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Fragile X Syndrome in Humans and Mice

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Fragile X syndrome is the most common cause of interited mental retardation in humans, with a frequency of approximately 1 in 1200 males and 1 in 2500 females [1]. It is second only to Down syndrome as a genetic cause of mental retardation, which has an overall frequency of 1 in 600. These frequency estimates suggest that fragile X syndrome accounts for approximately 3% of mental retardation in males, and perhaps as much as 20% in males with IQs between 30 and 55 [2]. The disease derives its name from the observation of a fragile site at Xq27.3 in cultured lymphocytes, fibroblasts and amniocytes [3].

The phenotype of the fragile X syndrome is mental retardation, usually with an IQ in the 4-70 range [4] and a number of dysmorphic features: long face, everted ears and large testicles [for review see ref. 5] (Fig. 1). Not every patient shows all the physical symptoms, which are generally more apparent after childhood. Macroorchidism is a common feature of fragile X syndrome in more than 90% of postpuberal males. Some patients show hyperactivity and attention deficits as well as avoidance behaviour similar to autism. Affected females generally have a less severe clinical presentation, and their IQ scores are generally higher, with typically borderline IQs or mild mental retardation.

No gross pathological abnormalities have been described in the brains of fragile X patients. Only a few post-mortem brain studies of fragile X males have been described and the information is very limited, presenting only non-specific findings such as brain atrophy, ventricular dilatation and pyramidal neurons with abnormal dendritic spines. It has been shown that the volume of the hippocampus was enlarged compared to controls [6], while a significantly decreased size of the posterior cerebellar vermis and increased size of the fourth ventricle was found [7]. Using magnetic resonance imaging it was shown that fragile X patients have an increased volume of the caudate nucleus [8]. The caudate volume is correlated with IQ and methylation status of the *FMR1* gene.

The syndrome shows unusual genetic characteristics in that the pattern of inheritance does not follow the classical Mendelian pattern for X-linked genes. In fragile X families, normal males are identified that can transmit the fragile X mutation to their daughters. These males are themselves clinically and cytogenetically normal and have been termed

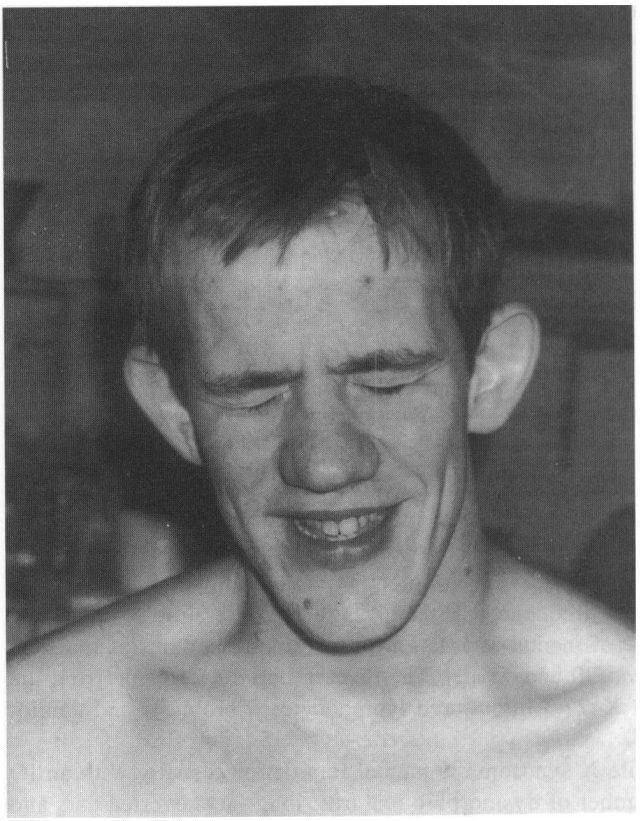

Fig. 1 - Mentally retarded patient with fragile X syndrome. Note long face with prominent forehead and ears.

normal transmitting males (NTM). The mutation in these males was called a premutation. The daughters of these males are always normal, but they have a high probability of having offspring who inherit the abnormal X chromosome to become clinically affected.

The *FMR1* gene

The cloning of the fragile X gene, *FMR1*, has uncovered the molecular basis for the inheritance pattern of fragile X syndrome. The *FMR1* gene, cloned by a Dutch-American collaboration, was isolated from a fetal brain cDNA library using cosmids that spanned the fragile site [9, 10].

The gene produces a transcript of approximately 4.8 kb, which can be found in almost all tissues, but is particular abundant in brain and testes. The gene consists of 17 exons spanning approximately 40 kb [11]. The CpG island in front of the *FMR1* gene is methylated in fragile X patients [12, 13], and the fragment containing this CpG island is increased in size [14-16]. Within the first exon of *FMR1*, a trinucleotide sequence, CGG, is found which expands beyond the normal range (6-52 repeats with a mean of 30 repeats) in individuals who are carriers of a premutation (55-200 repeats) [17]. In

patients, this repeat is expanded to over 200 copies. In FRAXA, expansion of the CGG repeat into the full mutation range leads to methylation of the repeat and the nearby CpG island [14, 18, 19]. As a result of this methylation the transcription of the *FMR1* gene is reduced [20, 21], and no *FMR1* protein (FRMP) is produced [22, 23] (Fig. 2). Detection of the full mutation in males and females can be routinely carried out by assessing the size of restriction-enzyme-generated DNA fragments encompassing the CGG repeat [14, 15, 24-28]. The full mutation is not only defined by size but also by methylation, which may sometimes act as an independent determinant of the phenotype. Some mild cases without mental impairment have been described with mutations of borderline size or even fully expanded mutations in the absence of (complete) methylation [28-30]. A test for the methylation status can be easily combined with Southern analysis by including a digestion with a methylation-sensitive restriction enzyme. An alternative technique is PCR amplification of the CGG repeat [17, 31]. Although adaptations of the PCR method have been described that do detect the full mutation [31], most laboratories involved in the diagnosis of fragile X syndrome patients and the detection of carriers employ a combination of a PCR-based method and Southern blotting.

