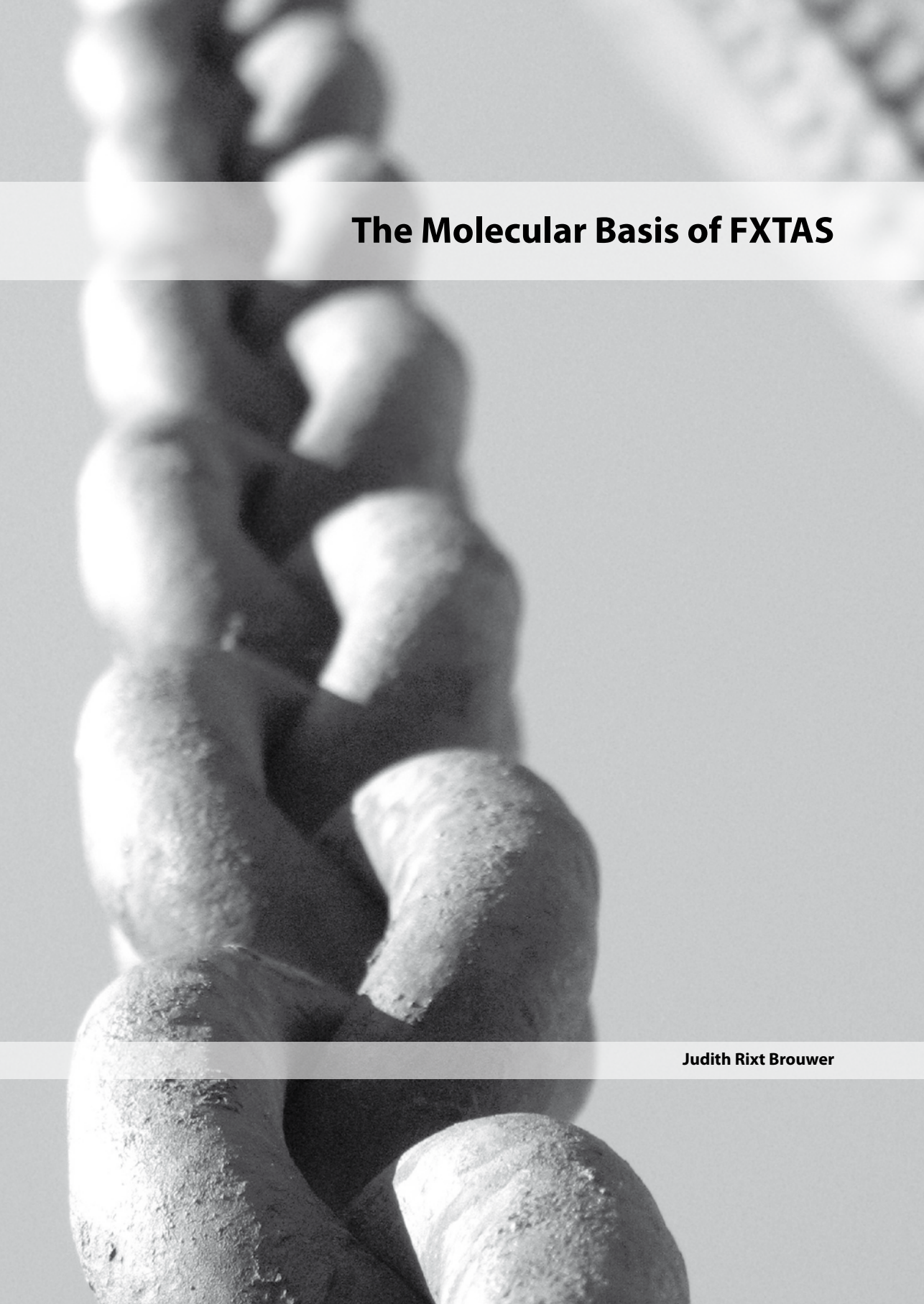

The Molecular Basis of FXTAS

Judith Rixt Brouwer

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De moleculaire basis van FXTAS

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List of abbreviations

$(CGG)_n$	CGG repeat
$(CGG)_n$ mice	*Fmr1* expanded (CGG)n knock in mice
ACTH	adrenocorticotrophin hormone
AMH	anti-Müllerian hormone
ANOVA	analysis of variance
ASD	autism spectrum disorders
AVP	arginin vasopressin
CACNA1A	α1A-subunit of voltage-dependent calcium channel
cAMP	cyclic adenosine 3'5'-monophosphatase
CBP	CREB-binding protein
CELF	CUG-BP/ETR-3-like family
CMTF2	Charcot-marie Tooth disease
CREB	cAMP response element binding protein
CRH	corticotrophin releasing hormone
CUGBP1	CUG-binding protein
DM	myotonic dystrophy
DMPK	myotonic dystrophy protein kinase
EGFP	enhanced green fluorescent protein
FEN1	flap endonuclease 1
FM	full mutation
FMR1	fragile X mental retardation 1
FMR2	fragile X mental retardation 2
FMRP	fragile X mental retardation protein
FRAXA	fragile x syndrome
FRDA	Friedreich ataxia
FSH	follicle stimulating hormone
FXTAS	fragile X associated tremor/ataxia syndrome
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GMD	geometrical mean difference
GR	glucocorticoid receptor
Gus	β-glucoronidase
HAT	histone acetyltransferase
HD	Huntington's disease
HDAC	histone deacetylase
HDL2	Huntington's disease-like 2
HNPCC	hereditary nonpolyposis colorectal cancer
hnRNP	heterogeneous nuclear ribonucleoprotein

