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Epigenetics of fragile X syndrome and fragile X-related disorders

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ABBREVIATIONS

FMR1	Fragile X mental retardation 1 gene
FMRP	Fragile X mental retardation protein

FREE1	Fragile X-related element 1
FREE2	Fragile X-related element 2
FXS	Fragile X syndrome
FXTAS	Fragile X-associated tremor/ataxia syndrome
mRNA	Messenger ribonucleic acid

The fragile X mental retardation 1 gene (*FMR1*)-related disorder fragile X syndrome (FXS) is the most common heritable form of cognitive impairment and the second most common cause of comorbid autism. FXS usually results when a premutation trinucleotide CGG repeat in the 5′ untranslated region of the *FMR1* gene (CGG 55–200) expands over generations to a full mutation allele (CGG >200). This expansion is associated with silencing of the *FMR1* promoter via an epigenetic mechanism that involves DNA methylation of the CGG repeat and the surrounding regulatory regions. Decrease in *FMR1* transcription is associated with loss of the *FMR1* protein that is needed for typical brain development. The past decade has seen major advances in our understanding of the genetic and epigenetic processes that underlie FXS. Here we review these advances and their implications for diagnosis and treatment for individuals who have *FMR1*-related disorders.

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Review

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- Improved analysis of DNA methylation allows better epigenetic evaluation of the fragile X gene.

- New testing techniques have unmasked interindividual variation among children with fragile X syndrome.
- New testing methods have also detected additional cases of fragile X.

[Main text]

Fragile X syndrome (FXS) and other disorders related to the fragile X mental retardation 1 gene (*FMR1*) are caused by expansion of a trinucleotide CGG repeat that is located adjacent to the *FMR1* gene promoter, with the normal CGG repeat range being less than 45 repeats. FXS is the most common single-gene cause of intellectual disability and autism, affecting 1 in 3600 males and 1 in 6000 females,[1] and occurs when the CGG repeat expands to at least 200 repeats, a repeat size that is termed 'full mutation'.[2] The full mutation CGG expansion triggers a series of epigenetic events that reduce or abolish transcription of the *FMR1* gene without altering its DNA sequence, in turn leading to reduced or absent production of the fragile X mental retardation protein (FMRP). *FMR1* plays a central role in human cortical development and is a key regulator of genes that have been implicated in autism spectrum disorders.[3]

Paradoxically, increased transcription of *FMR1* is associated with the late-onset disorders fragile X-associated tremor/ataxia syndrome (FXTAS) and fragile X-associated primary ovarian insufficiency. These two conditions are found primarily in individuals with expansions of between 55 and 200 CGG trinucleotides, termed 'premutation'. Premutation alleles do not cause FXS, but when maternally transmitted can expand to full mutation. Premutation alleles are common, affecting between 1 in 300 to 1 in 800 males, and between 1 in 200 to 1 in 370 females.[4–6] FXTAS has been reported in 45% of premutation males and 17% of premutation females over the age of 50 years,[7] and fragile X-associated primary ovarian insufficiency is found in 20% of premutation females.[8] These premutation-specific presentations have been linked to aggregation of proteins by overexpressed *FMR1* messenger ribonucleic acid (mRNA), leading to mitochondrial dysfunction and cell death.[9]

THE *FMR1* PROMOTER AND *FMR1* INTRON 1: STRUCTURE AND BIOLOGICAL SIGNIFICANCE

Transcription of *FMR1* is controlled from the *FMR1* promoter, the DNA sequence located directly upstream of the transcription start site (Fig. 1). The *FMR1* promoter was defined initially as a small region extending from 355 base pairs upstream (5′) to 60 base pairs

downstream (3′) of the CGG expansion, comprising the CGG repeat and the *FMR1* CpG island.[10] The *FMR1* CpG island contains 52 CpG dinucleotides that are methylated in FXS, resulting in silencing of *FMR1*.[11] In FXS, *FMR1* silencing occurs at 11 weeks of gestation, at which time full mutation repeat expansions trigger an 'epigenetic switch' comprising increased DNA methylation and modifications to histone proteins.[12]