FMRP

Multiple isoforms of FMRP are observed, which are the translation products of different mRNAs resulting from extensive alternative splicing (Fig. 2) [22, 32, 33]. No differences in RNA splicing have been detected between different tissues. It is not known whether different isoforms do have different functions. The *FMR1* gene is highly expressed in brain and gonadal tissues, consistent with the observed phenotype [23, 34, 35]. In adult

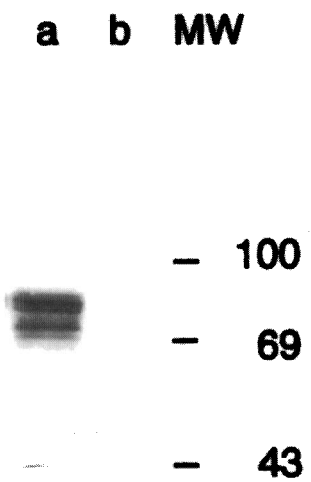

Fig. 2 - FRMP expression. Immunoprecipitation of lymphoblastoid cell lines with polyclonal antibodies [22] against FMRP of control (lane a) and patient (lane b).

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testes, FMRP was detected only in spermatogonia [23]. The intracellular localization of the *FMR1* gene products was cytoplasmic [22, 23]. In brain, FMRP is expressed in neurons and in Purkinje cells; expression in neurons located in the cortex is shown in Fig. 3a. In the patient, expression is seen in only 1% of the neuronal cells (Fig. 3b) which, probably, originate from a low percentage of cells that have a premutation allele [36].

The lack of *FMR1* expression is widely accepted as the cause of the fragile X syndrome. This relationship between loss of *FMR1* expression and fragile X syndrome is confirmed in patient with a deletion encompassing the *FMR1* gene [37-39]. However, this does not explain the presence of mosaic males with a premutation, and thus *FMR1* expression, in a proportion of the cells. The level of disease expression in females is presumably determined by X inactivation patterns. Cells that have an inactivated normal X chromosome do not produce FMRP. It is possible that the percentage of cells that do express FMRP is too low, indicating that expression is required in many cells. Another possible explanation could be that the leucocytes, in which the mosaicism is detected, are not representative for the tissues affected in fragile X syndrome.

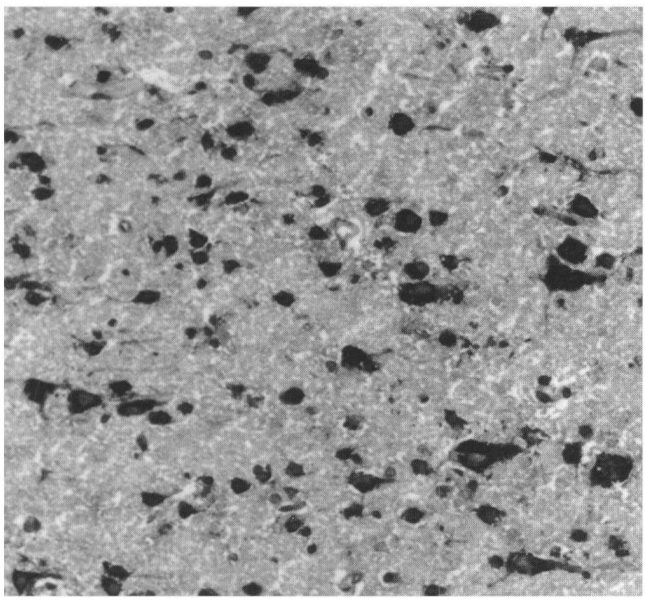
Function of *FMR1* Gene

Since the isolation of the gene in 1991, clues about the function of FMRP has been slowly accumulating. Homology has been observed with members of a class of proteins involved in RNA binding [40, 41]. Two different domains could be distinguished: a KH domain which is shared among several genes involved in RNA splicing, and an RGG domain which is found in the C terminal part of FMRP. RNA binding of FMRP has been demonstrated in vitro with both its own mRNA as well as with approximately 4% of mRNAs from human fetal brain [41]. In a patient with the fragile X phenotype but without the amplification of the CGG repeat, a point mutation has been described in one of the most highly conserved residues of the KH domain [42]. Impairment of RNA-binding activity in this patient provides strong evidence for an RNA-binding role at this position of FMRP [43, 44].

However, studies to localize FMRP suggest a primarily, cytoplasmic location [22, 23]. It could be hypothesized, given the RNA-binding activity and the cytoplasmic location, that FMRP is involved in translation of mRNA, with a role in directing selected mRNAs to cellular locations, or masking a subset of mRNAs from translation until required.

Other trinucleotide diseases

The expansion of trinucleotide repeat sequences within the transcribed regions of genes has been demonstrated to be the underlying genetic defect in a number of other inherited human disorders: spinal and bulbar muscular atrophy (SBMA) or Kennedy disease [45], myotonic dystrophy (DM) [46, 47], Huntington disease (HD) [48], spinocerebellar ataxia type 1 (SCA1) [49], dentatorubral-pallidoluysian atrophy (DRPLA) [50, 51], FRAXE mental retardation [52], Haw River syndrome (HRS) [53] and Machado-Joseph syn-



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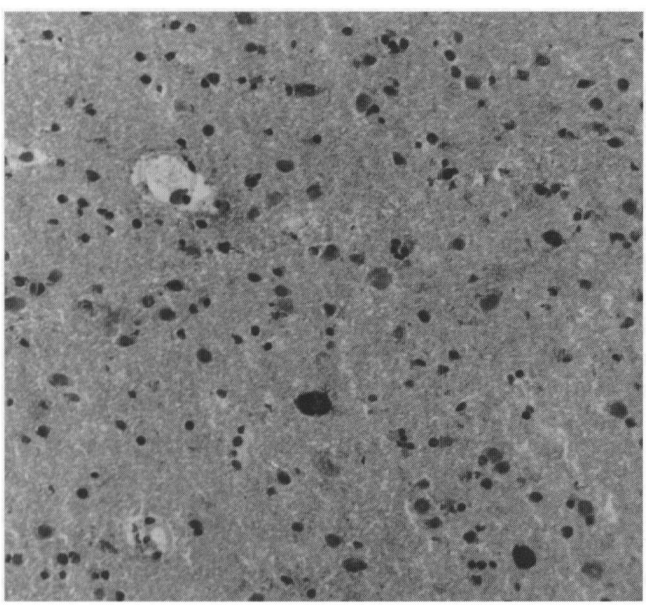

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Fig. 3 - Expression of FMRP in the cortex. Light microscope micrographs of cryostat sections from the cortex of a control individual (*a*) and a patient (*b*) using monoclonal 1A1 [23, 36].

