing method to decrease *MSH3* expression specifically in neurons in target brain areas. Delivery of an ASO to the brain via intrathecal (IT) injections into the CSF is currently being pursued for multiple CNS indications, and this approach has been clinically validated in spinal muscular atrophy (SMA) with IT injections of the unconjugated ASO nusinersen.[125]

However, the remaining challenge for HD and other CNS REDs is that therapeutically relevant distribution to the striatum of an unconjugated ASO injected IT has not been demonstrated to date. Rather, in NHPs, even repeat IT administration of high doses of an ASO yielded good concentrations in the cortex, but limited distribution to the caudate nucleus and almost no distribution to the putamen, key HD-relevant areas that

are part of the striatum.[126] These preclinical data are in line with pPharmacokinetics (PK) modeling and simulation for tominersen, another unconjugated ASO (designed to lower *HTT*) injected via repeat IT injections in clinical trials in people with HD. Early results of these trials indicate markedly greater distribution to cortex than striatum and rapid distribution to plasma.[97] (https://chdifoundation.org/2020-conference/#schobel) In 2021, dosing with tominersen in HD clinical trials washas been discontinued due to an unfavorable benefit/risk assessment. The interested reader is referred to a comprehensive review of different *HTT* lowering approaches, including tominersen (Tabrizi *et al. Lancet. Neurol. in press* (2022)).

Factors to be considered for delivery to the deep brain include both neuroanatomy and CSF flow dynamics. An ASO that is injected into the lateral cerebral ventricle will be distributed to the brain following the physiological CSF flow, with one part flowing towards the foramina of Monro and then caudally through the ventricular system and around the convexity of the brain, while another smaller part remains in the lateral ventricles and moves through the brain parenchyma rostrally and therefore reaches the relevant deep brain sites.[127] This transependymal flow facilitates transport of the injected ASO, or any drug, to the brain parenchyma.[127]
The accuracy of the predicted flow dynamics has been demonstrated in rats after ICV injections of an ASO yielding broad and bilateral distribution.[128]
ICV injections confer the key advantage of effective delivery to deep brain regions, and for longer-term treatment also provide greater convenience to patients, while limiting distribution to peripheral tissues. With an implanted cerebroventricular port, the drug can be administered non-invasively, which in theory could extend to the possibility of administration in the patient's home (**Table 3**). Though implantation of an ICV device has been used for over 60 years, and repeatedly in the same people, for almost 20 years, this approach has typically been employed to administer chemotherapeutic agents for oncological indications.[129] Recently, the feasibility of repeat ICV administration for a neurological disorder has been demonstrated by the approval of Brineura®, in US and Europe, via biweekly ICV infusion, for a pediatric form of Batten disease:
https://www.accessdata.fda.gov/drugsatfda_docs/nda/2017/761052Orig1s000TOC.cfm

Summary and Outlook for HD Therapeutics

Significant progress in the understanding of HD pathophysiology has opened the door for novel therapeutic targets. Genetic modifiers, such as *MSH3*, operate through modulation of somatic expansion in a gene-dose dependent manner in HD and other REDs. Somatic expansion has been detected in the striatum in people with HD around the same time as first subtle clinical signs, *e.g.* caudate and putamen volume loss, CSF NfL elevations, and cognitive symptoms have been described, *i.e.* more than ten years before full motor manifestation.[5,32,130] The hypothesis has been put forward that occurrence of somatic expansion is a necessary second step in a two-step process for

disease manifestation, with step one being the inheritance of an expanded disease gene.[60]

As somatic expansion operates upstream of somatically expanding disease genes, it is an attractive therapeutic target with the prospect of treating 30+ CNS REDs with the same molecule and using the same route of administration. Of note, somatic expansion has been detected in the striatum and cortex in multiple CNS REDs (**Table 1**) and for many CNS REDs, disease manifestation is attributed to those changes in the striatum and cortex.[4,36,90,131,37,39–41,51,59,63,89] Using the ICV route, the investigational ASO TTX-3360 has been safely delivered to the striatum and cortex in more than 125 NHPs to date, resulting in pronounced and sustained reduction of *MSH3* expression ~~(https://chdifoundation.org/2021-conference/#antonijevic)~~.