More recently it has become evident that methylation changes associated with full mutation expansions extend beyond the boundaries of the CpG island and the previously defined *FMR1* promoter. A distinct DNA-methylation boundary has been identified 650 to 800 nucleotides upstream of the CCG repeat,[13,14] and in healthy individuals this boundary separates the normally methylated DNA located upstream of the *FMR1* promoter from the unmethylated *FMR1* promoter. In individuals with FXS full mutation repeat expansions, this boundary is lost, allowing methylation to move across a region called fragile X-related element 1 (FREE1) and into the *FMR1* CpG island, where *FMR1* transcription start sites are located (Fig. 1). Methylation of FREE1 is correlated with FMRP deficit in the blood of individuals with FXS.[14,15] A similar epigenetic boundary exists 3′ of the *FMR1* promoter, located within intron 1 of the *FMR1* gene, and this boundary is also lost in individuals with FXS, allowing methylation to extend into intron 1 of the *FMR1* gene, and into a region called fragile X-related element 2 (FREE2).[14,16] Methylation at FREE1, FREE2, and the *FMR1* CpG island is consistent between different tissues, and in the presence of a full mutation it leads to decreased expression of FMRP.[14]

The discovery of FREE1 and FREE2 has allowed the development of new diagnostic tests that can target these regions using technologies such as high-resolution melt,[17] pyrosequencing,[18] and the matrix-assisted laser desorption/ionization-time of flight mass spectrometry EpiTYPER system.[14] These new tests are faster and less cumbersome than the traditional diagnostic approach of methylation-sensitive Southern blot analysis. In addition, by assaying a much greater number of CpG sites with higher sensitivity, these tests have unmasked previously unrecognized interindividual variability, at single CpG resolution, in the methylation signatures between individuals with FXS.[14,17]

EPIGENETIC SIGNATURES OF *FMR1* DISORDERS AND DOWNSTREAM EFFECTS

The threshold above which *FMR1* gene silencing occurs has traditionally been regarded as 200 CGG repeats; however, recent evidence suggests that full mutation expansions between 200 and 400 repeats are less likely to be fully methylated and that the threshold for stable

silencing of full mutation alleles might be greater than 400 rather than over 200.[19] The sequence of events whereby full mutation expansions lead to *FMR1* silencing is not well understood, but the process is thought to be mediated through the formation of secondary structures of DNA and/or RNA that occur when large CGG repeat tracts are present. These secondary structures are likely to affect the equilibrium between factors that favour an active chromatin configuration, and factors that contribute to a closed chromatin configuration.[20] In unaffected males with normal-size alleles, the CGG repeats and CpG dinucleotides in the promoter region are unmethylated and the associated chromatin is enriched with active chromatin markers (such as acetylated histones H3 and H4 and trimethylated histone H3 at lysine 4) and low in inhibitory chromatin markers (such as trimethylated histone H3 at lysine 9). The resulting 'open' chromatin confirmation allows access of transcription factors to the *FMR1* promoter, leading to transcription of *FMR1*. In contrast, in individuals affected by FXS, DNA methylation moves into the region containing the promoter and CGG repeats and the associated chromatin adopts a compacted conformation that is enriched with markers of inactive chromatin. The result is that transcription factors are denied access to the promoter and *FMR1* transcription does not occur. In addition to these epigenetic factors, mRNA transcribed from the expanded CGG repeat tract is itself thought to contribute to *FMR1* silencing by binding to the complementary CGG portion of the *FMR1* gene.[12]

The downstream effects of *FMR1* silencing also seem to be mediated epigenetically. FXS human pluripotent stem cells have been used to model the early stages of neurogenesis in FXS and have revealed patterns of increased or decreased methylation, specific to FXS, at more than 1600 locations across the genome.[21] These loci are enriched for genes that are associated with developmental signalling, cell migration, and neuronal maturation, and gene networks that have been implicated in autism spectrum disorders. Although most of these aberrations are probably mediated through loss of FMRP, it is also possible that some are independent of FMRP. It is now recognized that the *FMR1* locus, in addition to being home to the *FMR1* gene, accommodates several long non-coding RNAs that are transcribed but not translated into protein. In FXS, transcription of several of these, including *ASFMR1*/*FMR4* (Peschansky et al.)[22] and *FMR6*,[23] is suppressed epigenetically, potentially contributing to the FXS phenotype by altering expression of other genes elsewhere in the genome (Fig. 2).