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Table 1 - Unstable Triplet repeat diseases

Disease	Reference	Chromo some	Repeat	Location	Normal allele	Affected allele	Change in function
Fragile X syndrome (FRAXA)	this study	Xq27.3	CGG	5' UTR	6-55	200->2,000	loss
FRAXE mental retardation	52	Xq28	GCC	?	6-25	200->850	?
Kennedy disease (SBMA)	45	Xq11	CAG	ORF	12-30	40-62	gain
Huntington disease (HD)	48	4p16.3	CAG	ORF	10-36	38-100	gain
Spinocerebellar ataxia (SCA1)	49	6p22-23	CAG	ORF	9-39	41-81	gain
Dentatorubral-pallidoluysian atrophy (DRPLA)[1]	50, 51	12p12-13	CAG	ORF	7-23	49-75	gain
Haw River syndrome (HRS)[1]	53	12p12-13	CAG	ORF	3-13	63-68	gain
Machado-Joseph (MJD)	54	14q32.1	CAG	ORF	13-36	68-79	gain
Myotonic dystrophy (DM)	46, 47	19q13.3	CTG	3' UTR	5-37	50-2,000	mRna stability?
FRA16A[2]	55	16p13.11	CCG	?	16-49	>1,000	?
FRAXF[2]	56	Xq28	GCC	?	6-29	300-500	?

UTR = Untranslated repeat; ORF = open reading frame.

[1] The same gene is involved in the Japanese population (DRPLA) and African-American population (HRS).

[2] No known disease association.

drome (MJD) [54] (Table 1). All except one have neuronal cells as their primary target. The triplet repeats show length variation within the normal population. In the patient populations, the repeat has expanded to beyond the range found in the normal population. In SBMA, HD, SCA1, DRPLA, HRS and MJD the expansions of the CAG repeat are quite small, whereas as in FRAXA, in DM and FRAXE, the expansion may be very large. FRAXE mental retardation is associated with the expansion of a GCC repeat located 600 kb distal to the *FMR1* gene [52]. The expansion coincides with the methylation of a CpG island. However, the gene affected has not been cloned. Two further fragile sites, FRA16A and FRAXE, have been found to be caused by repeat amplifications [55, 56]. It is not known whether nearby genes are affected by this hypermethylation, and no disorder has been associated with these expansions. Of the four fragile sites defined at the molecular level, all are associated with expansion of a CGG/GCC repeat.

The dominant behaviour of a number of triplet repeat diseases has been suggested to result from so-called "gain of function" mutations in which the affected locus acquires a new functional characteristic rather than losing a function. For instance, the hypothesis is supported for SBMA by the observation that disruptions of the AR gene do not cause SBMA [45].

Anticipation

Transmission through females is essential to generate an expansion to a full mutation in the *FMR1* gene resulting in a disease phenotype. No expansion through a male meiosis from a premutation to a full mutation has been found. There must be factors present either during oogenesis or as an imprint during early embryogenesis that are involved in this amplification of the *FMR1* gene on the maternal X chromosome.

The triplet repeat diseases display anticipation, that is increasing severity and earlier age of disease onset in successive generations. However, according to this definition, in fragile X syndrome there is no strict anticipation. In fragile X syndrome, the anticipation observed is an increasing penetrance in successive generations, which was known as the Sherman paradox [57, 58]. Once the molecular basis of the disease became known, the resolution of this phenomenon became apparent [17]. It was shown that the CGG repeat in the *FMR1* gene tends to increase in size from mother to child. The increasing penetrance of the disease in successive generations correlates with increasing size of the CGG repeat. Smaller premutation alleles tend to stay in the premutation range despite an increase in size, while larger premutation alleles have a higher risk of expanding to a full mutation.

Timing of amplification

There is no evidence for any recent mutation in the CGG repeat in fragile X syndrome; all mothers of males with fragile X syndrome have been found to be carriers. Richards et al. [59] presented haplotype evidence for a founder effect in the fragile X mutation. Studies of other populations also showed that allele distributions are different on fragile X chromosomes compared to normal X chromosomes, giving further support to the sug-

gestion of a fragile X founder effect [60, 64]. The data argue for a limited number of independent mutations that provided the origin of most of the present-day fragile X chromosomes.

It is not known when the expansion of the repeat occurs. The repeat expansion in affected individuals is often mosaic, suggesting that the repeat is unstable in somatic tissues. However, the pattern of mosaicism was strictly identical in three pairs of monozygotic twins, indicating that the somatic heterogeneity and abnormal methylation are established early in embryonal development [65]. This could suggest that either the expansions has already taken place in the germ-line and is unstable in somatic cells or that transition does not occur in the germ-line and has taken place in the early embryo. Daughters of affected males are normal and have a premutation [66] and it was shown that sperm cells of affected males show only a premutation [67]. This was confirmed by demonstrating the presence of FMRP in the tubuli of the testes in early spermatogonia [36]. The finding that sperm cells of male patients with a full mutation in their blood cells only contain a premutation, has led to two hypotheses for the possible timing of the CGG repeat amplification [67]. In the first mode, the amplification is assumed to occur during oogenesis leading to a full mutation in all somatic cells (Fig. 4). In a low number of cells, both sperm and somatic cells, the full mutation regresses to a premutation and due to a selection mechanism, only the sperm cells with a premutation can proliferate. This selection mechanism could be the necessity of *FMR1* expression for proper spermatogenesis. This selection mechanism is not based on the absence or presence of FMRP, since recent breeding studies with mice lacking FMRP have demonstrated normal litter size and thus no requirement of FMRP for spermatogenesis [68]. In addition, a family has been described in which a 1.6-kb deletion of the *FMR1* promoter region, resulting in the absence of *FMR1* mRNA cosegregated with the fragile X syndrome phenotype in all affected males [39]. This deletion was originally derived from a male with a progeny of five children, confirming that the expression of FMRP is not required for spermatogenesis. Another selection mechanism, however, is still possible. It was shown that full-mutation alleles replicate much later than normal or premutation alleles [69]. Therefore, sperm cells containing a premutation might replicate and divide faster than sperm cells carrying a full mutation. This then results in the detection of a premutation in the majority of sperm cells. In a second model (Fig. 4B), the extensive amplification of the repeat occurs during postzygotic proliferation, after separation of the germ line, which occurs early in the blastocyst stage. This model suggests a two-step expansion in the somatic cells. In the first step, the premutation expands mildly. The second step, after separation of the germ line, involves an expansion to a full mutation in the majority or all of the cells. This model can explain the single-size premutation allele found in sperm [67], while the mutation in somatic tissues is very diverse, resulting in a smear of full-mutation alleles on a Southern blot.