Based on compelling preclinical data for both safety and efficacy, the first clinical trial with TTX-3360 will enroll premanifest and early manifest people with HD, equivalent to stages 1 (biomarker of pathogenesis, such as increases in NfL and caudate and putamen volume loss) and 2 (biomarkers and signs and symptoms, such as deterioration in cognitive and motor measures) in the new integrated staging system for HD (HD-ISS).[132] Once safety and target engagement have been demonstrated for TTX-3360 in people with HD, the indication spectrum could be rapidly expanded to include multiple CNS REDs. Each new indication will provide data that informs each other indication and will increase the safety data, allowing for efficient development of TTX-3360.

The insights into the role of certain genes in the DNA repair pathway for somatic expansion and disease phenotypes were only possible because of the participation of thousands of people with HD in non-interventional studies. The Enroll-HD registry has organized disperse data into a rich single database to inform drug development at multiple levels, *e.g.* from identification of new molecular target, such as *MSH3*, to disease staging and disease trajectories, and providing populations that can serve to augment control groups in clinical trials.

The enthusiastic participation of people with HD in clinical research for many decades has been indispensable for the progress made in our understanding of HD, as well as other REDs. The result is an ever-growing number of clinical trials aiming to demonstrate disease modification in HD, while there were very few such trials just a decade ago. Though the attrition rate of clinical trials is unfortunately high, more trials increase the chance for identifying effective therapeutics for people in HD over the next decade (**Table 4**).

With this perspective in mind, we should not forget families in low-income countries, such as Venezuela, that provided DNA leading to the discovery of the HD-causing mutation, and are a sad example of the havoc HD can wreak particularly on poor societies. The organization Factor-H is commended for working with impoverished families affected by HD in Colombia, Peru, and Venezuela to improve medical care and family planning (https://factor-h.org/).

As a scientific and drug development community, we must ensure that people with HD, all around the world, will get access to safe and effective drugs once approved.

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Table 1: REDs with described somatic expansion in striatum and/or cortex

RED	Gene with repeat expansion	Pathogenic repeat length threshold	Predominant repeat sequence	Inheritance pattern	Somatic expansion in striatum and/or cortex	References
Huntington disease (HD)	*HTT* (Huntingtin)	~36	CAG	Autosomal dominant	Yes	4
Spinocerebellar ataxia type 1 (SCA1)	*ATXN1* (Ataxin 1)	~40	CAG	Autosomal dominant	Yes	4
SCA2	*ATXN2* (Ataxin 2)	~32	CAG	Autosomal dominant	Yes	91
SCA3	*ATXN3* (Ataxin 3)	~52	CAG	Autosomal dominant	Yes	63
SCA7	*ATXN7* (Ataxin 7)	~33	CAG	Autosomal dominant	Yes	80
Dentatorubral-pallidoluysian-atrophy (DRPLA)	*ATN1* (Atrophin 1)	~48	CAG	Autosomal dominant	Yes	63,70,74
Myotonic dystrophy type 1 (DM1)	*DMPK* (DM protein kinase)	~50	CTG	Autosomal dominant	Yes	83
Fragile X syndrome (FXS)	*FMR1* (Fragile X mental retardation 1)	~200	CGG	X-linked recessive	Yes	56,133
Fragile-X linked Tremor Ataxia Syndrome (FXTAS)	*FMR1* (Fragile X mental retardation 1)	~55	CGG	X-linked dominant	Yes	133,134
Friedreich ataxia (FDRA)	*FXN* (Frataxin)	~70	GAA	Autosomal recessive	Yes	56,135
X-linked dystonia parkinsonism	*TAF1* (TATA-box binding protein associated factor 1)	~30	CCCTCT	X-linked recessive	Yes	136

Table 2: Unique features of therapeutic targeting of *MSH3* to treat HD and other REDs

Category	Unique features of therapeutic *MSH3* lowering
Target biology and disease relevance	• Genetic modifier with strong human and animal genetics validation • Gene-dose effect in humans and animals • Allele selectivity not applicable • Acts upstream of individual disease gene • Relevant for HD and 30+ other CNS REDs
Assumed mechanism	• 50% lowering of mRNA halts or substantially reduces somatic expansion at time of intervention • Without further somatic expansion, ever increasing toxicity of mHTT is stopped • Halting or slowing somatic expansion is expected to also reduce CAG-length dependent production of the aberrantly spliced exon 1/intron 1 transcript of *mHTT*
Required knockdown level	• 50% in striatum and cortex • No safety concerns described in humans with heterozygous loss of function
Target population	• No known interference with brain development or brain function, and therefore the intervention can be administered to young people with HD