HPA axis	hypothalamo-pituitary-adrenal axis
HPG axis	hypothalamo-pituitary-gonadal axis
HSP	heat shock protein
IF	intermediate filament
IVF	*in vitro* fertilisation
JPH3	junctophilin-3
KH domain	heterogeneous nuclear ribonucleoprotein K homology domain
MBNL	muscleblind-like protein
MBP	myelin basic protein
MCP	middle cerebellar peduncles
MDD	major depressive disorder
MEF	mouse embryonic fibroblast
MMR	mismatch repair
NER	nucleotide excision repair
OPMD	oculopharyngeal myotonic dystrophy
PABP2	poly(A)-binding protein 2
PC12 cells	pheochromocytoma 12 cells
PGC	primordial germ cell
PM	premutation
POF	premature ovarian failure
POI	premature ovarian insufficiency
polyQ	polyglutamine
POMC	proopiomelanocortin
PSP	progressive supranuclear palsy
RGG box	domain containing repeats of an Arg-Gly-Gly (RGG) motif
RNA-BP	RNA binding protein
RNP	ribonucleoprotein
SBMA	spinal and bulbar muscle atrophy
SCA	spinocerebellar ataxia
ssDNA	single stranded DNA
TBP	TATA-binding protein
UPS	ubiquitin proteasome system
UTR	untranslated region
wt	wild type
YAC	yeast artificial chromosome
ZNF9	zinc finger 9

CHAPTER 1

The *FMR1* gene and fragile X-associated tremor/ataxia syndrome

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The *FMR1* gene and the unstable CGG-repeat | 1.1

Fragile X syndrome has been recognised as the most common inherited form of mental retardation [1]. Its name refers to a fragile site, which was discovered on the long arm of the X chromosome of four mentally retarded patients [2]. This fragile site was later found to be inducible when culturing cells in folic acid-deficient medium. This facilitated the establishment of a link between expression of the fragile site (at Xq27) and the clinical phenotype [3,4]. Cloning of the gene responsible for fragile X syndrome, *FMR1* (fragile X mental retardation 1), revealed the presence of an expanded ($(CGG)_n$) in the 5'UTR. In fragile X syndrome this repeat exceeds 200 trinucleotides (full mutation: FM) [5-7]. *FMR1* consists of 17 exons, spanning a 38 kb region [8]. The $(CGG)_n$ in the *FMR1* gene is considered a 'dynamic mutation'; the phenomenon that a trinucleotide repeat becomes dramatically unstable once it exceeds a certain threshold length [9]. Depending on the length of the $(CGG)_n$, different clinical outcomes can develop (figure 1.1). Observations and hypotheses on when instability takes place are discussed in chapter 2.

Analysis of repeat length variation in the normal population showed a range of allele sizes between 6 to 54 CGGs [10], with an average of 29 to 30 repeats [11]. Alleles of between 45 and 54 CGGs form a distinct category. They were named grey zone alleles, when it was appreciated that these alleles are more likely to be unstable upon transmission, than are $(CGG)_n$ shorter than 45 trinucleotides [10,12]. Grey zone alleles frequently contain a long and uninterrupted $(CGG)_n$ [12]. The longest tract of uninterrupted $(CGG)_n$ appears to determine instability and susceptibility to disease, whereby the presence of a pure CGG-tract of more than 33 triplets appeared to greatly enhance the chance of unstable transmission. Thus, AGG interruptions might stabilise a $(CGG)_n$ [13]. This could be through prevention of formation of stable secondary RNA structures such as hairpins, as has been shown to occur in *in vitro* studies [14]. Hairpins might decrease the efficiency of FMRP translation initiation [15]. AGG interruptions, through influencing RNA structure, predominantly determine the extent of instability of long normal and short premutation alleles [14].

The remaining repeat range of 55 to 200 CGGs has been defined as the premutation (PM) [16]. Initially, it was thought that the only risk associated with the PM was for the $(CGG)_n$ to expand to an FM allele, as alleles in this size range are quite unstable upon maternal transmission [17]. The risk of expansion from a female PM carrier to an FM in her progeny increases with repeat length [18]. In few cases, a contraction of maternal origin is seen. Inheritance of a $(CGG)_n$ of paternal origin shows a less consistent pattern, as expansions, contractions and stable transmissions occur. Expansion of a PM to an FM is restricted to maternal transmission. Also, daughters of a male FM carrier, affected with fragile X, never show clinical or cytogenetical signs of fragile X syndrome. This was explained by the finding that FM males only carry PM alleles in their sperm [19]. Thus, both allele size and the parent's sex determine the risk of expansion from parent to offspring [20].

One in 813 males and one in 259 females of the general population in North America were described to carry the PM [11,21]. A recent study in 40 000 women in Israel revealed a prevalence of 1 in 154. No difference in carrier frequency was seen between women with and without family history of mental retardation and developmental abnormalities [22]. However, it is now known that different ethnic groups show a different prevalence, for example it is less common in Asian populations [23], while it is more prevalent in Mediterranean groups [24,25].

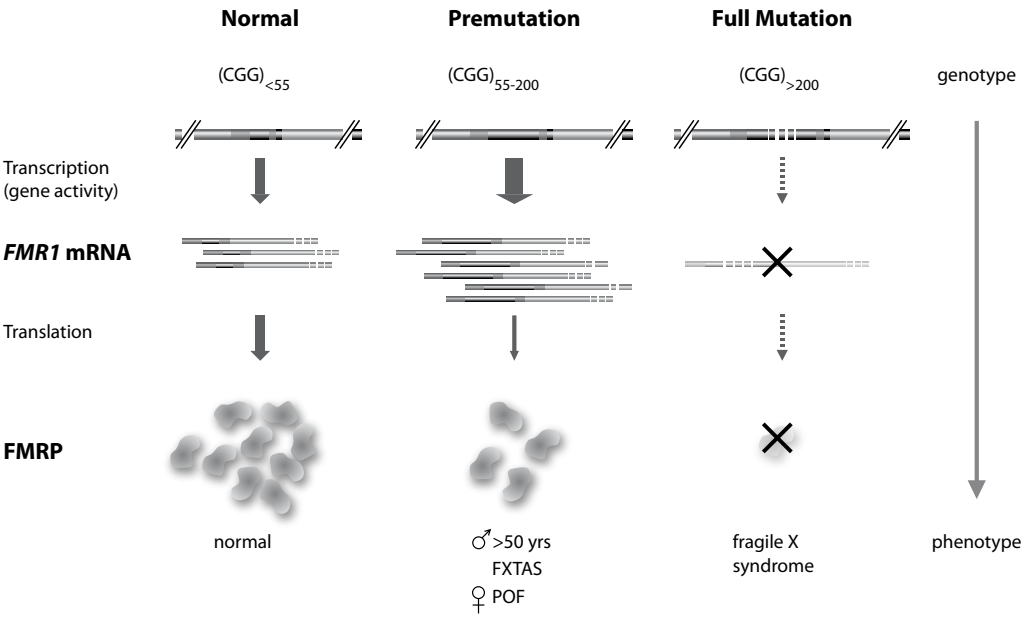

Figure 1.1 | $(CGG)_n$ length and *FMR1* expression and clinical outcome. This figure shows the consequences of the different repeat length categories on transcription, translation and clinical phenotype. (Adapted from [76] with permission of the authors.) See page 163 onwards for colour version of this figure.

