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Intergenerational and striatal CAG repeat instability in Huntington's disease knock-in mice involve different DNA repair genes

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Abstract

Modifying the length of the Huntington's disease (HD) CAG repeat, the major determinant of age of disease onset, is an attractive therapeutic approach. To explore this we are investigating mechanisms of intergenerational and somatic *HD* CAG repeat instability. Here, we have crossed *HD* CAG knock-in mice onto backgrounds deficient in mismatch repair genes, *Msh3* and *Msh6*, to discern the effects on CAG repeat size and disease pathogenesis. We find that different mechanisms predominate in inherited and somatic instability, with Msh6 protecting against intergenerational contractions and Msh3 required both for increasing CAG length and for enhancing an early disease phenotype in striatum. Therefore, attempts to decrease inherited repeat size may entail a full understanding of Msh6 complexes, while attempts to block the age-dependent increases in CAG size in striatal neurons and to slow the disease process will require a full elucidation of Msh3 complexes and their function in CAG repeat instability.

Keywords

Huntington's disease; trinucleotide; instability; repeat; striatum; repair; mouse; knock-in; pathogenesis

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Introduction

Huntington's disease (HD) is a dominantly inherited neurodegenerative disorder caused by the expansion of a CAG repeat within the *HD* gene encoding huntingtin (Huntington's disease collaborative research group 1993). The *HD* CAG expansion initiates a protracted cascade of events that results initially in the degeneration of medium-spiny neurons in the striatum, motor, cognitive and psychiatric decline and eventual death (Vonsattel et al. 1985; Harper 1999). The mutant *HD* CAG repeat is remarkably unstable when transmitted to subsequent generations, with a bias towards expansion in transmissions from fathers (Duyao et al. 1993; MacDonald et al. 1993; Zuhlke et al. 1993; Telenius et al. 1994; Wheeler et al. 2007). The repeat also exhibits somatic instability, undergoing expansion particularly in the striatum (Telenius et al. 1994; Kennedy et al. 2003; Shelbourne et al. 2007; Veitch et al. 2007; Gonitel et al. 2008). Significantly, age of onset and disease severity are highly dependent on CAG length (Duyao et al. 1993; Andrew et al. 1993; Snell et al. 1993; Stine et al. 1993; Gusella et al. 1996). Therefore, as intergenerational instability alters the inherited *HD* CAG repeat length and somatic instability further alters repeat length in the target tissue, understanding the factors that influence both intergenerational and striatal instability is critical as these factors may modify the disease.

HD homologue *(Hdh)* CAG knock-in mice (White et al. 1997; Wheeler et al. 1999) that replicate the genetic mutation in HD patients provide ideal models in which to study the instability of the *HD* CAG repeat in its appropriate genomic context. We have shown that Hdh^{Q111} knock-in mice recapitulate many features of repeat instability seen in patients, namely intergenerational repeat length changes with a paternal expansion bias at frequencies seen in humans, and somatic expansion that is predominant in striatum (Lloret et al. 2006; Wheeler et al. 1999). Importantly, Hdh^{Q111} mice, exhibiting accurate expression of mutant huntingtin, also display early presymptomatic phenotypes that exhibit key features of the human disease mechanism, namely, dominant inheritance, CAG length and time dependence, and striatal specificity, allowing modifiers of the *HD* CAG pathogenic process to be tested (Fossale et al. 2002; Gines et al. 2006; Gines et al. 2003; Wheeler et al. 2000; Wheeler et al. 2002; Wheeler et al. 2003;). Significantly, the CAG repeat length dependence of knock-in phenotypes provides the possibility of testing directly whether factors that alter repeat length also alter the pathogenic process.

We previously investigated the role of mismatch repair gene *Msh2* in *HD* CAG repeat instability and the *HD* CAG pathogenic process in Hdh^{Q111} mice (Wheeler et al. 2003). In paternal transmissions *Msh2* was required for intergenerational Hdh^{Q111} CAG repeat expansions and protected against contractions. *Msh2* was also required for striatal Hdh^{Q111} CAG repeat instability and dramatically enhanced an early histological phenotype, the accumulation or epitope-accessibility of a conformation of full-length mutant huntingtin in striatal neurons, as revealed by nuclear immunostaining with the EM48 anti-huntingtin antibody. This finding strongly suggested that striatal instability contributes to the Hdh^{Q111} pathogenic process.

Here, taking advantage of the opportunity afforded by Hdh^{Q111} mice to dissect both intergenerational and somatic instability as well as *HD* CAG pathogenesis, we have investigated Msh2's binding partners, Msh3 and Msh6 (Acharya et al. 1996), in these processes, as well as nucleotide excision repair gene *Xpc* with the aim of exploring the roles of other DNA repair pathways. We have crossed Hdh^{Q111} mice onto backgrounds deficient in either *Msh3*, *Msh6* or *Xpc* to determine whether: 1. these genes influence *HD* CAG striatal and intergenerational instability in a precise genetic model of HD, 2. the same or different mechanisms underlie striatal and intergenerational instability, and 3. these genes are modifiers

of an early, dominant, CAG length-dependent phenotype, nuclear mutant huntingtin immunostaining in striatal neurons.

Materials and Methods

Mice

Hdh^{Q111} knock-in mice (White et al. 1997; Wheeler et al. 1999) were maintained on a CD1 (Charles River Laboratories) background. *Msh3* and *Msh6* knockout mice (Edelmann et al. 2000; Edelmann et al. 1997) (C57BL/6J) were obtained from Dr. Winfried Edelmann and *Xpc* knockout mice (Cheo et al. 1997) (C57BL/6J) were obtained from Dr. Errol Friedberg. *Msh2* knockout mice (129Ola/FVB) were from Dr. Hein te Riele (Toft et al. 1999). *Hdh*$^{Q111/+}$ mice were crossed with each DNA repair knockout strain to generate mice heterozygous for each mutation. These mice were then crossed to generate *Hdh*$^{Q111/+}$ littermates that were wild-type (+/+), heterozygous (+/-) or homozygous mutant (-/-) for each DNA repair gene. These mice were either used for assessment of somatic effects (instability, nuclear huntingtin immunohistochemistry), or were the transmitting parents for the intergenerational instability experiments. Genetic background can modify instability and nuclear huntingtin accumulation (Lloret et al. 2006). However, assuming that such modifier genes segregate independently from the repair genes being analyzed they will occur equally in the repair gene mutants and their wild-type littermates. Thus, sufficient numbers of mice of each genotype will allow identification of effects of the repair gene mutants over and above those due to genetic background. Therefore, to minimize potential confounding effects of genetic background all genotypic comparisons for intergenerational instability, somatic instability and EM48-immunohistochemistry were performed using littermates. The number of mice we have used in each analysis was based on numbers of mice needed to detect modifier effects of the *Msh2* gene on a mixed genetic background (Wheeler et al. 2003). All analyses were with heterozygous *Hdh*^{Q111}/*Hdh*$^{+}$ mice. All animal procedures were carried out to minimize pain and discomfort, under an approved Institutional Animal Care and Use Committee protocol.