Mechanism

The exact mechanism involved in the generation of expansions is not clear. Hypotheses have largely centreed on errors during replication. One possible mechanism is based on the misalignment of replicating DNA with subsequent slippage of the polymerase [70].

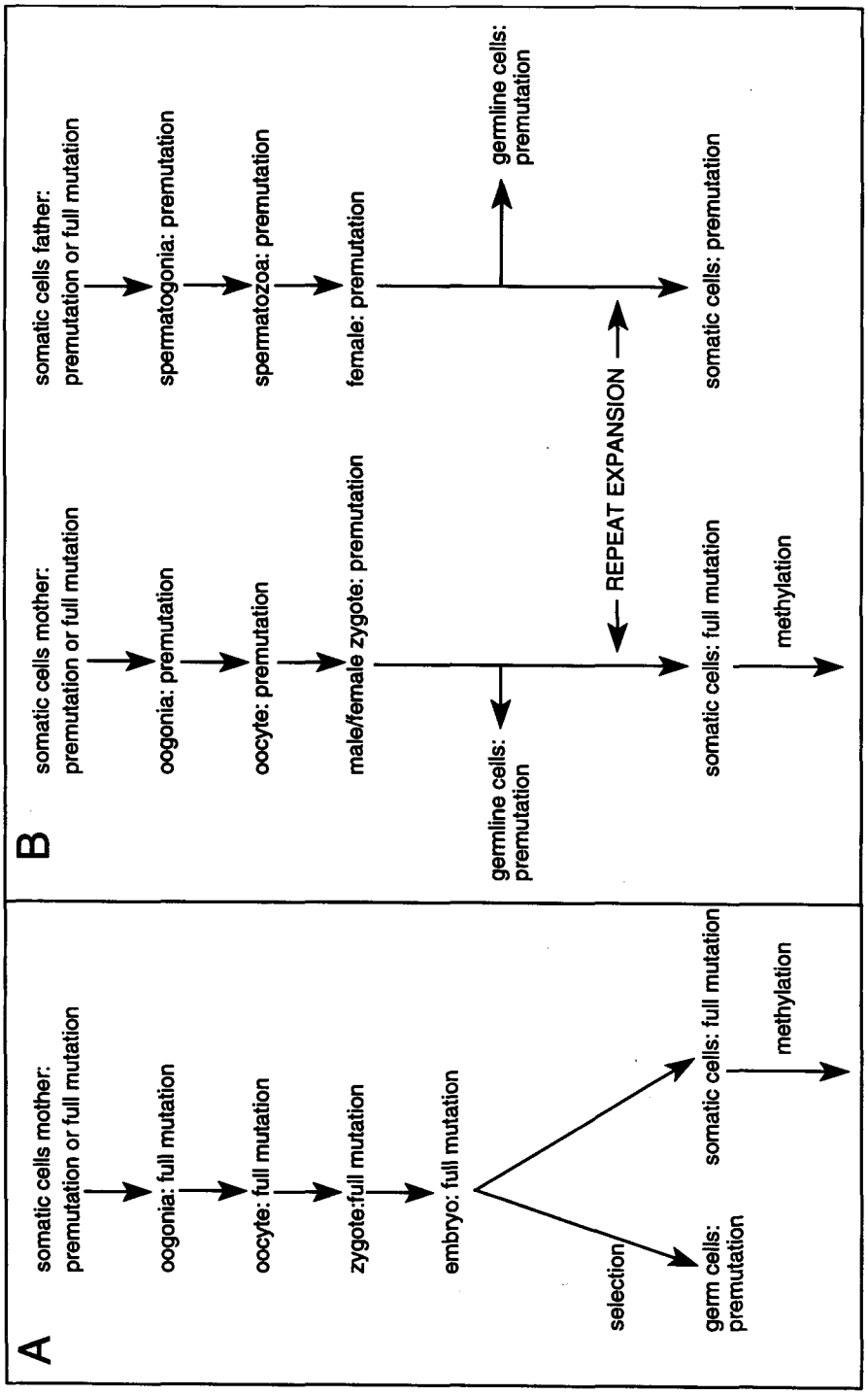

Fig. 4 - Models for timing of repeat amplification.