In contrast to full mutation alleles, premutation alleles are associated with increased transcription, and there is a direct relationship between the repeat number and *FMR1* mRNA levels.[20] Little is known about why transcription is increased from premutation alleles; however, it is hypothesized that CGG repeats in the premutation range alter the local

chromatin environment to make it more accessible to transcription factors and/or to factors that inhibit gene silencing.[20] The clinical features of FXTAS are thought to arise primarily from a toxic gain of function of elevated levels of *FMR1* mRNA contained the long CGG tract.[24] In addition, evidence has emerged that aberrant proteins, comprising tandemly repeated amino acids, can also be translated from expanded CGG/CCG trinucleotides, even in the absence of an AUG start codon. This 'repeat-associated non-AUG' translation of potentially toxic proteins has been associated with neuronal inclusions in patients with FXTAS, and may contribute to the FXTAS phenotype.[25] Finally, transcription of *ASFMR1*/*FMR4* is also increased in premutation carriers and may contribute to premutation phenotypes.

EPIGENOTYPE–PHENOTYPE CORRELATIONS IN FRAGILE X-RELATED DISORDERS

The observation of interindividual variability in methylation profiles at the *FMR1* CpG island and FREE1 and FREE2 regions in patients with FXS, combined with the clinical heterogeneity among individuals with full mutation expansions, has led to the question of whether there is a correlation between fragile X epigenotype and phenotype severity. However, in males, detecting such a correlation is challenging because most of those with FXS have both high methylation levels and a severe phenotype, and study sample sizes have been small. In one small and heterogeneous cohort of FXS males, intellectual functioning was found to correlate inversely with *FMR1* CpG island methylation, as quantified using Southern blot.[26] Other studies have assayed methylation within the FREE2 region in full mutation males and demonstrated a gradient epigenotype–phenotype relationship when assessing phenotype using the Aberrant Behaviour Checklist[27] and intellectual function;[28] however, larger confirmatory studies are needed.

In theory, an association between *FMR1* promoter methylation and phenotype should be easier to detect in full mutation females than in full mutation males, given their broader spectrum of severity and variable patterns of X-inactivation. However, the results have been inconsistent. Earlier work that analysed *FMR1* CpG island methylation in full mutation females found that it was correlated inversely with intellectual functioning;[29] however, another study with similar outcome measures did not detect a correlation.[30] More recent studies that have assayed *FMR1* promoter methylation at the *FREE2* locus have detected a more convincing inverse relationship between methylation and cognition in full mutation females.[31,32]

Epigenetic factors have also been hypothesized to play a role in the phenotypes associated with premutation expansions in females. One might expect that premutation phenotypes would be more prominent in women in whom a higher proportion of cells harboured the premutation on the active X chromosome. This seems to be the case in relation to the clinical and radiological features of FXTAS;[33,34] however, in contrast, there is no evidence for a relationship between skewed X-inactivation and fragile X-associated primary ovarian insufficiency.[35] Interestingly, in premutation females, FREE2 methylation, which is sensitive to X-inactivation changes, has been found to be inversely correlated both with performance on tasks of executive function[36] and with increased grey matter volume of cortical structures that have known roles in executive function, such as frontal and parietal gyri.[37]

MOSAICISM AND FXS

Fragile X epigenotypes have traditionally been classified into unmethylated premutation expansions and fully or partly methylated full mutation expansions. However, these classifications are complicated by the existence of mosaicism, defined as the presence of two or more different cell populations within a given individual. In FXS, two main types of mosaicism exist: methylation mosaicism and repeat size mosaicism.