Genotyping and HD CAG repeat length determination

Genomic DNA was isolated from tail biopsies at weaning (for routine genotyping and intergenerational instability analysis) or from adult tail and striatum dissected from adult mice brains (for somatic instability analysis) using the PureGene DNA isolation kit (Gentra, Minneapolis, MN, USA). Genotyping of the *Hdh*^{Q111} knock-in allele was carried out using a combination of a human-specific PCR assay that amplifies the *HD* CAG repeat from the knock-in allele but does not amplify the mouse sequence, and a wild-type mouse-specific assay that generates a PCR product from the wild-type mouse allele but not from the knock-in allele. The human-specific assay has been previously described (Mangiarini et al. 1997). For the wild-type mouse-specific assay primers 5′-CCTGGAAAAGCTGATGAAGG (forward) and 5′-TGGACAGGG AACAGTGTTGGC (reverse) were used in a PCR buffer containing 67 mM Tris-HCl pH 8.8, 16.7 mM $(NH_4)_2SO_4$, 2 mM MgCl2, 0.17 mg/mg BSA, 10 mM 2-mercaptoethanol, 10% DMSO, 200μM dNTPs, 5 ng/μl primers with 0.5 U *Taq* polymerase (Perkin Elmer). Cycling conditions were 94°C 90 sec, 35 cycles of 94°C 30 sec, 56°C 30 sec, 72°C 90 sec, followed by 10 minutes at 72°C. Products were resolved in 0.8% agarose gels. DNA repair knockout mice were genotyped as described (Cheo et al. 1997; Edelmann et al. 2000; Edelmann et al. 1997; Toft et al. 1999).

HD CAG repeat size was determined using the human *HD* CAG repeat-specific PCR assay (Mangiarini et al. 1997). The forward primer was fluorescently labeled with 6-FAM (Perkin Elmer) and products were resolved using either the ABI 377 or AB1 3730xl automated DNA analyzer (Applied Biosystems). GeneScan and Genotyper software packages with GeneScan

500-TAMRA as internal size standard (ABI 377) or GeneMapper v3.7 with GeneScan 500-LIZ as internal size standard (ABI 3730) were used to assign repeat size. Runs included the same control DNAs of known *HD* CAG repeat size. The *HD* CAG size was assigned as the highest peak in the GeneScan trace.

Analysis of intergenerational instability

For assessment of intergenerational instability, breeding pairs were established between 2-4 different Hdh^{Q111}/Hdh^{+} mice of each sex and DNA repair gene genotype (+/+, +/- and -/-) and $Hdh^{+/+}$ wild-type littermates that were either +/+ or +/- for the DNA repair gene mutation. Genomic DNA was extracted from tail biopsies of $Hdh^{Q111/+}$ transmitting parents and $Hdh^{Q111/+}$ progeny at weaning. Amplification of the *HD* CAG repeat and repeat size analysis were carried out as described above. Intergenerational instability was determined by comparing *HD* CAG repeat size in the $Hdh^{Q111/+}$ transmitting parent with those in the $Hdh^{Q111/+}$ progeny. Parent and pups were compared in the same ABI automated DNA sequencer run.

Immunohistochemistry

Immunohistochemistry was carried out on 7 μm paraffin-embedded coronal sections of brains perfused with periodate-lysine-paraformaldehyde as described (Wheeler et al. 2002). Immunostaining with polyclonal anti-huntingtin antibody EM48 (amino acids 1-256) (Gutekunst et al. 1999) was as described (Wheeler et al. 2002). All EM48 immunostaining experiments were performed under identical conditions. Diffuse EM48 immunostaining was quantified in striata from 3-5 $Hdh^{Q111/+}$ mice of each DNA repair genotype (+/+, +/-. -/-) to be investigated at 5 months of age. The mean nuclear stain intensity was determined in four 750μm × 500μm regions of dorsal striatum, matched in terms of their anterior/posterior location, using the 'histogram' function in Adobe Photoshop to convert the signal intensity in all stained nuclei to arbitrary units. Background signal, the mean value for 10 fields within each 750μm × 500μm region analyzed, was subtracted from the nuclear signal. To quantify the total amount of nuclear stain in a way that reflects both the immunostaining intensity and the number of immunostained nuclei, a staining index (SI) was calculated as the product of the mean nuclear stain intensity and the number of stained nuclei for each 750μm × 500μm region analyzed.

Statistical analyses

Intergenerational instability—Progeny genotype has been found to influence intergenerational instability (Savouret et al. 2003). Therefore we first assessed the influence of progeny genotype (+/+, +/-, -/-) on intergenerational instability in transmissions from heterozygous parents. There was no effect of progeny genotype on intergenerational instability for any of the genes analyzed (data not shown), therefore progeny genotype was not accounted for in subsequent comparisons of parental genotype. Although we attempted to match transmitting parents as closely as possible for CAG length and age, we nevertheless controlled for these variables in our statistical analyses to ensure that these factors did not confound the outcome.

We used four statistical models in order to assess the influence of parental genotype on repeat length changes transmitted to progeny. To compare overall repeat size change distributions we used a multinomial distribution with a cumulative logit function to determine the probability of having a cumulative higher change than the cumulative lower changes. We also compared the overall frequencies of changed versus unchanged alleles using a binomial distribution. As additional tests we used binomial distributions to model the odds of expanded alleles compared to grouped unchanged and contracted alleles ('Risk' model) as well as the odds of contracted alleles compared to grouped unchanged and expanded alleles ('Protective' model). Analyses in all cases were carried out using Generalized Estimating Equations (GENMOD procedure in

SAS v 9.1) (SAS Institute 1999), controlling for parental CAG repeat size, parental age and for the correlation between pups from the same parent. We set the alpha level to 0.01, since a Bonferroni adjustment for multiple comparisons is too conservative given the correlated nature of the tests performed.

Immunohistochemical staining—Statistical analysis to compare quantified immunohistochemical staining index values between different genotypes was carried out using Generalized Estimating Equations (GENMOD procedure in SAS) adjusting for CAG size and for repeated observations from each mouse.

Results

Intergenerational repeat instability

The *Hdh*^{Q111} CAG repeat exhibits an expansion bias in paternal transmissions and a contraction bias in maternal transmissions (Lloret et al. 2006; Wheeler et al. 1999; Wheeler et al. 2003). We previously showed that the absence of Msh2 abolished paternal *Hdh*^{Q111} CAG expansions and resulted in an increased frequency of contractions (Wheeler et al. 2003). Msh2's binding partners Msh3 and Msh6 (Acharya et al. 1996) might therefore also play a role in generating paternal repeat expansions and/or protecting against paternal contractions. We were also interested to test whether *Xpc*, encoding the major DNA recognition protein in global genome nucleotide excision repair (Volker et al. 2001), influenced intergenerational instability, particularly the maternal repeat length changes and paternal repeat contractions that did not depend on Msh2 (Wheeler et al. 2003). To determine the roles of these genes in intergenerational *Hdh*^{Q111} CAG repeat instability the *Hdh*^{Q111} allele was crossed onto *Msh3*-, *Msh6*- and *Xpc*-deficient mouse strains and the size of the *Hdh*^{Q111} CAG repeat was determined in heterozygous *Hdh*$^{Q111/+}$ male and female parents and their progeny.