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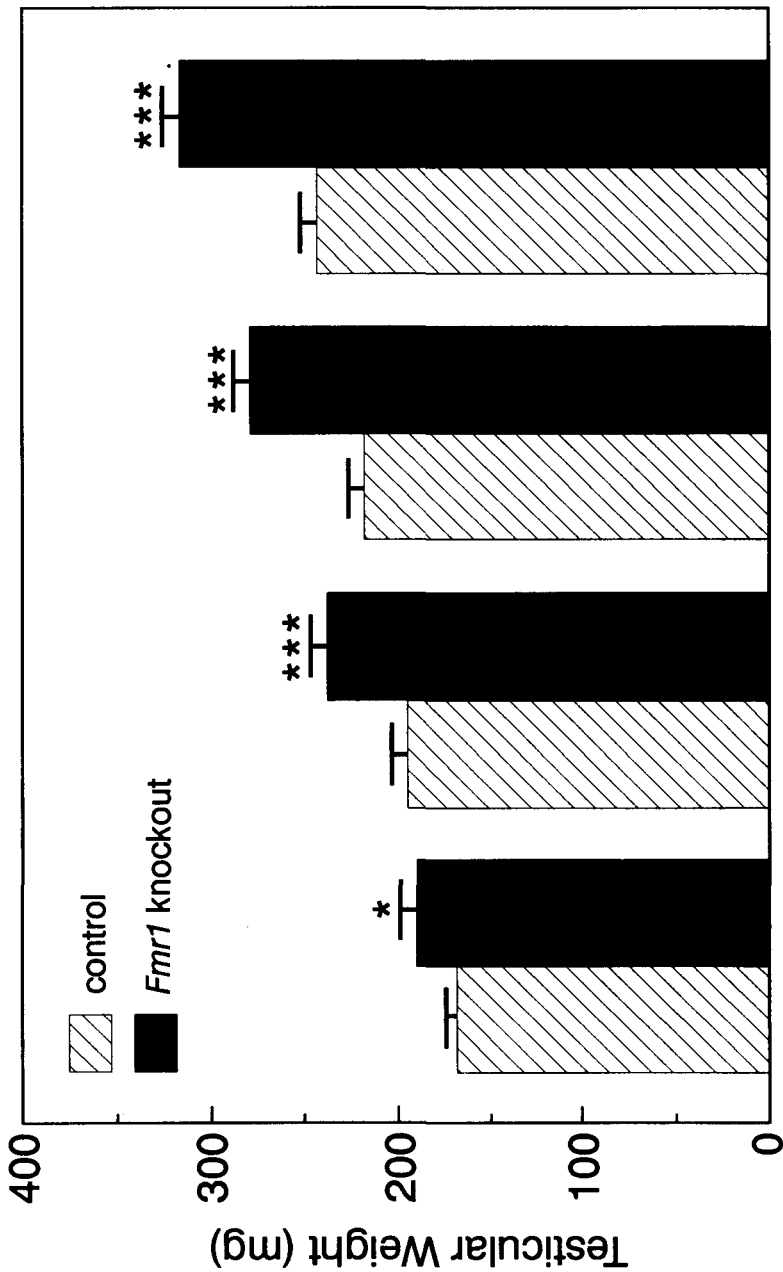

Fig. 5 - Testis size. Combined testicular weight of both testes of knockout versus control mice. The error bars display $SE_{\bar{x}}$. Asterisks indicate statistical differences between the mutant and age-matched normal litter mates (* $p < 0.05$; *** $p < 0.001$), calculated by Student's t test.

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The involvement of factors acting in either cis or trans has been postulated but there is little evidence thus far. It has been speculated that the expansion may be dependent on the repeat length, especially if the repeat has a length greater than 70: it could be contained in one Okazaki fragment [71]. Due to the nature of the repeat, this fragment is not well anchored and slippage of the repeat could occur. The nature of the repeat has been shown to be different in premutations compared to controls. In control alleles, the CGG repeat is occasionally interrupted by AGG triplets [72, 73]. These AGG repeats are found at the 5' end of the repeat, while most of the length variation occurs at the 3' end where the longest tracts of pure CGGs are found. In premutation alleles, this stretch of pure CGGs is increased and it is postulated that the threshold for instability is about 34 pure CGGs [74].

Instability of microsatellites is a common phenomenon in hereditary polyposis colon cancer, and is caused by mutations in one of the mismatch repair genes *MSH2, MLH1, PMS1, and PMS2* [75a77]. Whether these enzymes are involved in the instability of trinucleotide repeats is unknown.

Animal model

The *FMR1* gene is highly conserved among species [9] and the murine homologue Fmr1 shows 95% homology at the nucleic acid level and 97% homology in amino acid sequence [33]. The murine Fmr1 gene also contains a CGG repeat that is polymorphic among different mouse strains with an average repeat length of 10 CGGs. The expression pattern of *FMR1* at the mRNA and protein level is almost identical in different tissues of humans and mice [23, 34, 35, 78, 79], which makes the mouse a good animal model to study the fragile X syndrome.

A mouse has been developed in which the Fmr1 gene was inactivated [68]. As in fragile X patients, these mice lack normal Fmr1 RNA and protein. No gross pathological abnormalities were observed in these mice, and the brain anatomy appears to be normal, but these mice have enlarged testes (Fig. 5). As in patients, testes enlargement develops gradually over time. The mutant mice show cognitive impairment in the form of deficits in learning, as shown in Morris water maze experiments. The mice had a significantly higher level of exploratory behaviour and a higher level of motor activity than control mice. The observation of a set of defects similar to human ones in the mutant mice suggests that they can be used as a model to study fragile X mental retardation.