Methylation mosaicism describes the presence, in an individual with a full mutation, of a population of cells that are methylated at the *FMR1* locus and a second population of cells that are unmethylated. Patients with methylation mosaicism may exhibit a milder phenotype than full mutation patients with non-methylation mosaicism, the extreme example being males with full mutation expansions that are completely unmethylated, and who typically have normal intellect (but are at risk of premutation phenotypes such as FXTAS).[38] Interestingly, males with unmethylated full mutation are more likely to have a CGG repeat size in the range 200 to 400, consistent with the threshold for stable methylation of full mutation alleles being greater than 400, rather than over 200, as previously documented.[19]

Females are, in a sense, all methylation mosaics because the *FMR1* locus is sensitive to X-inactivation, such that normal levels of *FMR1* promoter methylation are about 50%, and females with full mutation expansions typically have methylation levels above closer to 75%.[39,40]

Repeat size mosaicism is characterized by the presence of cell populations with different CGG repeat sizes, most commonly one cell population with a full mutation and another with a premutation. Other combinations of full mutation, premutation, grey zone (45–

54 CGGs), and normal CGG size have been also reported, and may not be as uncommon as previously thought.[17] Repeat size mosaicism is thought to arise, in most cases, from a full mutation conception, with subsequent contraction of the full mutation to a smaller size allele in one or more cell lineages. Full mutation/premutation mosaicism has been reported to be associated with a less severe phenotype, although not consistently, and in addition can be associated with premutation phenotypes such as FXTAS that are not typically associated with non-mosaic full mutation. From a diagnostic perspective, the presence of full mutation and smaller CCG repeat size mosaicism can lead to a missed diagnosis of FXS. The reasons for this are twofold. First, the traditional two-step testing protocols used for FXS initially look for a normal CCG repeat size (using polymerase chain reaction) and only proceed to look for an expanded CGG repeat (using Southern blot analysis) when no normal CGG repeat is detected. Second, very-low-level full mutation mosaics will not be detected by Southern blot, which will only detect full mutations when they are present in more than 20% of cells.[17] Recently modified 'long range' polymerase chain reaction tests have been developed that can amplify CGG repeats up to full mutation CCG repeat size and may mitigate the issue of potentially missed cases associated with the two-step protocol. However, analytical sensitivity for low-level mosaicism is still an issue with these tests, and a first-line DNA methylation analysis targeting the FREE2 region provides a more sensitive and cost-effective approach.[17]

EPIGENETIC APPLICATIONS IN EARLY DIAGNOSTICS AND NEWBORN SCREENING FOR FRAGILE X

FXS is a potential target for newborn screening, the principal benefits being the timely institution of early intervention and the opportunity for parents to avoid having a second affected child. However, a challenge to implementation has been that techniques utilizing CGG sizing are relatively costly and have the potential disadvantage of detecting premutation expansions that do not cause intellectual disability but which are associated with a risk of late-onset disorders.[41] These disadvantages can be circumvented using an epigenetic approach. The feasibility of this approach was demonstrated using a real-time polymerase chain reaction test to screen for abnormal methylation in 36 124 male newborn spots.[42] The study reported the prevalence of full mutation as 1 in 5161 in the US male general population, which was similar to prevalence estimates reported using CGG sizing. Among females known to have full mutation, the same methylation was able to detect 82%, but was not able to distinguish clinically affected females from non-penetrant full mutation carriers.

More recently, FREE2 methylation testing (using methylation-specific quantitative melt analysis) has been applied successfully to identify full mutation newborn blood spots from males and females, demonstrating high throughput, low cost, and the ability to predict prognosis.[32,39,42] Larger studies are now underway to validate the feasibility of this approach for FXS newborn screening for both sexes.