Early *in situ* hybridisation studies in mice revealed that *Fmr1* mRNA is abundant and widespread in early embryogenesis. Later in embryonic development a specific expression pattern develops, with high expression mainly in brain, testes and ovaries. Adult mice also showed high expression in brain and testes. No *Fmr1* expression was seen in mature ovaries, despite the high levels in the foetal ovaries, when proliferation of oogonia takes place. Thus, the *FMR1* gene might have a function during germ cell proliferation in both sexes [26-28]. *FMR1* mRNA expression in human foetuses was high in the nervous system, but also in some non-neuronal tissues such as cartilage, hepatocytes, the spinal cord and the retina [29,30].

In fragile X patients, the $(CGG)_n$ of over 200 trinucleotides (FM) and the upstream promoter region of the *FMR1* gene are usually hypermethylated [5,6,31]. As a result, gene transcription is silenced, thus the gene product fragile X mental retardation protein, FMRP, is not produced. The complete lack of FMRP in neurons is the cause of the mental retardation seen in fragile X patients [32]. The functions of FMRP and the consequences of the absence of FMRP in fragile X syndrome are discussed in more detail below.

Transcriptional silencing of the *FMR1* gene | 1.2

Many studies have addressed the question of how methylation of CpGs in the *FMR1* gene leads to gene silencing. Local chromatin changes as a result of methylation mediate indirect mechanisms, while a direct effect is the inhibition of binding of transcription factors to the promoter [33,34]. Methyl binding proteins (such as MeCP2) recognise methylated CpG residues, upon which a multiprotein complex is

recruited. This protein complex includes histone deacetylases (HDACs) [35,36]. HDACs remove acetyl groups from lysines of histone H3 and H4 amino terminal tails, which results in increased condensation of the chromatin (heterochromatin), which is associated with inactive gene regions [37]. The *FMR1* gene normally shows acetylated histones H3 and H4, while cells of patients with fragile X show reduced acetylation [38]. Experiments in cells of fragile X patients showed that methylation and deacetylation indeed cooperate in silencing chromatin domains, when histone hyperacetylating drugs could synergistically potentiate reactivation of the *FMR1* gene induced by a demethylating agent [33,39]. Further analysis of epigenetic modification in three different regions of the *FMR1* gene revealed that epigenetic codes relevant to the transcription of the gene are concentrated in the 5'UTR region [40].

Histones can also be modified in ways other than acetylation, for example by the addition or removal of phosphate or methyl groups [41]. Methylation of lysines 4 and 9 of histone 3 (H3K4 and H3K9, respectively) has been looked at intensively and it became clear that methylation of H3K4 is associated with a transcriptionally active state, while methylation of H3K9 correlates with transcriptional inactivation [42,43]. Methylated H3K9 has been suggested to recruit histone methyl transferases, associated with HDACs, which then results in histone deacetylation [44]. However, studies in cell lines suggest that methylation of H3K9 and partial deacetylation, normally associated with heterochromatin, can coexist with H3K4 methylation, which is correlated with active *FMR1* transcription [40].

Two brothers with expanded $(CGG)_n$ in the full mutation range have been described, who were spared from the fragile X phenotype. Although cytogenetical analysis revealed a fragile site, the *FMR1* promoter was unmethylated and they did produce *FMR1* mRNA and FMRP. This indicates that inactivation of *FMR1*, due to methylation and not the expansion itself, is the cause of the symptoms seen in fragile X syndrome [45]. Characterisation of a human embryonic stem cell line derived from a pre-implantation embryo with FM range repeat showed that the *FMR1* gene was unmethylated and expressed, despite the presence of an FM allele. More specifically, it displayed the features of active chromatin [46]. Thus, these results confirm that the $(CGG)_n$ by itself is not sufficient to cause transcriptional silencing of *FMR1* [31]. Upon differentiation (by inducing formation of teratomas in immunodeficient mice) this ES cell line underwent partial transcriptional silencing. Differentiation lead to reduced H3 tail acetylation, and H3K9 became methylated, while *FMR1* mRNA levels decreased. However, the 5'UTR of *FMR1* was still hypomethylated. Thus, the authors concluded that *de novo* methylation of the upstream regulatory region of *FMR1* follows transcriptional silencing, whereby the chromatin changes take place relatively early during differentiation. DNA methylation appears to contribute to the maintenance, rather than to the induction of transcriptional inactivation in somatic cells in patients with fragile X [46]. This role for DNA methylation has previously been described with regard to X-inactivation in females [47].

However, many questions remain to be addressed about the precise molecular mechanism by which *FMR1* transcription is regulated, of which the answers might prove valuable for developing therapeutical strategies involving reactivation of the *FMR1* gene in fragile X syndrome.

The gene product FMRP | 1.3

FMRP is an RNA-binding protein (RNA-BP), as it bears two KH-domains and an RGG-box; both are sequences with RNA-binding capacities [48-50]. Among many other mRNAs, FMRP can bind its own

message with high affinity *in vitro* [48]. Furthermore, a nuclear localisation signal and a nucleur export signal have been identified, suggesting that FMRP shuttles between the nucleus and the cytoplasm [51-54]. 85% of cellular FMRP is present on actively translating polyribosomes, underscoring the role of FMRP as an RNA-BP [55]. FMRP has been identified in ribonucleoprotein (RNP) particles. RNA was shown to bind in stoichiometric ratios, suggesting that each FMRP molecule bears two RNA-binding sites [48]. A minority of FMRP is found in the nucleus, either at the nucleopore, or associated with the nucleolus [55-57].