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REFERENCES

1. Webb TP, Bundey SE, Thake AI, Todd J: Population incidence and segregation ratios in the Martin-Bell syndrome. Am J Med Genet 1986; 23: 573-580.
2. Turner G, Daniel A, Frost M: X-linked mental retardation, macro-orchidism, and the Xq27 fragile site. J Pediatr 1980; 96: 837-841.
3. Sutherland GR: Fragile sites on human chromosomes: Demonstration of their dependence on the type of tissue culture medium. Science 1977; 197: 265-266.
4. Fish G, Arinami T, Froster-Iskenius U, Fryns JP, Curfs LM, Borggreaf M, Howard-Peebles PN, Schwartz CE, Simensen RJ, Shapiro LR: Relationship between age and IQ among fragile X males: A multicenter study. Am J Med Genet 1991; 38: 481-487.
5. Hagerman RJ: Physical and behavioral phenotype; in Hagerman RJ, Silverman AC (eds): Fragile X Syndrome: Diagnosis, Treatment and Research. Baltimore, John Hopkins University Press, 1991, pp 1-68.
6. Reiss AL, Lee J, Freund L: Neuroanatomy of fragile X syndrome: The temporal lobe. Neurology 1994; 44: 1317-1324.
7. Reiss AL, Aylwarth E, Freund LS, Joshi PK, Bryan RN: Neuroanatomy of fragile X syndrome: The posterior fossa. Ann Neurol 1991; 29: 26-32.
8. Reiss AL, Abrams MT, Greenlaw R, Freund L, Denckla MB: Neurodevelopmental effects of the *FMR-1* full mutation in humans. Nat Med 1995; 1: 159-167.
9. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, Reiner O, Richards S, Victoria MF, Zhang FP, Eussen BE, Van Ommen GJB, Blonden LAJ, Riggins GJ, Chastain JL, Kunst CB, Galjaard H, Caskey CT, Nelson DL, Oostra BA, Warren ST: Identification of a gene (*FMR-1*) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 1991; 65: 905-914.
10. Verkerk AJ, Eussen BH, Van Hemel JO, Oostra BA: Limited size of the fragile X site shown by fluorescence in situ hybridization. Am J Med Genet 1992; 43: 187-191.
11. Eichler EE, Richards S, Gibbs RA, Nelson DL: Fine structure of the human *FMR1* gene. Hum Mol Genet 1993; 2: 1147-1153.
12. Bell MV, Hirst MC, Nakahori Y, MacKinnon RN, Roche A, Flint TJ, Jacobs PA, Tommerup N, Tranebjaerg L, Froster-Iskenius U, Kerr B, Turner G, Lindebaum D, Winter R, Pembrey M, Thibodeau S, Davies KE: Physical mapping across the fragile X: Hypermethylation and clinical expression of the fragile X syndrome. Cell 1991; 64: 861-866.
13. Vincent A, Heitz D, Petit C, Kretz C, Oberle I, Mandel JL: Abnormal pattern detected in fragile-X patients by pulsed-field gel electrophoresis. Nature 1991; 349: 624-626.
14. Oberlé I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, Boue J, Bertheas MF, Mandel JL: Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. Science 1991; 252: 1097-1102.
15. Yu S, Pritchard M, Kremer E, Lynch M, Nancarrow J, Baker E, Holman K, Mulley JC, Warren ST, Schlessinger D, Sutherland GR, Richards RI: Fragile X genotype characterized by an unstable region of DNA. Science 1991; 252: 1179-1181.
16. Rousseau F, Heitz D, Tarleton J, Macpherson J, Malmgren H, Dahl N, Barnicoat A, Mathew C, Mornet E, Tejada I, Maddalena A, Spiegel R, Schinzel A, Marcos J, Schorderet DF, Schaap T, Maccioni L, Russo S, Jacobs PA, Schwartz C, Mandel JL: A multicenter study on genotype-phenotype correlations in the fragile X syndrome, using direct diagnosis with probe StB 12.3: The first 2,253 cases. Am J Hum Genet 1994; 55: 225-237.
17. Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJ, Holden JJ Fenwick R Jr, Warren ST, Oostra BA, Nelson DL, Caskey CT: Variation of the CGG repeat at the fragile X site results in genetic instability: Resolution of the Sherman paradox. Cell 1991; 67: 1047-1058.
18. Hansen RS, Gartler SM, Scott CR, Chen SH, Laird CD: Methylation analysis of CGG sites in the CpG island of the human *FMR1* gene. Hum Mol Genet 1992; 1: 571-578.

19. Homstra IK, Nelson DL, Warren ST, Yang TP: High resolution methylation analysis of the *FMR1* gene trinucleotide repeat region in fragile X syndrome. Hum Mol Genet 1993; 2: 1659-1665.
20. Pieretti M, Zhang FP, Fu YH, Warren ST, Oostra BA, Caskey CT, Nelson DL: Absence of expression of the *FMR-1* gene in fragile X syndrome. Cell 1991; 66: 817-822.
21. Sutcliffe JS, Nelson DL, Zhang F, Pieretti M, Caskey CT, Saxe D, Warren ST: DNA methylation represses *FMR-1* transcription in fragile X syndrome. Hum Mol Genet 1992; 1: 397-400.
22. Verheij C, Bakker CE, de Graaff E, Keulemans J, Willemsen R, Verkerk AJ, Galjaard H, Reuser AJ, Hoogeveen AT, Oostra BA: Characterization and localization of the *FMR-1* gene product associated with fragile X syndrome. Nature 1993; 363: 722-724.
23. Devys D, Lutz Y, Rouyer N, Bellocq JP, Mandel JL: The *FMR-1* protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat Genet 1993; 4: 335-340.
24. Rousseau F, Heitz D, Biancalana V, Blumenfeld S, Kretz C, Boue J, Tommerup N, Van Der Hagen C, DeLozier-Blanchet C, Croquette MF, Gilgenkranz S, Jalbert P, Voelckel MA, Oberle I, Mandel JL: Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. N Engl J Med 1991; 325: 1673-1681.
25. Sutherland GR, Gedeon A, Komman L, Donnelly A, Byard RW, Mulley JC, Kremer E, Lynch M, Pritchard M, Yu S, Richards RI: Prenatal diagnosis of fragile X syndrome by direct detection of the unstable DNA sequence. N Engl J Med 1991; 325: 1720-1722.
26. Verkerk AJ, de Vries BB, Niermeijer MF, Fu YH, Nelson DL, Warren ST, Majoor-Krakauer DF, Halley DJ, Oostra BA: Intragenic probe used for diagnostics in fragile X families. Am J Med Genet 1992; 43: 192-196.
27. Oostra BA, Jacky PB, Brown WT, Rousseau F: Guidelines for the diagnosis of fragile X syndrome: National Fragile X Foundation. J Med Genet 1993; 30: 410-413.
28. Rousseau F, Robb LJ, Rouillard P, Derkaloustian VM: No mental retardation in a man with 40% abnormal methylation at the *FMR-1* locus and transmission of sperm cell mutations as premutations. Hum Mol Genet 1994; 3: 927-930.
29. McConkie-Rosell A, Lachiewicz A, Spiridigliozzi GA, Tarleton J, Schoenwald S, Phelan MC, Goonewardena P, Ding X, Brown WT: Evidence that methylation of the *FMR1* locus is responsible for variant phenotypic expression of the fragile X syndrome. Am J Hum Genet 1993; 53: 800-809.
30. Hagerman RJ, Hull CE, Safanda JF, Carpenter I, Staley LW, Oconnor RA, Seydel C, Mazzocco M, Snow K, Thibodeau SN, Kuhl D, Nelson DL, Caskey CT, Taylor AK: High functioning fragile X males: Demonstration of an unmethylated fully expanded *FMR-1* mutation associated with protein expression. Am J Med Genet 1994; 51: 298-308.
31. Brown WT, Houck GE, Jeziorowska A, Levinson FN, Ding X, Dobkin C, Zhong N, Henderson J, Brooks SS, Jenkins EC: Rapid, fragile-X carrier screening and prenatal diagnosis using a nonradioactive PCR test. JAMA 1993; 270: 1569-1575.
32. Verkerk AJ, De Graaff E, De Boulle K, Eichler EE, Konecki DS, Reyniers E, Manca A, Poustka A, Willems PJ, Nelson DL, Oostra BA: Alternative splicing in the fragile X gene *FMR1*. Hum Mol Genet 1993; 2: 399-404.
33. Ashley CT, Sutcliffe JS, Kunst CB, Leiner HA, Eichler EE, Nelson DL, Warren ST: Human and murine *FMR-1*: Alternative splicing and translational initiation downstream of the CGG-repeat. Nat Genet 1993; 4: 244-251.
34. Abitbol M, Menini C, Delezoide AL, Rhyner T, Vekemans M, Mallet J: Nucleus basalis magnocellularis and hippocampus are the major sites of *FMR-1* expression in the human fetal brain. Nat Genet 1993; 4: 147-153.
35. Bachner D, Steinbach P, Wohrle D, Just W, Vogel W, Hameister H, Manca A, Poustka A: Enhanced Fmr-1 expression in testis. Nat Genet 1993; 4: 115-116.
36. De Graaff E, Willemsen R, Zhong N, De Die-Smulders CEM, Brown WT, Freling G, Oostra BA: Instability of the CGG repeat and expression of the *FMR1* protein in a male fragile X patient with a lung tumour. Am J Hum Genet, in press.