Interestingly, and of relevance to early diagnostics and newborn screening, studies have suggested that *FMR1* methylation patterns in blood are not fixed, but can change over time. In females there is evidence for positive selection with age in favour of cells containing the normal *FMRI* allele on the active X chromosome.[30,43] In contrast, in males, detailed cross-sectional analysis of *FMR1* methylation suggests that it may increase gradually with age.[40] These findings have important implications for our understanding of epigenetic modifiers of *FMR1* activity, and for assessing the utility of quantitative analysis of *FMR1* methylation as part of diagnostic testing, especially in FXS females. Future studies should establish whether these changes in methylation also occur in other tissues, such as the central nervous system, and whether methylation changes over time have prognostic use.

EPIGENETIC-BASED THERAPIES FOR FXS

The fact that the epigenetic marks that inactivate *FMR1* are potentially reversible has led to the notion that they might be targeted therapeutically for the treatment of FXS. The hope that removal of one or more of the epigenetic marks that cause *FMR1* transcriptional repression might restore function is supported by the observation, as noted earlier, of rare individuals with unmethylated full mutation and normal intellect.[38] In FXS, the objective of treatment is to remove the DNA methylation and/or histone modifications that are maintaining the *FMR1* locus in a transcriptionally inactive state. Removal of DNA methylation can be achieved using the drugs 5-azacytidine and 5-azadeoxycytidine. Both drugs have been used for the treatment of myeloid leukaemia, where they elicit their therapeutic effect by desuppressing tumour suppressor genes that have been silenced inappropriately. When applied to cell lines from patients with FXS, these drugs have been shown to remove DNA methylation at the *FMR1* locus, shift histone modifications towards a transcriptionally active configuration, and partly restore FMRP production.[44–46] Similar studies have been performed in FXS-induced pluripotent stem cells, and treatment with 5-azacytidine has resulted in partial DNA demethylation, histone modification, and partial transcriptional activation of *FMR1*.[47] Yet there are several problems with the approach: the induced epigenetic changes are transient, with methylation returning to normal within a month of drug withdrawal,[11] and these agents

are cytotoxic, mutagenic, lack cell specificity, and only incorporated into rapidly dividing cells, which is not the case with neurons.

An alternative approach is to target histone modifications by inducing histone acetylation using histone deacetylation inhibitors such as 4-phenylbutyrate, sodium butyrate, and trichostatin A; however, use of histone deacetylase inhibitors alone does not result in any reactivation of *FMR1* (although it does have a synergistic effect when used with 5-azacytidine).[44] Two other widely used drugs, L-acetylcarnitine and valproic acid, also act as histone deacetylators and have been considered as potential therapies in FXS. In FXS lymphoblastoid cells, sodium valproate has been shown to have a modest effect on histone modifications at the *FMR1* locus but not to affect DNA methylation or to have a significant effect on transcriptional reactivation.[48] Despite these findings, in an open label trial, treatment with valproic acid has been observed to reduce hyperactivity and improve adaptive behaviour in males with FXS.[49] Acetylcarnitine is another medication that has been shown to deacetylate histones at the *FMR1* locus, but not to induce DNA demethylation.[46] A double-blind placebo-controlled comparison of acetylcarnitine with placebo in male children with FXS demonstrated a modest reduction in hyperactivity and prosocial behaviour in those with FXS treated with acetylcarnitine, but no effect on intellectual function.[50]

Ultimately a more targeted approach is required that allows selective modulation of *FMR1* with minimal cytotoxicity. The development of the CRISPR/Cas9 gene editing system has provided a valuable tool in this regard, and two approaches have shown promise. First, CRISPR/Cas9 gene editing has been used to excise the CGG repeat expansion in patient-derived FXS-induced pluripotent stem cells, with resultant demethylation of the *FMR1* promoter and reactivation of FMRP production.[51,52] More recently, the Cas9 system has been adapted to allow targeted editing of DNA methylation without altering the DNA sequence.[53] Using this approach, studies in FXS-induced pluripotent stem cells have demonstrated demethylation of both the *FMR1* CGG expansion and the FREE1 region, with consequent de-repression of *FMR1* chromatin, restoration of FMRP expression, and the rescue of electrophysiological abnormalities in FXS neurons.[53]