Immunocytochemical studies in mice revealed that Fmrp is ubiquitously expressed up until day 14 of embryonic development. In later embryonic development a more specific pattern arises with tissues of ectodermal origin (brain, ganglia, hair follicles, sensory cells, adrenal medulla) and the gonads (mesodermal origin) showing the highest expression levels [58]. FMRP expression in human embryos (3-7 weeks) was consistent with the murine expression pattern, showing highest expression in brain and testes [29,30,59,60]. In adult human brain, FMRP is present in high quantities in neurons, while little or no FMRP is detected in glia and oligodendrocytes [28,30,59,61,62]. High levels of FMRP expression in human tissue coincide with clinical involvement in fragile X Syndrome [59,60,63,64]. The absence of evident neuropathological abnormalities of fragile X foetuses suggests that the *FMR1* gene product FMRP is not crucial in early stages of development of the nervous system [29].

After identification of the *FMR1* gene research has focused on the cellular function of FMRP. The RNA-binding capacities, association with ribosomes and the cytoplasmic localisation in dendrites pointed towards a function for FMRP in mRNA transport or translation. mRNA transport and translation in dendrites is important for neuronal function, including modulation of synaptic plasticity. This is essential in memory consolidation and learning [65,66]. Altered spine morphology (long and thin dendritic spines) has been observed in *post mortem* brains of fragile X patients [67-69] and in *FMR1* KO mice [70-72] Target mRNAs of FMRP that are involved in synaptic function or dendritic growth have been identified, which is very interesting in light of the aberrant spine maturation [49]. FMRP was found to strongly inhibit translation of several mRNAs *in vitro*, through inhibition of the assembly of the 80S ribosomal complexes on target mRNA. The second KH-domain appears crucial in this process, as the I304N mutant FMRP, with a mutation in this RNA-binding domain, failed to inhibit translation [73]. Thus, a model has been proposed that FMRP binds specific mRNAs and mediates the targeting of these transcripts into the dendrite. During transport they remain translationally inactive until appropriate synaptic input allows translation. Thus, FMRP might have a role in transport and/or translational efficiency of specific mRNAs at the synapse. The absence of FMRP would then lead to translational dysregulation of a subset of mRNAs at the synapse. Translational dysregulation of mRNAs normally associated with FMRP is proposed to be the underlying cause of mental retardation in fragile X [49,74,75].

Clinical symptoms associated with the premutation | 1.4

As mentioned above, it was long thought that carriership of the PM was not associated with clinical problems, other than the risk of expansion to an FM upon transmitting the PM allele to the next generation. However, a small subgroup of PM carriers was reported to have mild learning disabilities and social phobias or anxiety disorder [76]. Furthermore, it became apparent that 20% of female PM carriers manifest premature ovarian failure (POF: cessation of menstruation at or before 40 years of age)[77].

Women with the PM, even if they are still cycling, have higher levels of follicle stimulation hormone (FSH) than do healthy women [78]. It has recently been suggested that Anti-Müllerian Hormone (AMH) may be a better marker of ovarian decline. AMH is expressed only in growing follicles, thus serves as a marker for the size of the primordial follicle pool in women. Indeed, female PM carriers, with $(CGG)_n$ lengths beyond 70, had lower AMH levels than did female PM carriers with $(CGG)_n$ shorter than 70 nucleotides. Thus, lower AMH levels are suggestive of early ovarian decline in women with $(CGG)_{>70}$ [79]. Penetrance and age at onset of POF, as well as the increase of FSH levels, correlate with $(CGG)_n$ length [80]. However, a non-linear relationship has been described for age at menopause and premutation size, in which premutations in the mid-size range are at greatest risk for POF, while larger repeat tracts are associated with a lower risk [81]. It has been proposed that premature ovarian insufficiency (POI) is a more accurate term for the disorder, to describe the broad range of clinical manifestations associated with what used to be classified as POF [82].

Over the past few years, it has become apparent that PM carriers are also at risk of developing a progressive neurodegenerative disorder, which is clinically and neuropathologically entirely distinct from fragile X syndrome [83-85]. In 2001, five male PM carriers were described who suffered from progressive action tremor, causing executive function deficits, cerebellar dysfunction, cognitive decline and Parkinsonism associated with generalised brain atrophy. Also autonomic dysfunction and peripheral neuropathy were reported [83]. Since it had already been established that PM carriers have elevated *FMR1* mRNA levels in their lymphocytes, but only somewhat reduced FMRP levels [86], it was then hypothesised that the progressive nervous system degeneration as seen in those five male PM carriers might be the result of the elevated *FMR1* mRNA levels [83]. The new syndrome was called fragile X-associated tremor/ataxia syndrome (FXTAS) [84]. However, not all PM carriers develop FXTAS; of male PM carriers ascertained through family members with fragile X syndrome, more than a third of men older than 50 years showed both tremor and ataxia [87]. Penetrance increases with age, to at least 50% in men aged 70-90 years. It has now been suggested that penetrance increases with CGG repeat length. For instance; most people with FXTAS had $(CGG)_n$ with over 70 CGGs, while in the general population only 22% of PM alleles are of this size or larger [88]. Based on the findings of Jacquemont *et al*. [88], Allen and co-workers defined alleles with over 70 CGGs as 'at risk' alleles, while a repeat shorter than 70 trinucleotides was considered a 'low risk' allele. Preliminary analyses of neuromotor tests did not reveal a difference between low-risk allele carriers and non-carriers [89].

It is as yet unknown whether specific prodromal signs exist for FXTAS. Although elevated *FMR1* mRNA levels in blood from PM carriers (devoid of FXTAS symptomatology) have been found to be associated with more psychological symptoms [90], naturally at the time it was unknown whether these PM carriers will later develop FXTAS. As awareness about FXTAS grows, monitoring of PM carriers (in known fragile X families) is likely to give insight into whether PM carriers are also at risk of developing problems before FXTAS develops and whether possible prodromal symptoms have predictive value for FXTAS development. This will of course also have importance for genetic counselling of fragile X families.