Table 3: Differentiation of ICV vs IT injections of an unconjugated ASO, such as TTX-3360, in NHPs

Category	ICV administration	IT administration
Exposure in HD-relevant brain regions	• Distribution follows physiological CSF flow direction • Desired ASO distribution to striatum and cortex can be achieved at safe and well tolerated dose levels while achieving ~50% target lowering • Much less peripheral tissue exposure compared to the brain	• Distribution involves non-physiological CSF flow direction • Exposure is minimal / low in striatum at safe and well tolerated dose levels; target lowering by ~50% is not feasible in the striatum • Rapid and marked peripheral tissue exposure
Human pharmacokinetic and pharmacodynamic (PK/PD) model development	• Frequent CSF sampling via an implanted ICV device is feasible and allows to establish a robust PK model in humans • Measurement of target mRNA in CSF collected from lateral ventricles (next to the caudate nucleus) is expected to reflect striatal target engagement	• Frequent CSF sampling is not easily feasible; such sampling requires an indwelling IT catheter • Target mRNA collected from lumbar CSF is expected to less well reflect striatal target engagement
Clinical dosing	• Infrequent clinical dosing can maintain desired target engagement in striatum and cortex	• More frequent dosing and/or higher doses needed to achieve sufficient target engagement in the striatum • Risk of ASO accumulation in cortex and potentially adverse effects

Table 4: Overview of rationally designed therapeutic programs for HD

Therapeutic mechanism	Therapeutic modality	Route of administration	Stage of development	Sponsor *Comments*
HTT lowering	ASO	Repeat IT (intrathecal)	Phase 3	Roche, Ionis *Dosing discontinued in March 2021.* *Post-hoc analyses indicate potential benefit in younger and earlier stage people with HD*
HTT lowering	Gene therapy (miRNA, AAV-5)	One time Intra-striatal	Phase 1/2	UniQure
mHTT lowering	ASO (mutant allele selective)	Repeat IT	Phase 1	Wave Life Sciences *First two trials with two distinct ASOs terminated in 2021*
HTT lowering	Small molecule	Oral	Phase 2	Novartis
HTT lowering	Small molecule	Oral	Phase 1	PTC Therapeutics *Phase 2 start expected soon*
HTT lowering	Small molecule	Not disclosed	Preclinical	Vertex (RiboMetrix) Design Therapeutics Expansion Therapeutics Arrakis SkyHawk Therapeutics
HTT lowering	Zinc-Finger Technology (genome engineering)	Not disclosed	Preclinical	Sangamo Therapeutics, Takeda
CAG targeting	ASO	IV (anticipated)	Preclinical: in IND-enabling studies	Neubase Therapeutics
CAG targeting	ASO	Not disclosed	Preclinical	VICO Therapeutics
MSH3 lowering	ASO	Repeat ICV (intracerebro-ventricular)	Preclinical: in IND enabling studies	Triplet Therapeutics
MSH3 lowering	Small molecule	Oral (anticipated)	Preclinical	LoQus23 Therapeutics

Figure 1: Illustration of key features of the proposed mechanism of somatic expansion and intervention via lowering MSH3

(A) It is hypothesized that normal duplex DNA in the region of the expanded repeats is disassociated, and that repeats re-annealed out of register to form small loop-outs. (B) These loops provide a substrate for binding of MutSβ ~~(dimer of MSH2 and MSH3)~~. When DNA strands separate for events such as transcription, binding of MutSβ to the loops is likely the first of multiple events of the inappropriate DNA repair process leading to the aberrant filling in of gaps and creating longer CAG repeats (C). As this process repeats, the number of repeats increases (D).
(E) MutSβ is proposed to play a critical initiating role in this process. Reducing one of its members, i.e. MSH3, is hypothesized to reduce dimer formation, thereby slowing or halting the process of adding repeats and thus somatic expansion.