To determine the influence of the DNA repair genes on intergenerational instability several statistical methods were used that captured the magnitude, frequency and direction of the repeat length changes (see Materials and Methods for details). First, the overall distribution of repeat length changes was modeled using a multinomial distribution to ascertain the likelihood of having a positive repeat size change. Second, the frequency of changed alleles (irrespective of direction) versus unchanged alleles was modeled using a binomial distribution. Finally, we wished to determine the effect of the DNA repair genes on the relative frequencies of expansions and contractions. As the exclusion of unchanged alleles resulted in loss of data we instead used two alternative binomial distributions: the first modeled the odds of positive change ('Risk' model) by comparing the frequency of expansions with the combined frequencies of contractions and unchanged alleles. The second distribution modeled the odds of negative change ('Protective' model) by comparing the frequency of contractions with the combined frequencies of expansions and unchanged alleles. In all statistical models odds ratios (OR) were determined for homozygous and heterozygous DNA repair gene knockout mice compared to wild-type controls. Table 1 and Supplementary Table 1 show the results of these analyses for paternal and maternal transmissions, respectively.

We first quantified repeat length changes in paternal transmissions from *Msh2*$^{+/+}$, and *Msh2*$^{-/-}$ males, as well as *Msh2*$^{+/-}$ males, which had not previously been evaluated (Wheeler et al. 2003). As shown in Figure 1A and Table 1, expansions were eliminated in transmissions from *Msh2*$^{-/-}$ males, as previously found (Wheeler et al. 2003), with a statistically significantly lower odds of having a higher repeat size change in transmissions from *Msh2*$^{-/-}$ compared to *Msh2*$^{+/+}$ males (OR=0.2, p=0.0065). In transmissions from *Msh2*$^{+/-}$ males, the frequency of expansions was also reduced compared to *Msh2*$^{+/+}$ males, but this trend did not reach statistical significance. Therefore, the absence of two *Msh2* alleles appears necessary to eliminate expansions in paternal transmissions of the *Hdh*^{Q111} CAG repeat.

We then investigated *Xpc* to assess whether the global genome nucleotide excision repair pathway was involved in intergenerational instability. The effect of *Xpc* on paternal and maternal transmissions of the Hdh^{Q111} CAG repeat is shown in Figure 1B. There was no significant effect of *Xpc* genotype on maternally inherited Hdh^{Q111} CAG repeat length changes. The absence of *Xpc* did result in a slight reduction in the frequency of paternal contractions with a corresponding increase in the frequency of unchanged alleles. However, these data did not reach statistical significance (Table 1), and, given the large number of paternal transmissions analyzed (84 $Xpc^{+/+}$ and 55 $Xpc^{-/-}$), we propose that Xpc is unlikely to contribute in any significant way to paternal Hdh^{Q111} CAG repeat contractions.

We then analyzed Msh2's binding partners Msh6 and Msh3. The effects of *Msh6* and *Msh3* genotype on paternal and maternal Hdh^{Q111} transmissions are shown in Figures 1C and 1D respectively. There was no significant effect of *Msh6* or *Msh3* genotype on maternally inherited Hdh^{Q111} CAG repeat length changes, consistent with the lack of involvement of Msh2 in intergenerational instability in female transmissions (Wheeler et al. 2003). In transmissions from $Msh3^{+/-}$ and $Msh3^{-/-}$ males, there was a shift from expansions to unchanged and contracted alleles compared to transmissions from $Msh3^{+/+}$ males (Figure 1D). However, this did not reach statistical significance at the pre-specified significance level of 0.01 (Table 1), suggesting that the majority of Msh2-mediated paternal expansions occur independently of Msh3. *Msh6* genotype also did not significantly influence the expansion frequency in paternal transmissions (Figure 1C, Table 1). Therefore, Msh2-mediated paternal expansions of the Hdh^{Q111} CAG repeat also occur independently of Msh6. However, in transmissions from $Msh6^{+/-}$ males there was a statistically significant increase in contractions compared to $Msh6^{+/+}$ males (changed versus unchanged OR=4.98, $p<0.0001$; 'Protective' model OR=4.28, $p=0.009$), suggesting that Msh6 may protect against paternal contractions (Figure 1C, Table 1). Interestingly, in transmissions from $Msh6^{-/-}$ males, the distribution of repeat length changes was similar to wild-type, suggesting that other mechanisms may compensate for the increase in contractions caused by the absence of Msh6. As Msh2 also protected against paternal repeat contractions (Figure 1A, Table 1), our results suggested that this protective effect is likely to be mediated by Msh2-Msh6 dimers.

Somatic repeat instability

Hdh^{Q111} mice display age-dependent, expansion-biased somatic instability of the Hdh^{Q111} CAG repeat in the striatum that becomes apparent by 5 months of age. In contrast, tail DNA does not exhibit somatic instability at this age (Wheeler et al. 1999). We previously showed that Hdh^{Q111} CAG striatal instability was eliminated in $Msh2^{-/-}$ mice (Wheeler et al. 2003). We have now determined striatal instability of the Hdh^{Q111} CAG repeat in heterozygous $Msh2^{+/-}$ mice to assess the effects of removing a single *Msh2* allele. DNA was extracted from dissected striata of $Hdh^{Q111/+}$ mice at 5 months of age and repeat size determined by GeneScan analysis of the PCR-amplified Hdh^{Q111} CAG repeat. As shown in Figure 2A, $Msh2^{+}/Msh2^{+}$ striatum displayed somatic instability as indicated by a characteristic bimodal repeat size distribution, not seen in somatically stable tail DNA at the same age. $Msh2^{+/-}$ striatum showed a similar bimodal repeat size distribution to $Msh2^{+}/Msh2^{+}$ striatum, in contrast to $Msh2^{-/-}$ striatum previously shown to have no somatic instability (Wheeler et al. 2003). Therefore, the absence of a single *Msh2* allele was not sufficient to eliminate somatic instability in Hdh^{Q111} striatum.

To delve further into the underlying mechanism and to explore an additional DNA repair pathway, we then evaluated the effects of eliminating *Xpc*, *Msh6* and *Msh3* on striatal Hdh^{Q111} CAG repeat instability in $Hdh^{Q11/+}$ mice at 5 months of age. As shown in Figure 2B, $Xpc^{+/+}$ and $Xpc^{-/-}$ striata both displayed a bimodal repeat size distribution characteristic of somatically unstable Hdh^{Q111} CAG repeats, showing that *Xpc* does not play a major role in

Hdh^{Q111} CAG repeat striatal instability. Similarly, as shown in Figure 2C, *Msh6*$^{+/+}$, *Msh6*$^{+/-}$ and *Msh6*$^{-/-}$ striata all displayed a bimodal repeat size distribution characteristic of somatically unstable *Hdh*^{Q111} CAG repeats. Therefore, Msh6 does not play a major role in *Hdh*^{Q111} CAG repeat striatal instability.

In contrast, as shown in Figure 2D, while *Msh3*$^{+/+}$ striatum displayed a bimodal repeat size distribution characteristic of somatically unstable *Hdh*^{Q111} CAG repeats, the GeneScan trace in *Msh3*$^{-/-}$ striatum was similar to that seen in tail, showing that Msh3 was required for striatal instability. Interestingly, in *Msh3*$^{+/-}$ striatum the GeneScan trace was not bimodal but was slightly broader than that in *Msh3*$^{-/-}$ striatum, showing that striatal instability is greatly reduced in the absence of only a single *Msh3* allele. These results indicate that *Hdh*^{Q111} CAG striatal instability is likely to be mediated via Msh2-Msh3 dimers. Furthermore, the finding that striatal instability is significantly reduced in *Msh3*$^{+/-}$ heterozygotes but not in *Msh2*$^{+/-}$ heterozygotes indicates that Msh3 levels are a limiting factor in this process.