Clinical aspects of FXTAS | 1.5

FXTAS patients generally present with cerebellar gait ataxia and intention tremor [84], but may develop other neurological symptoms such as Parkinsonism, autonomic dysfunction and peripheral neuropathy and may suffer from cognitive decline ranging from mild frontal executive and memory deficits to global dementia [91]. The dementia resembles cognitive performance in the frontal variant of frontotemporal dementia, but not dementia in Alzheimer's disease [92,93]. There is substantial variation in clinical presentation, as illustrated by the broad range of diagnoses given to patients who were later diagnosed with FXTAS, once the syndrome had been identified and described [94].

Evidence is accumulating that the severity of some aspects of the disorder is correlated with the length of the $(CGG)_n$. For instance, age at death negatively correlated with the number of $(CGG)_n$ [95]. $(CGG)_n$ length was also reported to negatively correlate with age at onset of the major clinical motor symptoms, action tremor and gait ataxia [96]. Two earlier studies had not found any association between $(CGG)_n$ length and age at onset of clinical symptoms [84,91]. This difference was explained by cohort size and the width of the repeat range tested. Age at onset of symptoms was not correlated to *FMR1* mRNA levels, nor to FMRP levels [96]. This was explained by the finding that *FMR1* mRNA levels were measured in peripheral lymphocytes. The increase of *FMR1* mRNA levels in brain tissue from one FXTAS patient relative to a control subject is smaller than when *FMR1* mRNA levels in blood are compared. This is due to the higher abundance of *FMR1* mRNA in brain versus in blood (measured relative to GUS mRNA), also in controls. Differences in transcript levels in brain vary considerably over different regions, although they are driven by the same $(CGG)_n$ length, which is generally consistent throughout the brain [97]. It is possible that region-specific transcriptional regulation by transcription factors determines the variable *FMR1* expression levels throughout the brain. A lack of correlation between *FMR1* transcription and onset of motor problems is not surprising, as FMRP levels are not deviant from normal controls [96]. $(CGG)_n$ length was also described to correlate with increased cognitive and functional impairment in PM carriers [91]. Total brain volume reductions and increased white matter disease correlated with the number of CGGs [98,99]. Similar, though smaller volumetric changes were seen in PM carriers without symptoms of FXTAS, suggesting that neuropathological changes take place before clinical symptoms become apparent [98].

It has been described that the abnormal elevation of *FMR1* mRNA is associated with increased psychological symptoms such as anxiety, depression and irritability. Thus, increased psychopathology is not confined to FXTAS patients, but can occur in all adult PM carriers, although especially in males [85,90,100,101]. However, the observed neuropsychiatric phenotypes do not show a very consistent pattern, due to methodological limitations. Therefore, very recently a controlled study into neuropsychiatric functioning was conducted in male PM carriers, who did not show any signs of FXTAS, who were compared to controls who have a similar family environment (care of a family member with fragile X) and other non-PM carrier controls. This study did not find significant differences in psychopathology between PM carriers and non-PM carrier family and other controls, except that PM carriers were impaired in utilising working memory and accessing recall check. Also, when comparing PM carriers with normal controls, but not family controls, PM carriers showed more alcohol abuse [102]. A recent study investigated the prevalence of mood and anxiety disorders in PM mothers of at least

one child with fragile X syndrome. The most prominent finding was a higher rate of lifetime major depressive disorder (MDD). This did not appear to be fully explained by the stress of raising a child with fragile X syndrome. Interestingly, a longer $(CGG)_n$ was associated with less likely occurrence of lifetime MDD. Preliminary analysis might suggest a non-linear relationship between $(CGG)_n$ length and MDD, such that women with mid-range lengths are at increased risk, while females that carry the longest repeats have a lower risk [103]. Although speculative, this is in line with recent findings for POI prevalence in PM females [81,104]. As yet, no controlled study has been performed for PM carriers with FXTAS. A study which thoroughly investigated cognitive functioning in PM carriers with and without FXTAS, found substantial executive impairment and dysfunction in a wide range of aspects of cognitive functioning in patients with FXTAS, while unaffected PM carriers performed worse than controls on executive cognitive functioning and declarative learning and memory [105]. All relevant clinical aspects of FXTAS have been nicely summarised in a recent review by Berry-Kravis and colleagues [106].

In 2004, the first female cases with FXTAS were described. Female PM-carriers are much less likely to develop FXTAS, probably because of a protective effect due to the expression of the normal allele in part of their neurons. Also, oestrogen might have an alleviating effect on the disease mechanism [107]. The diluting effect of the presence of a normal *FMR1* allele is also the likely cause of the less severe clinical outcome seen in female FXTAS patients, as compared to male patients with FXTAS [107,108]. Positively skewed X-inactivation, wherein the normal allele is preferentially active, could lessen the toxic effects of elevated *FMR1* mRNA levels. In these first known females with FXTAS, no skewed X-inactivation was seen [107]. However, another study described two sisters who are both PM-carriers, displaying a different degree of clinical involvement, which correlated with the pattern of X-inactivation. The sister who had a skewed X-inactivation pattern, favouring a higher percentage of cells expressing the PM-allele, suffered from more severe symptomatology. Although genetic background may influence severity of FXTAS, this is not likely a major factor in these sisters. Thus, this study provides a clear view on the effect of X-inactivation on the clinical outcome in FXTAS [109].

Diagnostic criteria for FXTAS | 1.6

MRI studies of the first patients identified to suffer from FXTAS revealed generalised brain atrophy and enlarged ventricles [83]. Later, neuropathological *post mortem* studies of patients with manifestations of FXTAS generally showed global brain atrophy, as well as cerebellar and subcortical cerebral white matter disease. Also substantial Purkinje cell dropout with Bergmann gliosis is seen [110]. Another prominent feature is the so-called MCP sign, namely increased signal intensities in T2-weighted MRI recordings of the middle cerebellar peduncles (MCPs), which is caused by spongiosis of the deep cerebellar white matter [84,111]. This MCP sign is observed in about 60% of PM carriers with tremor and/or ataxia and was therefore included in the diagnostic criteria for FXTAS. Diagnosis is based on the following criteria, presented in table 1.1, on top of the mandatory criterion of a $(CGG)_n$ length between 55 and 200 [112]. Testing guidelines for FXTAS, as well as the proposed diagnostic criteria have recently been described and summarised in a review by Berry-Kravis *et al*. [106].