Another approach is to use small molecules that target histone methylation. At present, no such molecule has been identified for FXS, but recent and notable progress has been made using a comparable approach in Prader–Willi syndrome, where researchers have identified a small molecule that selectively inhibits histone methylation at the Prader–Willi syndrome locus on chromosome 15, restoring expression of Prader–Willi syndrome-

associated genes and improving growth and survival in a mouse model of Prader–Willi syndrome.[54]

Finally, it is notable that any therapy increasing transcription of expanded *FMR1* alleles may increase the risk of FXTAS. In fact, to treat FXTAS, where the pathogenesis involves increased transcription of *FMR1*, an opposite epigenetic approach is required. Histone deacetylase inhibitors – small molecules that inhibit the acetylation of histone proteins – provide the opportunity to repress *FMR1* chromatin, restore *FMR1* transcription to normal levels, and have shown promise in a fruit fly model of FXTAS.[55]

CONCLUSION

In FXS, expansion of the *FMR1* CGG repeat to a full mutation triggers a cascade of epigenetic events including methylation of the *FMR1* promoter and modification of associated histones. Downstream effects are then mediated elsewhere in the genome, through the absence of FMRP, and possibly by the silencing of other non-coding RNAs that are normally transcribed from the *FMR1* locus. An improved understanding of these events provides a possible explanation for the interindividual variation found among children with FXS, and has opened the door to the development of new epigenetic-based diagnostic tests that are highly sensitive, may help predict long-term prognosis, and have the potential for use in newborn screening. Larger-scale studies are still required to fully evaluate the clinical use of these new tests, in particular to define epigenotype–phenotype relationships. Epigenetic therapies for fragile X have produced some promising results in cell model systems, but are not currently ready for in vivo use.

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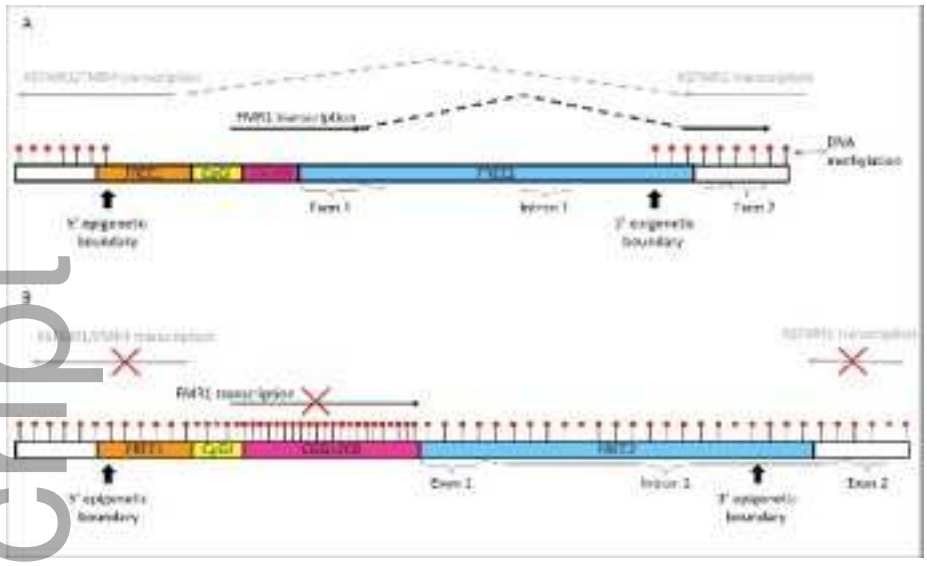
Figure 1: DNA methylation and bidirectional transcription at the *FMR1* promoter in males. (a) In individuals with normal *FMR1* alleles with CGG repeat sizes less than 44 repeats, the promoter region is flanked by the 5′ and 3′ epigenetic boundaries. While DNA is methylated (red circle) on either side of these boundaries, no methylation can be found within the *FMR1* promoter spanning from the FREE1 region (orange), through the CpG island (CpGI, yellow), CGG repeat (pink), exon 1, and the intron 1 portion of the FREE2 region (blue). The associated chromatin adopts an open confirmation that allows transcription of the *FMR1*, *ASFMR1*, and *FMR4* genes. (b) In most individuals with a full mutation, both the 5′ and 3′ epigenetic boundaries are lost, with DNA methylation moving into the promoter region. The associated chromatin adopts a closed conformation around the CGG full mutation expansion, preventing transcription factor binding to transcription factor binding sites located within the CpG island for *FMR1*, and the FREE1 region for *ASFMR1*/*FMR4*.