Nuclear mutant huntingtin immunostaining

The time-dependent immunostaining of mutant huntingtin in the nuclei of striatal neurons is an early, dominant, CAG length-dependent phenotype in *Hdh*^{Q111} mice that is delayed in the absence of Msh2 (Wheeler et al. 2003). We hypothesized that Msh2 influenced nuclear huntingtin immunostaining as a result of its role in mediating somatic instability in the striatum. The distinct effects of *Msh2*, *Msh3*, *Msh6* and *Xpc* genes on striatal instability now afforded the opportunity to explore further the relationship between somatic *Hdh*^{Q111} CAG instability and the early disease process in *Hdh*^{Q111} striatum. We asked whether heterozygous loss of *Msh2* was sufficient to delay this early phenotype, and explored whether homozygous knockout of *Xpc* and homozygous and heterozygous knockouts of *Msh6* and *Msh3* might modify this phenotype. Thus, we examined nuclear mutant huntingtin immunostaining using the EM48 anti-huntingtin polyclonal antibody (Gutekunst et al. 1999) in striata of 5-month heterozygous *Hdh*$^{Q111/+}$ mice with the same *Msh2*, *Xpc*, *Msh6* and *Msh3* genotypes analyzed above for striatal instability. This antibody specifically detects a conformation of mutant huntingtin in striatal nuclei of *Hdh*^{Q111} mice (Wheeler et al. 2000).

Figure 3 shows the quantified staining indices, reflecting the immunostaining intensity and number of immunostained nuclei, for each DNA repair gene analyzed. The staining index was not significantly altered in the absence of a single *Msh2* allele (Figure 3A), in the complete absence of *Xpc* (Figure 3B), or in the absence of either one or two *Msh6* alleles (Figure 3C). By contrast, the staining index was significantly decreased by the loss of either one ($p=0.02$) or two ($p<0.001$) *Msh3* alleles. In contrast to Msh2, reduction of Msh3 levels, rather than elimination of Msh3, was sufficient to diminish nuclear mutant huntingtin immunostaining. *Msh3* genotype did not alter the total amount of huntingtin or the amount of mutant huntingtin in striatum (Supplementary Figure 1). Our findings that only Msh2 and Msh3 were unequivocal contributors to both striatal instability and to nuclear huntingtin immunostaining implicate Msh2-Msh3 dimers in both these events, and provide strong support for the hypothesis that somatic instability in the striatum contributes to the *HD* CAG pathogenic process.

Discussion

The length of the *HD* CAG repeat is the single most critical determinant of HD pathogenesis. Therefore, understanding the factors responsible for both intergenerational instability, which alters inherited repeat length, and somatic instability, which further alters repeat length in the striatum, may lead to novel therapeutic targets. Here, using a precise genetic model of HD, *Hdh*^{Q111} knock-in mice, we have explored mechanisms of intergenerational and striatal instability by performing a detailed analysis of the roles of DNA repair genes *Msh3*, *Msh6* and *Xpc*, and have asked directly whether these genes are modifiers of an early disease phenotype.

Intergenerational instability

Xpc did not play a significant role in *Hdh*^{Q111} CAG repeat intergenerational instability. This finding was in agreement with the lack of an effect of *XPC* on CAG repeat contractions in cultured human cells (Lin and Wilson 2007). In particular, *Xpc* did not significantly influence *Hdh*^{Q111} maternal repeat length changes or paternal contractions, neither of which depend on Msh2, implicating pathways other than mismatch repair and global genome nucleotide excision repair in these events.

Msh2 is required for paternal *Hdh*^{Q111} CAG repeat expansions and protects against paternal *Hdh*^{Q111} CAG repeat contractions, implicating Msh2's binding partners Msh3 and/or Msh6 in these events. We found that paternal expansions did not require Msh6, and were largely independent of Msh3, strongly contrasting with an absolute requirement for Msh2. These findings suggest that while some paternal expansions of the *Hdh*^{Q111} CAG repeat may be mediated by Msh2-Msh3 dimers, the majority are mediated by Msh2-dependent mechanisms that are independent of both Msh6 and Msh3. Interestingly a myotonic dystrophy type 1 (DM1) mouse model also exhibited intergenerational CTG expansions that were Msh2-dependent, but independent of both Msh6 and Msh3 (Foiry et al. 2006). However, these only accounted for a minority of the expansion events, with most being dependent on both Msh2 and Msh3. Therefore, it is likely that the effects of the mismatch repair proteins, perhaps at the level of repeat binding (Owen et al. 2005; Pearson et al. 1997), may be modulated by repeat sequence (CAG versus CTG), repeat length and genomic location.

We report a novel role for Msh6 in protecting against paternal intergenerational repeat contractions. As Msh2 also protected against repeat contractions, our results suggests that the protection is afforded by Msh2-Msh6 dimers. However, although the absence of a single *Msh6* allele resulted in an increased contraction frequency, this effect was not seen in the absence of two *Msh6* alleles. These results were intriguing, and we propose the following model to explain the effects of Msh2 and Msh6 (Figure 4). Two Msh2-dependent mechanisms play a role in paternal intergenerational *Hdh*^{Q111} CAG repeat instability: Msh2-dependent pathway (s) mediate expansions and Msh2-Msh6 protects against contractions (Figure 4A). The model assumes that repeat length can be influenced sequentially by each mechanism. Thus, the sum of each effect determines whether the repeat ultimately undergoes an expansion or a contraction, and the distribution of inherited repeat length changes depends on the relative influence of each mechanism. In transmissions from *Msh2*$^{-/-}$ fathers both mechanisms are eliminated, resulting in the loss of expansions and an increase in contraction frequency (Figure 4B). In transmissions from *Msh6*$^{+/-}$ fathers, the protective mechanism is reduced, resulting in an increased contraction frequency (Figure 4C). In transmissions from *Msh6*$^{-/-}$ fathers although contractions are increased, we hypothesize that the complete loss of Msh6 shifts the balance of Msh2 complexes in favor of Msh2-dependent pathway(s) that generate expansions sufficient to compensate for the increased number of contractions. Dynamic interactions between different Msh2 complexes have been described previously (Chang et al. 2000;Foiry et al. 2006;van den Broek et al. 2002). The net result of these two effects in *Msh6*$^{-/-}$ transmissions is a distribution of inherited repeat size changes that is similar to that in *Msh6*$^{+/+}$ transmissions (Figure 4D and Figure 1C). Therefore, from a therapeutic perspective, our results suggest that it may be possible to shorten the inherited *HD* CAG repeat length, the major determinant of onset age in HD, by reducing, but not eliminating, Msh6.

Striatal instability

Striatal *Hdh*^{Q111} CAG repeat instability is dependent on Msh2 (Wheeler et al. 2003). We show that striatal instability does not require Xpc or Msh6; however, it is completely dependent on Msh3, indicating that it is mediated via Msh2-Msh3 dimers. These results are consistent with roles of Msh2 and Msh3 in generating somatic CAG and CTG repeat expansions in other tissues

in HD and DM1 mouse models (Foiry et al. 2006; Manley et al. 1999; Owen et al. 2005; Savouret et al. 2003; van den Broek et al. 2002). Furthermore, our finding that Msh3 was a limiting factor in somatic instability was also reported in a DM1 mouse model (Foiry et al. 2006). Therefore, similar roles of Msh2-Msh3 in HD and DM1 mouse models suggest a common mechanism of somatic expansion for CAG and CTG repeat diseases. Significantly, we have shown here that Msh3 determines *HD* CAG repeat length in the striatum, the target of HD pathogenesis.