Criteria	Clinical		Radiological		Neuropathological (*post mortem*)
Diagnosis	**major**	**minor**	**major**	**minor**	
Definite FXTAS	1: intention tremor OR gait ataxia **AND:**		MCP sign **OR:**		Presence of intra-nuclear inclusions
Probable FXTAS	2: intention tremor AND gait ataxia **OR:**	Parkinsonism **AND:**	MCP sign		
Possible FXTAS	1: intention tremor OR gait ataxia **AND:**			Cerebral white-matter lesions, generalised atrophy	

Table 1.1 | Diagnostic criteria for FXTAS (mandatory criterion: 55-200 CGGs in *FMR1*) (adapted from: [112]). The table should be read from left to right, for each level of probability of the diagnosis.

Molecular correlates of FXTAS | 1.7

The development of FXTAS is restricted to carriers of the premutation, who have a transcriptionally fully active *FMR1* gene [113]. PM carriers, irrespective of showing signs of FXTAS, have been shown to have up to eight-fold elevated *FMR1* mRNA levels in peripheral blood leucocytes, despite close to normal FMRP levels [86,114-116]. Relative *FMR1* mRNA levels in peripheral blood leucocytes are more elevated in comparison with normal controls than are relative *FMR1* mRNA levels in brain [97]. The level of *FMR1* mRNA in blood leukocytes is highly and positively correlated to $(CGG)_n$ length in the PM range [86,114,115,117]. The mechanism underlying the elevated *FMR1* mRNA levels is unknown. The observation that increased transcript levels were also measured when constructs bearing the *FMR1* 5'UTR with PM-sized $(CGG)_n$, fused to a luciferase reporter were transfected into two different cell lines [15], suggests that the expanded $(CGG)_n$ itself, rather than the reduced FMRP levels, is responsible for increased transcription [75,114,115]. Multiple *FMR1* transcriptional initiation sites have been identified, with different efficiencies, dependent on the length of the $(CGG)_n$ in the 5'UTR downstream of the initiation sites. Thus, it appears that the $(CGG)_n$ exerts some effect at the level of transcription initiation [118]. Cell culture studies on the decay of *FMR1* mRNA indicated that increased stability is not the cause of elevated *FMR1* mRNA levels [86]. Later, it was shown that increased *FMR1* mRNA levels are due to increased transcriptional activity of both spliced and unspliced mRNA. Also, the majority of *FMR1* mRNA was found to be localised in the cytoplasm, thus excluding the possibility that nuclear retention of *FMR1* mRNA contributes to the higher levels (as occurs in myotonic dystrophy, which will be discussed later)[113].

Although different methods and different repeat ranges tested show somewhat different results, it seems that within the PM range, FMRP levels gradually decrease with increasing $(CGG)_n$ length [86,114-116]. The reduced levels have been found to reflect impaired translational efficiency of the $(CGG)_n$ containing *FMR1* mRNA [119]. Since none of the characteristic features of FXTAS have been observed in FM carriers, who express little or no FMRP, it is very unlikely that this syndrome originates from a protein-deficiency.

The neuropathological hallmark of FXTAS | 1.8

As can be seen in table 1.1, apart from generalised atrophy, another neuropathological feature has been found to be characteristic for FXTAS brain pathology. Neurohistological examination of brains of PM carriers who displayed the neurological phenotype which would later be diagnosed as FXTAS, revealed the presence of eosinophilic, intranuclear inclusions in neurons and astrocytes (figure 1.2). These inclusions are discrete, round bodies, 2 to 5 µm large, and were seen in various regions throughout the cerebrum and brain stem [110]. They are most numerous in the hippocampus. A strong correlation was found between repeat length and number of intranuclear inclusions, in neurons as well as astrocytes [95]. The intranuclear inclusions stain positively with antibodies against ubiquitin. No positive staining in inclusions was obtained with antibodies against tau, cytokeratin, desmin, αB-crystallin, vimentin, GFAP or neurofilament. The antibody against neurofilament, however, showed swollen axons in the granular cell layer of the cerebellum (Purkinje axonal torpedoes). The GFAP-antibody revealed Bergmann gliosis. Furthermore, Purkinje cell drop out was seen in the cerebellum. No inclusions were observed in Purkinje cells. Brains of two female PM carriers unaffected by FXTAS were also examined; none of the neuropathological changes associated with FXTAS were observed [110].

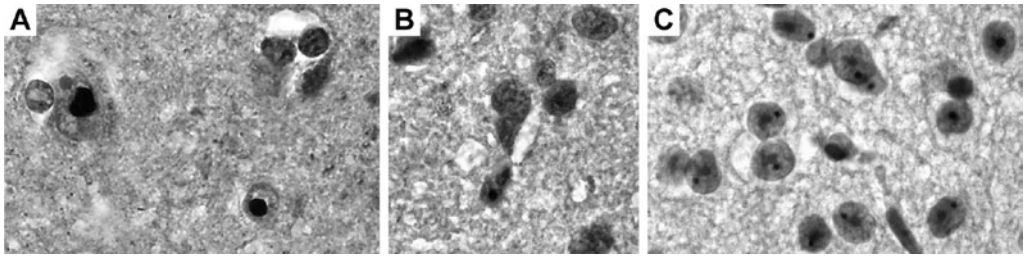

Figure 1.2 | Ubiquitin-positive intranuclear inclusions, the neuropathological hallmark of FXTAS in A: human neurons, B: human astrocytes, C: murine neurons. No inclusions have been seen in murine astrocytes. See page 163 onwards for colour version of this figure.