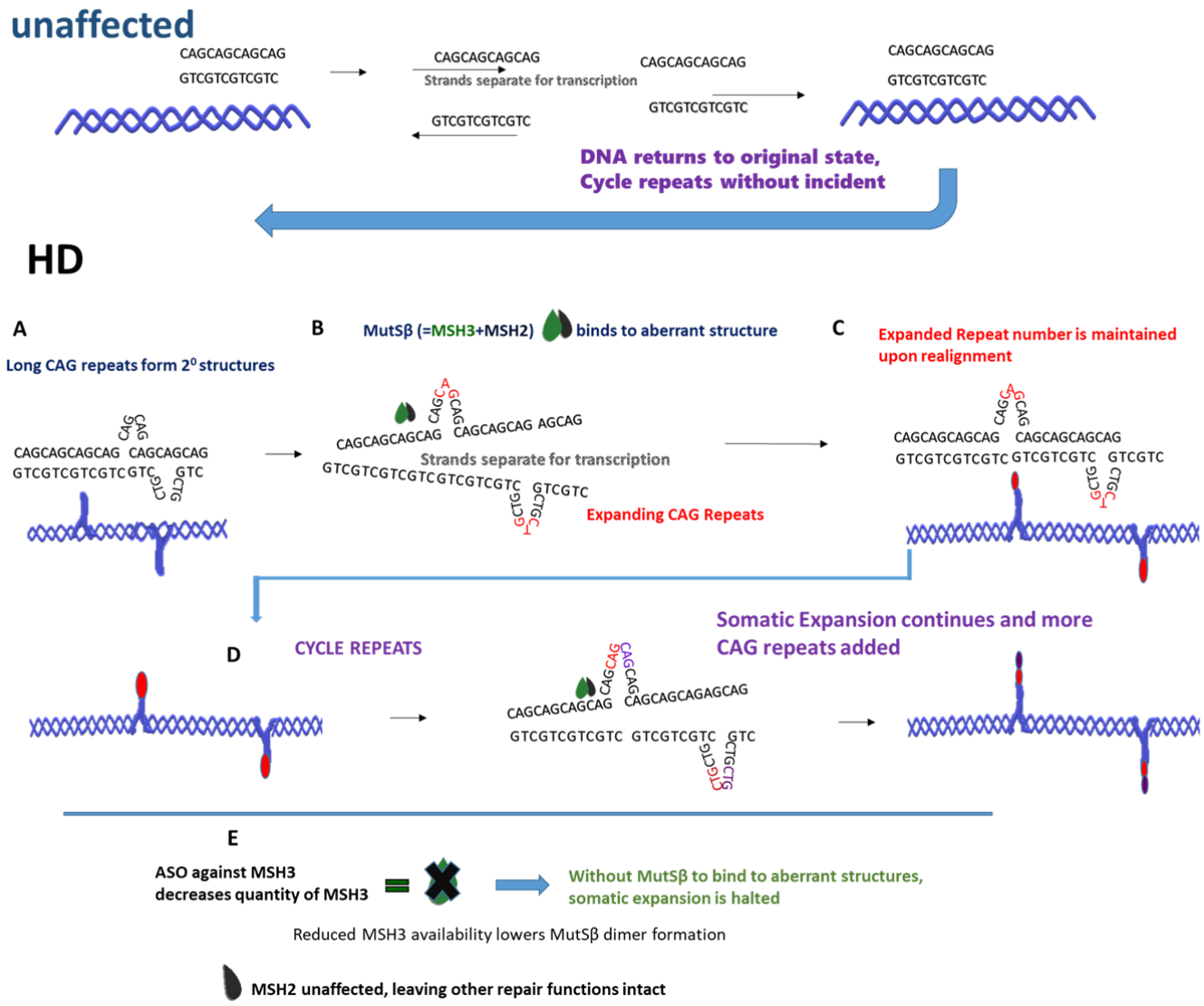
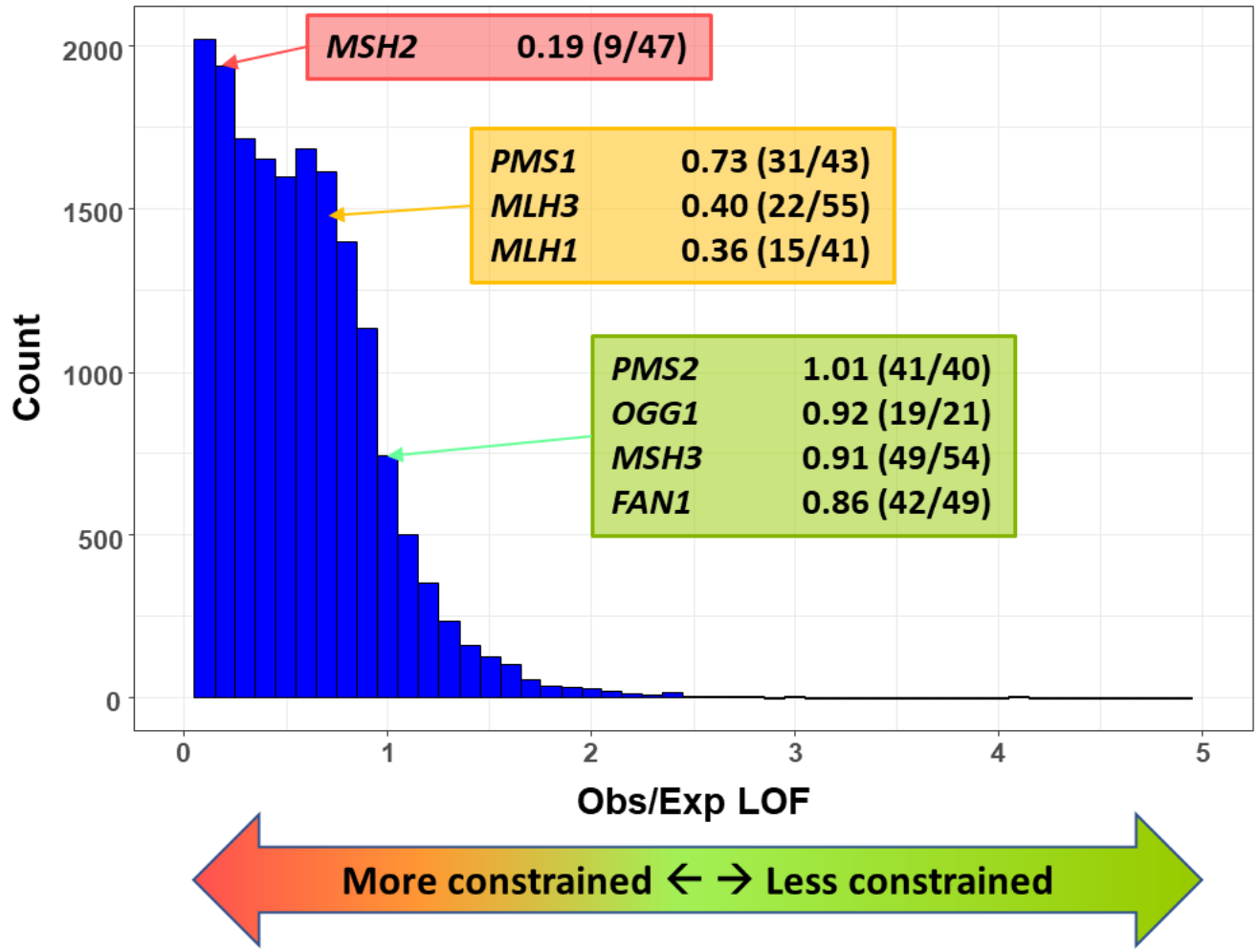