Msh2-Msh3 dimers have been shown to bind expanded CAG repeat hairpin structures *in vitro* (Owen et al. 2005), although this has not been demonstrated *in vivo*. Msh2-Msh3 binding may mediate the formation of such structures (Panigrahi et al. 2005), which are subsequently processed to generate expansions. It has been proposed that Msh2-Msh3 binding subverts the mismatch repair machinery to result in expansion (Owen et al. 2005). However, it is also possible that expansions occur as a consequence of active engagement of the mismatch repair process (Gomes-Pereira et al. 2004). Further experiments are needed to distinguish these possibilities.

A requirement for Msh3 in generating somatic expansions contrasted with the relatively minor role of Msh3 in generating intergenerational expansions. Msh2 homodimers and homomultimers have been identified (Acharya et al. 1996), and purified Msh2 can bind to mismatched bases (Alani et al. 1995), Holliday junctions (Alani et al. 1997) and CAG/CTG repeat structures (Pearson et al. 1997). The different requirements for Msh3 in striatal expansions and intergenerational expansions may reflect the binding affinities of Msh2 and Msh2-Msh3 to different repeat structures that may form in striatal cells and in spermatogenic cells. *eg.* purified Msh2 binds more strongly to CAG repeat secondary structures than CTG repeat secondary structures (Pearson et al. 1997). Thus, the formation of CTG versus CAG secondary structures in striatal and spermatogenic cells could result in the differential binding of Msh2, and potentially of Msh2-Msh3 dimers. Alternatively, the different requirements for Msh3 could reflect levels of expression of Msh3 in striatal versus sperrmatogenic cells. Interestingly, Msh2 and Msh3 expression patterns differed during mouse spermatogenesis (Richardson et al. 2000), suggesting that, at least for some activities, these two proteins may be functionally uncoupled. Our results imply that drugs that interfere with Msh2 binding to expanded CAG/CTG repeats may inhibit intergenerational expansions, whilst drugs that interfere with Msh2-Msh3 binding to expanded CAG/CTG repeats, or dimerization of Msh2-Msh3 may inhibit striatal expansions.

Nuclear mutant huntingtin immunostaining

Msh3 also modified nuclear mutant huntingtin immunostaining in the striatum, as found previously for Msh2 (Wheeler et al. 2003). As postulated for Msh2, Msh3 could either modify this early phenotype as a consequence of its effect on somatic instability, or via an alternative mechanism, unrelated to its role in somatic instability. In the present study, in which we have examined both somatic instability and nuclear huntingtin immunostaining in striata from mice of many different DNA repair genotypes, we observed a very good correlation between striatal instability and nuclear mutant huntingtin immunostaining. These data provide strong support for a direct contribution of somatic instability to this phenotype. Furthermore, the finding that Msh3 was a limiting factor both in somatic instability and in nuclear mutant huntingtin immunostaining implies a shared underlying mechanism. Thus, we propose that Msh2-Msh3-dependent striatal instability is itself a modifier of the *HD* CAG pathogenic pathway, accelerating CAG repeat length-dependent striatal phenotypes. Our results, therefore, suggest both Msh2 and Msh3 as potential therapeutic targets. Msh3 is of particular interest as cancer-causing mutations in Msh3 have not been found, and because eliminating only a single allele's worth of Msh3 was sufficient to slow the Hdh^{Q111} CAG pathogenic process.

Conclusions

Reducing *HD* CAG repeat length is an attractive therapeutic strategy. To determine potential therapeutic targets it is important to understand mechanisms underlying both intergenerational and striatal instability, as both process alter *HD* CAG repeat length. We have found that distinct Msh2-dependent mechanisms contribute to intergenerational and striatal *HD* CAG repeat instability. Our studies suggest Msh6 as a target for increasing intergenerational contractions, thus reducing the inherited *HD* CAG repeat length, and Msh3 as a target for eliminating striatal expansions. Importantly, we show directly that Msh3 is a modifier of the *HD* CAG pathogenic process, supporting a role of somatic instability in the disease, and suggesting novel routes of intervention in this devastating disorder.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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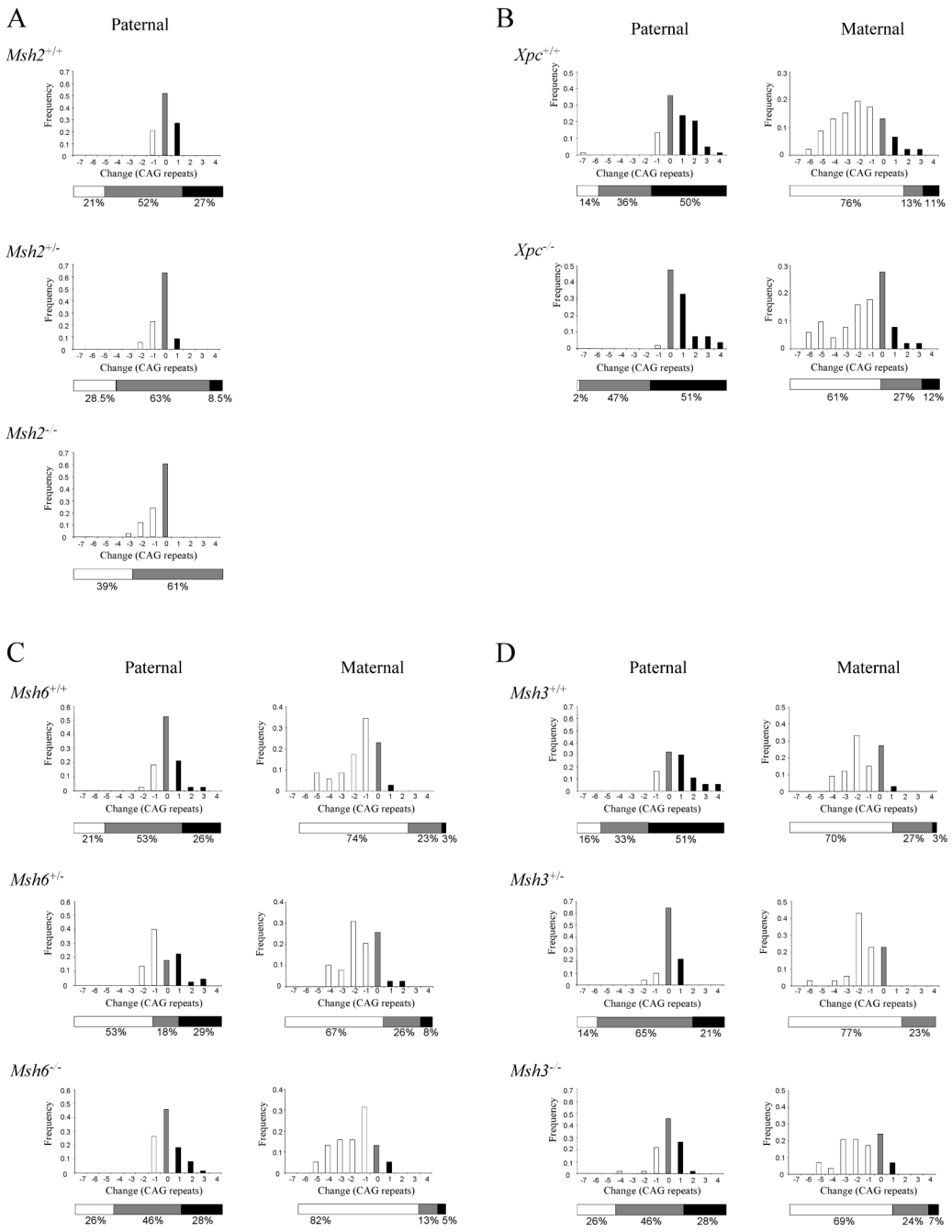

Figure 1.