Previous neuropathological examinations of brains of elderly carriers of the FM did not show inclusions [68,120]. Some neuroanatomical changes do, however, overlap between PM carriers with FXTAS and FM carriers; Purkinje cell loss and Bergmann gliosis have also been described in an older male with the FM [120]. Also, shrinkage of the temporal lobe [121] and enlarged ventricles [122] have been seen in FM carriers. However, overall brain volume tends to be greater in FM carriers [121,123]. Thus, the intranuclear inclusions seem to represent a neuropathological hallmark specific for FXTAS and their presence is therefore used as a *post mortem* diagnostic criterion (table 1.1).

Based on the toxic RNA gain-of-function model proposed for FXTAS, which will be described in detail later, it was predicted that *FMR1* mRNA is present within the intranuclear inclusions. Indeed, *FMR1* mRNA was detected in inclusions isolated from brain of a 70-year old patient. This finding serves as evidence for an association between elevated *FMR1* transcript levels and formation of intranuclear inclusions. Since the presence of inclusions is associated with the development of FXTAS, these results strongly point towards an RNA gain-of-function mechanism [97]. Another prediction of the model is

that RNA-BPs interfere with the abundant $(CGG)_n$ containing mRNA which then ultimately leads to the clinical phenotype [124]. To investigate which proteins might mediate the toxic effects of the expanded-repeat *FMR1* mRNA, a systematic analysis was undertaken into the composition of purified intranuclear inclusions of FXTAS brain. Over twenty proteins were found to co-localise with ubiquitin-positive inclusions, among which αB-crystallin, Hsp27 and Hsp70, muscleblind-like protein 1 (MBNL1), several intermediate filaments and microtubule components, as well as myelin associated proteins and the RNA-BP heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) [124]. Another group demonstrated co-localisation of Pur-α and ubiquitin in intranuclear inclusions in human FXTAS brain [125], which had not been identified in the purified inclusions in the study by Iwahashi and colleagues [124]. Thus, the exact composition and formation of inclusions is as yet unknown as well as how they are related to the clinical symptoms seen in FXTAS.

The pathogenesis of FXTAS | 1.9

Most studies on the pathogenesis of FXTAS currently focus on the proposed RNA toxic gain-of-function model (figure 1.3). The evidence for this pathogenesis model, in which the *FMR1* mRNA itself is the cause of the neurological disease, originates from the observations that *FMR1* mRNA levels are elevated in peripheral blood leucocytes of PM carriers, that *FMR1* mRNA is present in the intranuclear inclusions in brain and that no symptoms of FXTAS are seen among older carriers with the FM ([83,84,110], reviewed in [112]). This model parallels the RNA toxic gain-of-function model proposed for another non-coding expanded repeat disorder, myotonic dystrophy (DM: described in more detail in chapter 2). Myotonic dystrophy is either caused by expansion of a $(CTG)_n$ in the 3'UTR of the myotonic dystrophy protein kinase (*DMPK*) gene (DM1: mostly congenital), or by expansion of the $(CCTG)_n$ in intron 1 of the *ZNF9* gene (DM2: late onset form). In both forms of DM, RNA-BPs are sequestered to the mRNA that bears long C(C)TG-repeat tracts. One of these proteins is MBNL1. Nuclear foci are seen in DM, similar to intranuclear inclusions in FXTAS (reviewed in [126,127]). These foci contain the repeat-containing RNA involved in DM, as well as MBNL1. The effect of the sequestration of MBNL1 into the nuclear foci is dysregulation of splicing of several target mRNAs, which in most cases can explain symptoms seen in DM [128,129]. The toxic gain-of-function effect has been confirmed in transgenic mouse models in which either an expanded $(CTG)_n$ was placed within a non-coding region of a heterologous gene [126] or by introducing a transgene containing the human *DMPK* gene with an expanded $(CTG)_n$ [130]; both transgenic mouse lines developed myotonia. Thus, it has been proposed that the situation in FXTAS is similar to DM, based on the previously described observations. It has furthermore been considered that POF in female PM carriers might also arise from an RNA-mediated toxicity [131]. Cellular and animal models, which will be described in more detail hereafter, provide further evidence that indeed an RNA gain-of-function mechanism underlies the clinical syndromes in FXTAS. However, the protein targets that are sequestered by the $(CGG)_n$ containing mRNA remain to be elucidated, as well as the downstream effects. It will be difficult to answer these questions when solely investigating human samples, because in the case of the most interesting organ, the brain, one is limited to using patient material only at the end stage of the disease. Naturally, it is important to know about the onset and the course of the disease as well. Therefore, *in vivo* models as an alternative to conducting research in humans are necessary. Animal models have