37. Gedeon AK, Baker E, Robinson H, Partington MW, Gross B, Manca A, Kom B, Poustka A, Yu S, Sutherland GR, Mulley JC: Fragile X syndrome without CCG amplification has an *FMR1* deletion. Nat Genet 1992; 1: 341-344.
38. Wohrle D, Kotzot D, Hirst MC, Manca A, Kom B, Schmidt A, Barbi G, Rott HD, Poustka A, Davies KE, Steinbach P: A microdeletion of less than 250 kb, including the proximal part of the *FMR-1* gene and the fragile-X site, in a male with the clinical phenotype of fragile-X syndrome. Am J Hum Genet 1992; 51: 299-306.
39. Meijer H, Degraaff E, Merckx D, Jongbloed R, Dediesmulders C, Engelen J, Fryns JP, Curfs P, Oostra BA: A deletion of 1.6 kb proximal to the CGG repeat of the *FMR1* gene causes the clinical phenotype of the fragile X syndrome. Hum Mol Genet 1994; 3: 615-620.
40. Siomi H, Siomi MC, Nussbaum RL, Dreyfuss G: The protein product of the fragile X gene, *FMR1*, has characteristics of an RNA-binding protein. Cell 1993; 74: 291-298.
41. Ashley C Jr, Wilkinson KD, Reines D, Warren ST: *FMR1* protein: Conserved RNP family domains and selective RNA binding. Science 1993; 262: 563-568.
42. De Boulle K, Verkerk AJ, Reyniers E, Vits L, Hendrickx J, Van Roy B, Van den Bos F, de Graaff E, Oostra BA, Willems PJ: A point mutation in the *FMR-1* gene associated with fragile X mental retardation. Nat Genet 1993; 3: 31-35.
43. Siomi H, Choi M, Siomi MC, Nussbaum RL, Dreyfuss G: Essential role for KH domains in RNA binding: Impaired RNA binding by a mutation in the KH domain of *FMR1* that causes fragile X syndrome. Cell 1994; 77: 33-39.
44. Verheij C, De Graaff E, Bakker CE, Willemsen R, Willems PJ, Meijer N, Galjaard H, Reuser AJJ, Oostra BA, Hogeveen AT: Characterization of *FMR1* proteins isolated from different tissues. Hum Mol Genet, in press.
45. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH: Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 1991; 352: 77-79.
46. Brook JD, McCurrach ME, Harley HG, Buckler AJ, Church D, Aburatani H, Hunter K, Stanton VP, Thirion JP, Hudson T, Sohn R, Zemelman B, Snell RG, Rundle SA, Crow S, Davies J, Shelboume P, Buxton J, Jones C, Juvonen V, Johson K, Harper PS, Shaw DJ, Housman DE: Molecular basis of myotonic dystrophy: Expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. Cell 1992; 68: 799-808.
47. Mahadevan M, Tsilfidis C, Saborin L, Shutler G, Amemiya C, Jansen G, Neville C, Narang M, Barcelo J, OrHOy K, Leblond S, Earle-Macdonald J, de Jong P, Wieringa B, Komeluk RG: Myotonic dystrophy mutation: An unstable CTG repeat in the 3' untranslated region of the gene. Science 1992; 255: 1253-1255.
48. Huntington. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 1993; 72: 971-983.
49. Orr HT, Chung M, Banfi S, Kwiatkowski TJ, Servadio A, Beaudet A, McCall AE, Duvick LA, Ranum LPW, Zohgbi HY: Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. Nat Genet 1993; 4: 221-226.
50. Koide R, Ikeuchi T, Onodera O, Tanaka H, Igarashi S, Endo K, Takahashi H, Kondo R, Ishikawa A, Hayashi T, Saito M, Tomoda A, Miike T, Naito H, Ikuta F, Tsuji S: Unstable expansion of CAG repeat in hereditary dentatorubral-pallidoluysian atrophy (DRPLA). Nat Genet 1994; 6: 9-13.
51. Nagafuchi S, Yanagisawa H, Sato K, Shirayama T, Ohsaki E, Bundo M, Takeda T, Tadokoro K, Kondo I, Murayama N: Dentatorubral and pallidoluysian atrophy expansion of an unstable CAG repeat trinucleotide on chromosome 12p. Nat Genet 1994; 6: 14-18.
52. Knight SJ, Flannery AV, Hirst MC, Campbell L, Christodoulou Z, Phelps SR, Pointon J, Middleton-Price HR, Bamicoat A, Pembrey ME, Holland J, Oostra BA, Bobrow M, Davies KE: Trinucleotide repeat amplification and hypermethylation of a CpG island in FRAXE mental retardation. Cell 1993; 74: 127-134.
53. Burke JR, Wingfield MS, Lewis KE, Roses AD, Lee JE, Hulette C, Pericak-Vance MA, Vance JM: The Haw River syndrome: Dentatorubropallidoluysian atrophy (DRPLA) in an African-American family. Nat Genet 1994; 7: 521-524.