Hdh^{Q111} CAG repeat intergenerational instability.

Hdh^{Q111} mice were crossed onto backgrounds deficient in *Msh2* (A), *Xpc* (B), *Msh6* (C) or *Msh3* (D). The change in *Hdh*^{Q111} CAG repeat length upon transmission from heterozygous *Hdh*$^{Q111/+}$ fathers or mothers to their progeny was determined. The total number of transmissions analyzed for each genotype, the mean age and mean CAG repeat length of the transmitting parents were as follows: (A) Paternal: *Msh2*$^{+/+}$ n=48, 5.4 months, 88 CAGs; *Msh2*$^{+/-}$ n=35, 5.2 months, 87 CAGs; *Msh2*$^{-/-}$ n=33, 4.4 months, 86 CAGs.
(B) Paternal: *Xpc*$^{+/+}$ n= 84, 3.8 months, 103 CAGs; *Xpc*$^{-/-}$ n=55, 4.0 months, 103 CAGs. Maternal: *Xpc*$^{+/+}$ n=55, 4.1 months, 103 CAGs, *Xpc*$^{-/-}$ n=51, 4.3 months, 102 CAGs).

(C) Paternal: *Msh6*$^{+/+}$ n=38, 5.9 months, 101 CAGs; *Msh6*$^{+/-}$ n=45, 5.6 months, 101 CAGs: *Msh6*$^{-/-}$ n=61, 5.5 months, 101 CAGs. Maternal: *Msh6*$^{+/+}$ n=35, 5.4 months, 100 CAGs; *Msh6*$^{+/-}$ n=39, 5.7 months, 101 CAGs; *Msh6*$^{-/-}$ n=38, 5.5 months, 99 CAGs. (D) Paternal: *Msh3*$^{+/+}$ n=37, 5.1 months, 101 CAGs; *Msh3*$^{+/-}$ n=51, 5.0 months, 101 CAGs; *Msh3*$^{-/-}$ n=46, 4.8 months, 101 CAGs. Maternal: *Msh3*$^{+/+}$ n=33, 4.3 months, 101 CAGs; *Msh3*$^{+/-}$ n=35, 4.4 months, 101 CAGs; *Msh3*$^{-/-}$ n=29, 4.2 months, 100 CAGs. Bar graphs show frequency distributions of the inherited repeat length changes. The horizontal bars below the graphs depict the total percentage of expansions, contractions and unchanged alleles. Black: expansions; white: contractions; grey: unchanged alleles.

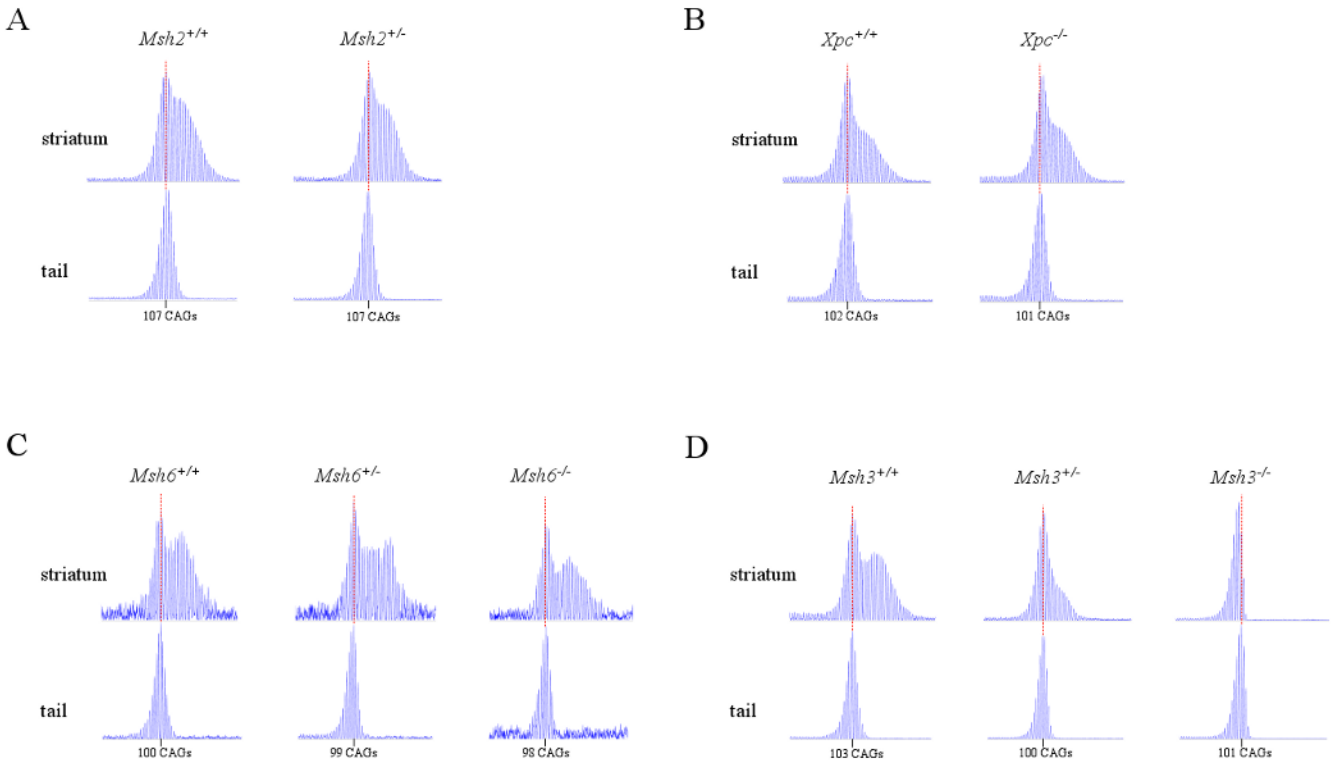

Figure 2.

Hdh^{Q111} CAG repeat striatal instability.

Hdh^{Q111} mice were crossed onto backgrounds deficient in *Msh2* (A), *Xpc* (B), *M*sh6 (C) or *Msh3* (D). ABI GeneScan traces are shown of the *Hdh*^{Q111} CAG repeat PCR-amplified from genomic DNA extracted from striata or tail of heterozygous *Hdh*^{Q111}/*Hdh*$^{+}$ mice at 5 months of age. The number of mice of each DNA repair genotype and the constitutive *HD* CAG repeat size determined from tail in each mouse is as follows: (A) *Msh2*$^{+/+}$ n=4, CAG 106, 107, 107, 107; *Msh2*$^{+/-}$ n=4, CAG 107, 107, 108, 111. (B) *Xpc*$^{+/+}$ n=3, CAG 100, 101, 102; *Xpc*$^{-/-}$ n=3, CAG 95, 99, 101. (C) *Msh6*$^{+/+}$ n=3, CAG 98, 100, 100; *Msh6*$^{+/-}$ n=3, CAG 99, 99, 99; *Msh6*$^{-/-}$ n=3, CAG 98, 99, 100. (D) *Msh3*$^{+/+}$ n=3, CAG 102, 103, 103; *Msh3*$^{+/-}$ n=4, CAG 100, 100, 101, 102; *Msh3*$^{-/-}$ n=3, CAG 94, 95, 101, 101. Mice of the same genotype had identical repeat profiles regardless of constitutive CAG repeat number. Representative GeneScan traces are shown. *HD* CAG repeat size in tail is indicated, the position of which is marked with a dotted red line.