Figure 2: The association between epigenotype and phenotype in fragile X-associated disorders. (a) Normal *FMR1* alleles with CGG repeat sizes of less than 44 repeats are associated with the absence of promoter methylation and normal transcription of *FMR1* and *ASFMR1*/*FMR4*, which in turn regulate the expression of hundreds of genes associated with normal neurodevelopment. (b) Premutation CGG repeats (55–200 repeats) are not associated with methylation of the promoter, but lead to increased transcription of *FMR1* mRNA, which is thought to lead to premutation phenotypes such as FXTAS and fragile X-associated primary ovarian insufficiency (FXPOI) via the mechanism of RNA toxicity. Paradoxically, in carriers of large premutations, production of FMR1 protein is reduced. Transcription of *ASFMR1*/*FMR4* is also increased in premutation carriers and may contribute to premutation phenotypes. (c) Full mutation CGG repeats (>200 repeats) are associated with methylation of

the FMR1 promoter and reduced or abolished transcription of *FMR1* RNA and translation of FMR1 protein. Transcription of *ASFMR1*/*FMR4* is also reduced, which may contribute to the phenotype of fragile X syndrome. (d) In rare individuals with full mutation CGG repeats, the FMR1 promoter remains unmethylated, allowing transcription of *FMR1* and *ASFMR1*/*FMR4*. Similar to the case with premutation CGG expansions, transcription of these expanded repeats is associated with increased production of RNA, which may lead to FXTAS and FXPOI through the mechanism of RNA toxicity. Conversely, production of FMRP is reduced. In females, the promoter region between the 5′ and 3′ boundaries is subject to X-chromosome inactivation, and variability in X-inactivation and related methylation of the promoter region has been correlated with premutation-related phenotypes in females.



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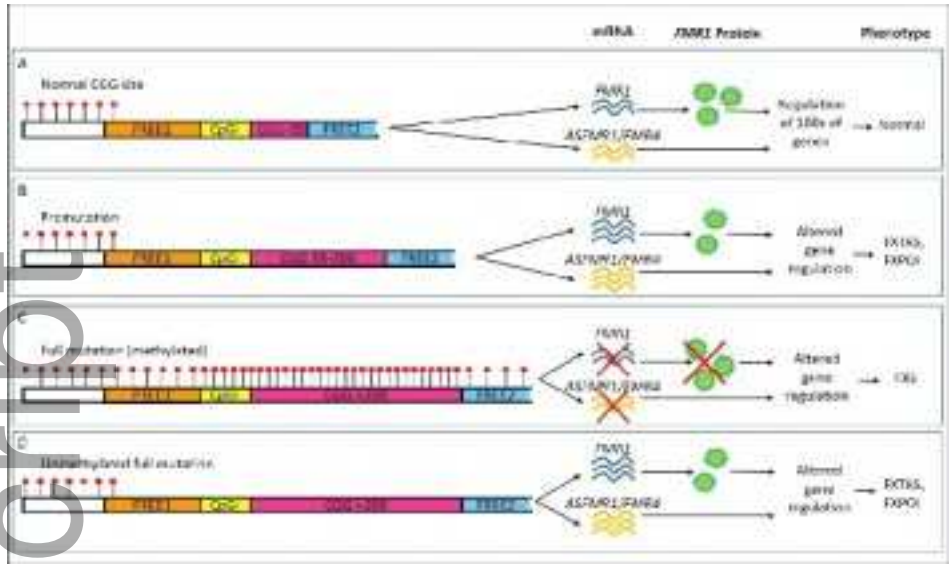

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