54. Kawaguchi Y, Okamoto T, Taniwaki M, Aizawa M, Inoue M, Katayama S, Kawakami H, Nakamura S, Nishimura M, Akiguchi I, Kimura J, Narumiya S, Kakizuka A: CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. Nat Genet 1994; 8: 221-228.
55. Nancarrow JK, Kremer E, Holman K, Eyre H, Doggett NA, Le Paslier D, Callen DF, Sutherland GR, Richards RI: Implications of FRA16A structure for the mechanism of chromosomal fragile site genesis. Science 1994; 264: 1938-1941.
56. Parrish JE, Oostra BA, Verkerk AJHM, Richards CS, Spikes AS, Shaffer LG, Nelson DL: Isolation of a GCC repeat showing expansion in FRAXF, a fragile site distal to FRAXA and FRAXE. Nat Genet 1994; 8: 229-235.
57. Sherman SL, Morton NE, Jacobs PE, Turner G: The marker (X) chromosome: A cytogenetic and genetic analysis. Ann Hum Genet 1984; 48: 21-37.
58. Sherman SL, Jacobs PA, Morton NE, Froster-Iskenius U, Howard-Peebles PN, Nielsen KB, Partington NW, Sutherland GR, Turner G, Watson M: Further segregation of the fragile X syndrome with special reference to transmitting males. Hum Genet 1985; 69: 289-299.
59. Richards RI, Holman K, Friend K, Kremer E, Hillen D, Staples A, Brown WT, Goonewardena P, Tarleton J, Schwartz C, Sutherland GR: Evidence of founder chromosomes in fragile X syndrome. Nat Genet 1992; 1: 257-260.
60. Oudet C, Mornet E, Serre JL, Thomas F, Lentes-Zengerling S, Kretz C, Deluchat C, Tejada I, Boue J, Boue A, Mandel JL: Linkage disequilibrium between the fragile X mutation and two closely linked CA repeats suggests that fragile X chromosomes are derived from a small number of founder chromosomes. Am J Hum Genet 1993; 52: 297-304.
61. Hirst MC, Knight SJ, Christodoulou Z, Grewal PK, Fryns JP, Davies KE: Origins of the fragile X syndrome mutation. J Med Genet 1993; 30: 647-650.
62. Oudet C, von Kuskull H, Nordstrom AM, Peippo M, Mandel JL: Striking founder effect for the fragile X syndrome in Finland. Eur J Hum Genet 1993; 1: 181-189.
63. Buyle S, Reyniers E, Vits L, De Boulle K, Handig I, Wuyts FL, Deelen W, Halley DJ, Oostra BA, Willems PJ: Founder effect in a Belgian-Dutch fragile X population. Hum Genet 1993; 92: 269-272.
64. Macpherson JN, Bullman H, Youings SA, Jacobs PA: Insert size and flanking haplotype in fragile X and normal populations – Possible multiple origins for the fragile X mutation. Hum Mol Genet 1994; 3: 399-405.
65. Devys D, Biancalana V, Rousseau F, Boue J, Mandel JL, Oberle I: Analysis of full fragile X mutations in fetal tissues and monozygotic twins indicate that abnormal methylation and somatic heterogeneity are established early in development. Am J Med Genet 1992; 43: 208-216.
66. Willems PJ, Van Roy B, De Boulle K, Vits L, Reyniers E, Beck O, Dumon JE, Verkerk A, Oostra B: Segregation of the fragile X mutation from an affected male to his normal daughter. Hum Mol Genet 1992; 1: 511-515.
67. Reyniers E, Vits L, De Boulle K, Van Roy B, Van Velzen D, de Graaff E, Verkerk AJMH, Jorens HZ, Darby JK, Oostra BA, Willems PJ: The full mutation in the *FMR-1* gene of male fragile X patients is absent in their sperm. Nat Genet 1993; 4: 143-146.
68. Bakker CE, Verheij C, Willemsen R, Vanderhelm R, Oerlemans F, Vermey M, Bygrave A, Hoogeveen AT, Oostra BA, Reyniers E, Deboulle K, Dhooge R, Cras P, Van Velzen D, Nagels G, Martin JJ, Dedeyn PP, Darby JK, Willems PJ: Fmrl knockout mice: A model to study fragile X mental retardation. Cell 1994; 78: 23-33.
69. Hansen RS, Canfield TK, Lamb MM, Gartler SM, Laird CD: Association of fragile X syndrome with delayed replication of the *FMR1* gene. Cell 1993; 73: 1403-1409.
70. Richards RI, Sutherland GR: Dynamic mutations: A new class of mutations causing human disease. Cell 1992; 70: 709-712.
71. Richards RI, Sutherland GR: Simple repeat DNA is not replicated simply. Nat Genet 1994; 6: 1 114-116.
72. Kunst CB, Warren ST: Cryptic and polar variation of the fragile X repeat could result in predisposing normal alleles. Cell 1994; 77: 853-861.

73. Hirst MC, Grewal PK, Davies KE: Precursor arrays for triplet repeat expansion at the fragile X locus. Hum Mol Genet 1994; 3: 1553-1560.
74. Eichler EE, Holden J, Popovich BW, Reiss AL, Snow K, Thibodeau SN, Richards CS, Ward PA, Nelson DL: Length of uninterrupted CGG repeats determines instability in the *FMR1* gene. Nat Genet 1994; 8: 88-94.
75. Leach FS, Nicolaides NC, Papadopoulos N, Liu B, Jen J, Parsons R, Peltomaki P, Sistonen P, Aaltonen LA, Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. Cell 1993; 75: 1215-1225.
76. Bronner CE, Baker SM, Morrison PT, Warren G, Smith LG, Lescoe MK, Kane M, Earabino C,: Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. Nature 1994; 368: 258-261.
77. Nicolaides NC, Papadopoulos N, Liu B, Wei YF, Carter KC, Ruben SM, Rosen CA, Haseltine WA,: Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. Nature 1994; 371: 75-80.
78. Hinds HL, Ashley CT, Sutcliffe JS, Nelson DL, Warren ST, Housman DE, Schalling M: Tissue specific expression of *FMR-1* provides evidence for a functional role in fragile X syndrome. Nat Genet 1993; 3: 36-43.
79. Bachner D, Manca A, Steinbach P, Wohrle D, Just W, Vogel W, Hameister H, Poustka A: Enhanced expression of the murine *FMR1* gene during germ cell proliferation suggests a special function in both the male and the female gonad. Hum Mol Genet 1993; 2: 2043-2050.

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