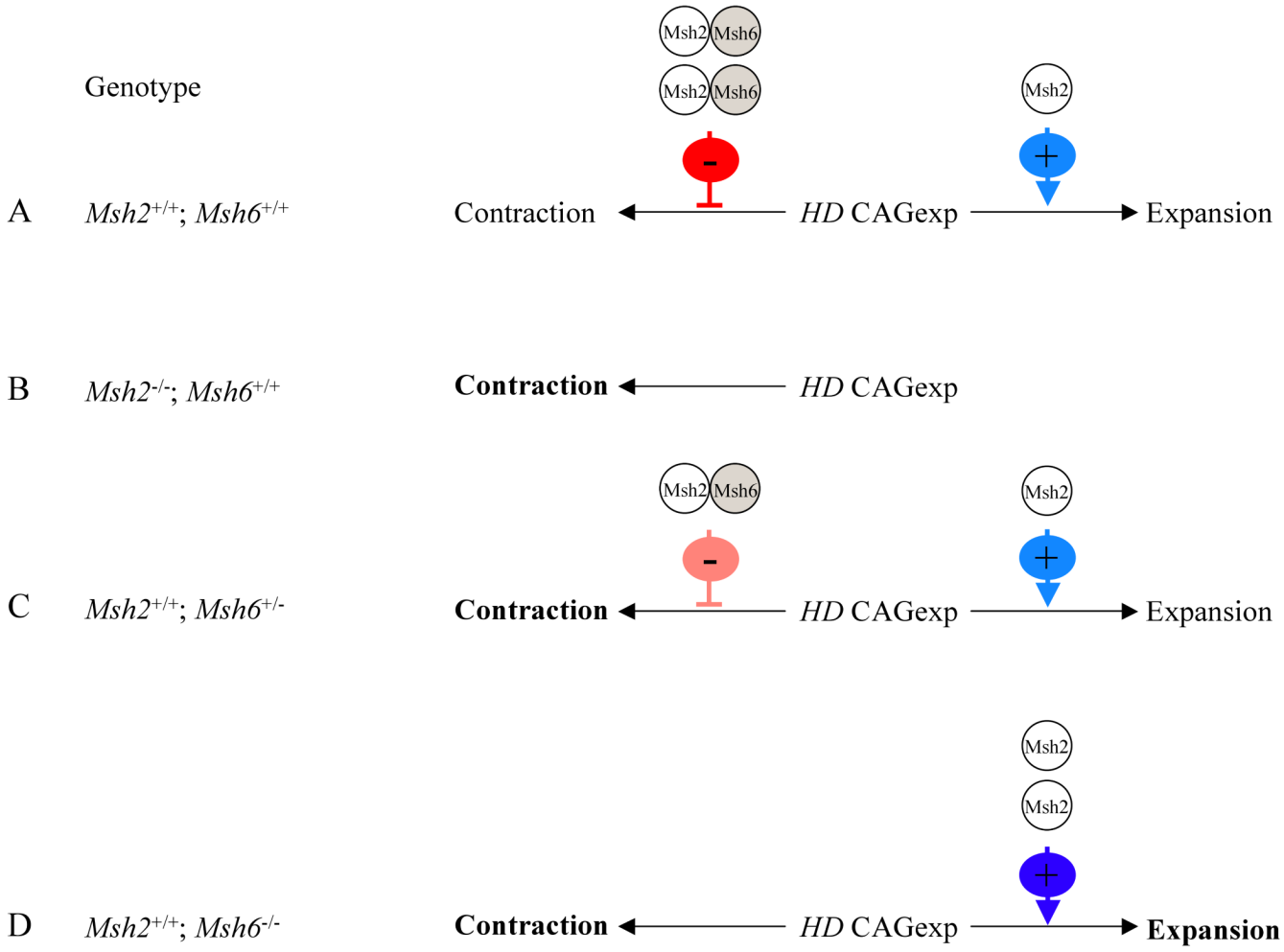

Figure 3.

Nuclear mutant huntingtin immunostaining in striatal neurons.

Hdh^{Q111} mice were crossed onto backgrounds deficient in *Msh2* (A), *Xpc* (B), *Msh6* (C) or *Msh3* (D). Striatal sections from *Hdh*$^{Q111/+}$ mice at 5 months of age were immunostained with anti-huntingtin antibody EM48 and a staining index (SI) calculated for each mouse as the product of the mean staining intensity and the number of immunostained nuclei (see Materials and Methods).

Left: Representative striatal histological sections immunostained with EM48.

Right: Bar graphs showing the mean SI for each genotype +/- standard error.

The number of mice of each DNA repair genotype (+/+, +/-, -/-) and the constitutive *HD* CAG repeat size determined from tail in each mouse is as follows: (A) *Msh2*$^{+/+}$ n=3, CAG 107, 108, 109; *Msh2*$^{+/-}$ n=4, CAG 106, 106, 107, 107. (B) *Xpc*$^{+/+}$ n=4, CAG 99, 101, 101, 101; *Xpc*$^{-/-}$ n=3, CAG 98, 101, 102. (C) *Msh6*$^{+/+}$ n=3, CAG 98, 99, ND$^{\#}$; *Msh6*$^{+/-}$ n=4, CAG 97, 98, 99, 99; *Msh6*$^{-/-}$ n=4, CAG 98, 99, 99, 99. (D) *Msh3*$^{+/+}$ n=5, CAG 98, 100, 100, 101, 102; *Msh3*$^{+/-}$ n=5, CAG 97, 97, 100, 101, 105; *Msh3*$^{-/-}$ n=3, CAG 99, 100, 102. * p=0.02, ** p<0.001. $^{\#}$A repeat value could not be determined for this mouse. Loss of two *Msh3* alleles had a slightly greater effect of loss of one *Msh3* allele but this effect was not statistically significant (p=0.11).