unaffected
CAGCAGCAGCAG
GTCGTCGTCGTC
Strands separate for transcription
DNA returns to original state,
Cycle repeats without incident
HD
A
Long CAG repeats form 2⁰ structures
B
MutSβ (=MSH3+MSH2) binds to aberrant structure
Strands separate for transcription
Expanding CAG Repeats
C
Expanded Repeat number is maintained upon realignment
D
CYCLE REPEATS
Somatic Expansion continues and more CAG repeats added
E
ASO against MSH3 decreases quantity of MSH3
Without MutSβ to bind to aberrant structures, somatic expansion is halted
Reduced MSH3 availability lowers MutSβ dimer formation
MSH2 unaffected, leaving other repair functions intact

Figure 2: Constraint on likely loss of function alleles in healthy people (gnomAD database: https://gnomad.broadinstitute.org/)

This database comprised at the time of analysis (2019) > 100,000 ~~healthy~~ human exome sequences from individuals with no known early onset disorders. Genes that tolerate loss of function (abbreviated LoF in the figure) are more likely to be safely inhibited or knocked down. MSH3 was among the top 15% least constrained genes in the genome.


MSH2 0.19 (9/47)
PMS1 0.73 (31/43)
MLH3 0.40 (22/55)
MLH1 0.36 (15/41)
PMS2 1.01 (41/40)
OGG1 0.92 (19/21)
MSH3 0.91 (49/54)
FAN1 0.86 (42/49)
Count
2000
1500
1000
500
0
0
1
2
3
4
5
Obs/Exp LOF
More constrained ← → Less constrained