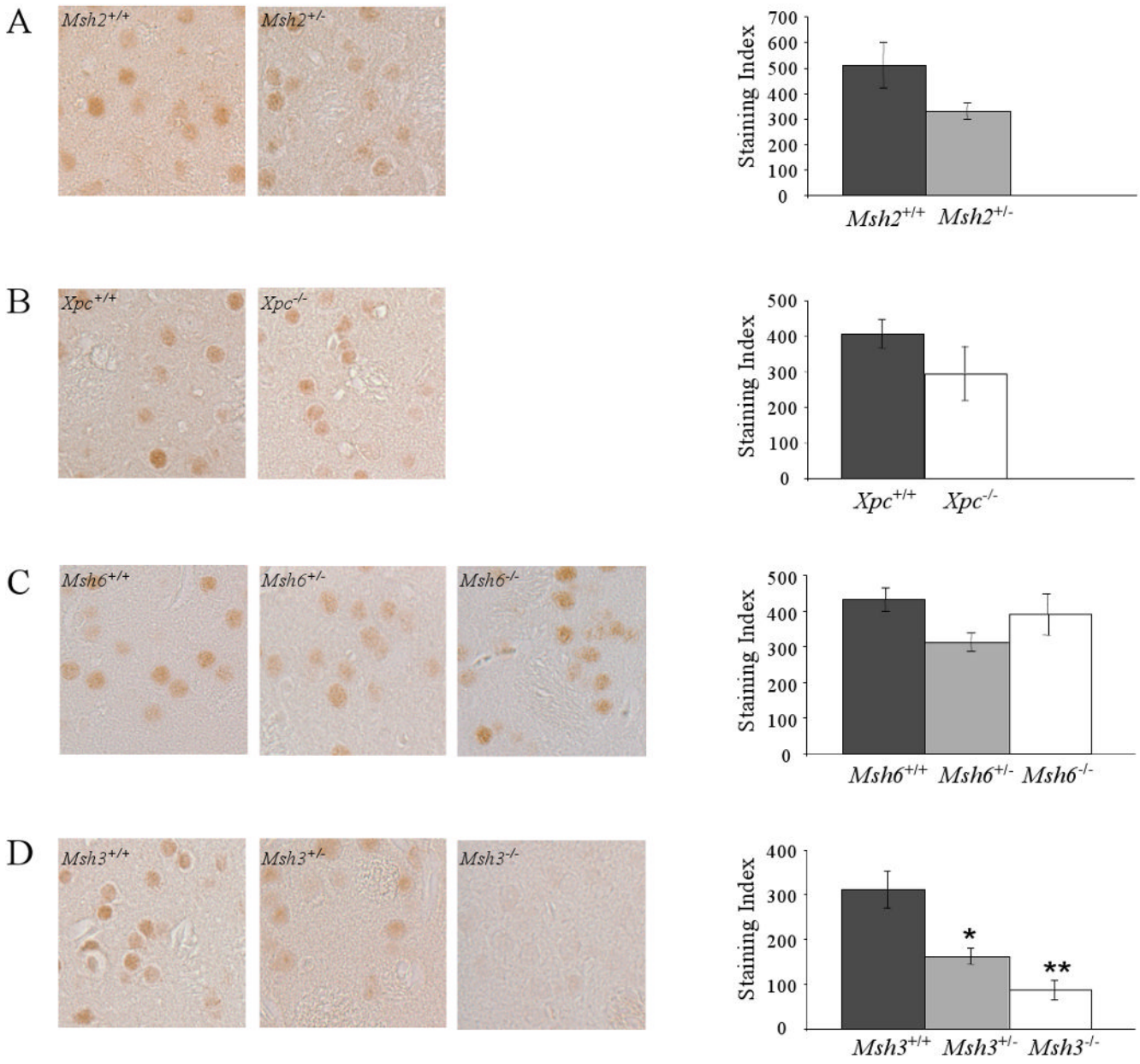

Figure 4.

Proposed model for the roles of Msh2 and Msh6 in *Hdh*^{Q111} CAG repeat intergenerational instability.

The length of the expanded *HD* CAG repeat (*HD* CAGexp) inherited from fathers is determined by the combined influences of two Msh2-dependent mechanisms: Msh2 mediates expansions and Msh2-Msh6 dimers protect against contractions. A) In mice wild-type for both *Msh2* and *Msh6* (*Msh2*$^{+/+}$/; *Msh6*$^{+/+}$) expansion (light blue '+' arrow) and inhibition of contraction (red '-' inhibitory symbol) both occur. B) In mice lacking Msh2 (*Msh2*$^{-/-}$; *Msh6*$^{+/+}$) both mechanisms are abolished, the net result being an increased contraction frequency (bold type). C) In mice lacking one *Msh6* allele (*Msh2*$^{+/+}$; *Msh6*$^{+/-}$) contraction frequency is increased (bold type) as a result of reduced Msh2-Msh6 inhibition (pink'-' inhibitory symbol) while expansions are unaffected. D) In mice lacking two *Msh6* alleles (*Msh2*$^{+/+}$; *Msh6*$^{-/-}$) contraction frequency

is increased (bold type) due to loss of Msh2-Msh6 inhibition. However, this is compensated by an increase in expansions (dark blue '+' arrow and bold type), as in the absence of Msh6 the balance of Msh2-dependent pathways is shifted in favor of those that mediate expansions.

Table 1

Paternal transmissions

Model	Parental genotype compared to +/+	Msh 2		Xpc		Msh 6		Msh 3	
		OR (99% CI)	**p-value**	**OR (99% CI)**	**p-value**	**OR (99% CI)**	**p-value**	**OR (99% CI)**	**p-value**
Multinomial (cumulative higher changes vs. cumulative lower changes)	-/-	0.20 (0.04, 0.92)	**0.0065**	1.03 (0.52, 2.02)	0.91	1.18 (0.46, 3.03)	0.65	0.44 (0.15, 1.35)	*0.061*
	+/-	0.41 (0.04, 4.54)	0.34	ND	ND	0.43 (0.07, 2.72)	0.24	0.51 (0.14, 1.84)	0.17
changed vs. unchanged	-/-	0.52 (0.18, 1.48)	0.11	0.55 (0.26, 1.20)	*0.05*	1.43 (0.67, 3.03)	0.23	0.88 (0.32, 2.42)	0.74
	+/-	0.54 (0.85, 3.45)	0.39	ND	ND	4.98 (2.72, 9.14)	< **0.0001**	0.35 (0.12, 1.04)	*0.013*
'Risk' expansions vs contractions +unchanged	-/-	NR	NR	0.88 (0.36, 2.16)	0.72	1.40 (0.63, 3.13)	0.28	0.59 (0.27, 1.31)	*0.089*
	+/-	NR	NR	ND	ND	1.16 (0.24, 5.52)	0.81	0.40 (0.13, 1.22)	*0.034*
'protective' contractions vs expansions +unchanged	-/-	2.13 (0.36, 12.64)	0.28	0.09 (0.003, 2.59)	*0.07*	1.15 (0.26, 5.11)	0.81	1.79 (0.31, 10.35)	0.39
	+/-	1.40 (0.06, 30.71)	0.78	ND	ND	4.28 (1.02, 18.00)	**0.009**	0.67 (0.09, 4.98)	0.61

Results of running four statistical models to determine the effect of *Msh2, Xpc, Msh6* and *Msh3* DNA repair genes on the intergenerational instability of the Hdh^{Q111} CAG repeat in paternal transmissions. Odds ratios (OR) with 99% confidence intervals (CI) and p-values are given for each model for comparisons between fathers that were deficient in either one (+/-) or two (-/-) copies of each of the DNA repair genes and the respective wild-type (+/+) controls. Values in bold are statistically significant. Values in italics indicate trends that did not reach significance. A p-value of 0.01 was used to determine significance (see Materials and Methods). NR: the 'Risk' model was not run as there were zero data points in the expansion group. ND: *Xpc* heterozygotes were not analyzed. Msh2: 116 total transmissions from 9 fathers; Xpc: 139 total transmissions from 12 males; Msh6: 144 total transmissions from 11 males; Msh3: 134 total transmissions from 12 males.