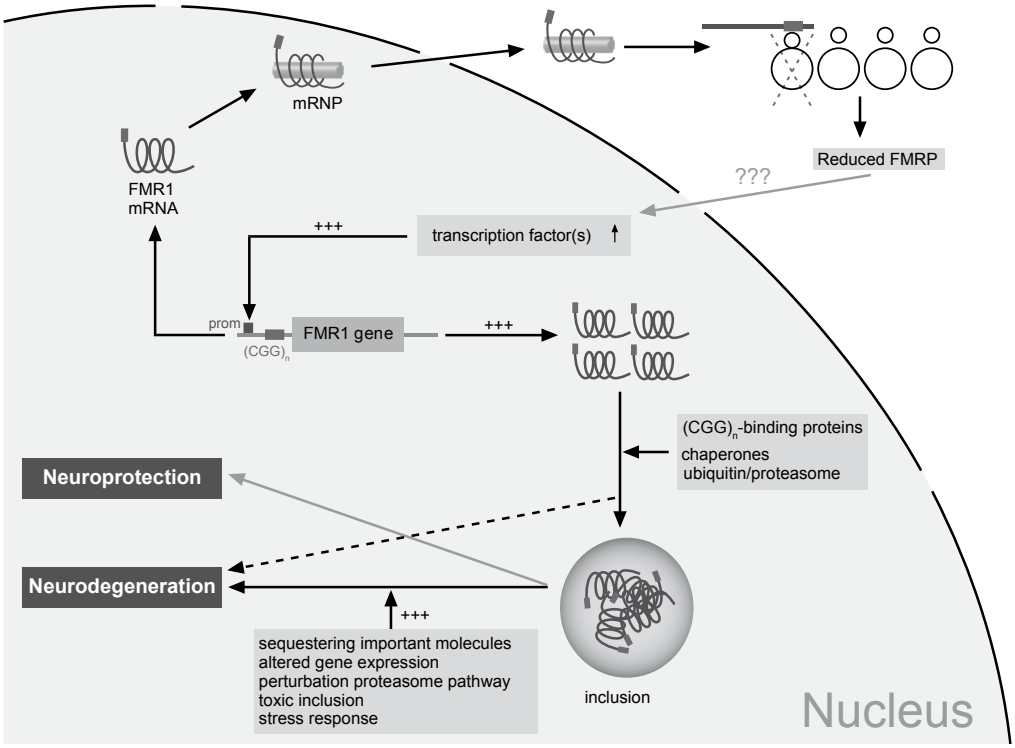

Figure 1.3 | A schematic representation of **the RNA gain-of-function mechanism proposed for the pathogenesis of FXTAS**. The *FMR1* gene is transcribed in the nucleus and transported to the ribosomes. The expanded $(CGG)_n$ present in the 5'UTR of the *FMR1* gene hampers translation, leading to lower FMRP levels. Through an as yet unknown mechanism, transcription is upregulated, leading to increased *FMR1* mRNA levels. In an attempt to get rid of the excess of *FMR1* mRNA, the cell might attract chaperones or elements of the ubiquitin/proteasome system. Also $(CGG)_n$-binding proteins might be recruited. These processes could lead to formation of intranuclear inclusions. Sequestration of proteins into the inclusions might prevent them from exerting their normal function, thereby disturbing cellular function, which in the end might cause neurodegeneration. Also, it cannot be excluded that neuroprotection takes place, such that cells that are capable of capturing the toxic transcripts in the inclusions are the cells that survive. See page 163 onwards for colour version of this figure.

proven and will continue to prove very helpful in answering questions about different stages of the disease and will be discussed in detail below.

Also cellular *in vitro* models can give valuable insight into the mechanisms of toxicity. Inducible expression of constructs with non-coding $(CGG)_n$ of different lengths in HEK293 cells showed higher levels of apoptosis with increasing repeat length. Non-induced cells, thus only expressing endogenous levels of *FMR1* mRNA, showed reduced cell death in comparison to the induced cells, suggesting that cell death is related to the amount of $(CGG)_n$ RNA that is transcribed [132]. Another cellular model mimics another aspect of FXTAS, namely the formation of inclusions, which occurred in human neuroblastoma cells after introduction of an expanded $(CGG)_n$. Another result of the expression of the $(CGG)_n$ containing RNA is disruption of lamin A/C architecture [133]. Lamin A/C was one of the intermediate filaments found to be a component of the intranuclear inclusions in FXTAS brain [124]. Controlled expression of $(CGG)_n$ tracts in cellular models might provide further insight on the altered cellular function as a result of toxic RNA species.

Animal models for FXTAS | 1.10

Several animal models have been generated in the past, in an attempt to mimic repeat instability as well as neurodegeneration associated with $(CGG)_n$ expansion in the *FMR1* gene. The different existing animal models and their contributions to our understanding of the pathogenesis of FXTAS are discussed.

Drosophila Melanogaster models for FXTAS | 1.10.1

A *Drosophila* model generated by Jin and co-workers [134] provided useful insight into the effect of ectopic expression of different amounts of different $(CGG)_n$ lengths in the fly. Strong expression of a construct with 90 CGGs in all neurons of the peripheral and central nervous system caused lethality, as did ubiquitous expression in the embryo. Epithelial expression of this construct did not cause any effect, which shows that neuronal cells suffer more from the toxic effect of the long $(CGG)_n$ containing RNA. Moderate or strong expression of constructs containing part of the human *FMR1* 5'UTR with either normal or PM-sized CGG-tracts was also specifically directed to the retina. Moderate expression of a tract of 60 CGGs hardly caused an aberrant phenotype in the eye, whereas 90 CGGs led to rough eye, loss of pigmentation and disturbed architecture. Strong expression of either repeat length caused a more severe phenotype, with an even stronger effect for the $(CGG)_{90}$, including major cell death. These experiments show that both the length of the CGG-tract and the expression level play a role in the severity of the neurodegenerative eye phenotype in *Drosophila*. Furthermore, disruption of the eye morphology increased with age in transgenic flies expressing 90 CGGs, mimicking the progressive nature of FXTAS. It was also shown that the phenotypes represent late onset symptoms rather than developmental defects. Immunohistochemical studies of the retina of flies strongly expressing the long repeat demonstrated the presence of inclusions, positive for ubiquitin, hsp70 and proteasome. These inclusions were found both in nuclei and cytoplasm, which is different from what is seen in humans and mice. Also, they were not as consistent in size and shape as in humans and mice. None of the aspects of the neurodegenerative phenotype could be induced with expression of a control construct containing EGFP (enhanced green fluorescent protein) only. Since hsp70 has been shown to be a universal suppressor of many neurodegenerative models caused by mutant proteins, for example polyglutamine-containing proteins due to an expanded $(CAG)_n$ in the coding region of a gene, flies were generated that co-express the $(CGG)_{90}$ and hsp70. Hsp70 was found to be capable of suppressing the $(CGG)_n$ RNA induced degeneration, despite the absence of a mutant protein, like in other neurodegenerative disorders where hsp70 could act as a chaperone in refolding mutant proteins. Thus, these results are promising since they provide a proof-of-principle that the RNA-induced phenotype can be rescued [134].

Subsequent studies in this model were aimed at testing the hypothesis that FXTAS is an RNA-mediated neurodegenerative disease caused by the titration of RNA-BPs by the $(CGG)_n$. A genetic screen of candidate RNA-BPs identified CUG binding protein (CUGBP1) as a modifier of the eye phenotype induced by the repeat tract of 90 CGGs. Overexpression of CUGBP1 was capable of suppressing the $(CGG)_n$ RNA-induced neurodegenerative eye phenotype. CUGBP1 was found to interact with the $(CGG)_n$ via hnRNP A2/B1 [135], a ribo-CGG binding protein, of which the presence has been demonstrated in inclusions in human FXTAS brain [124]. A paper published simultaneously demonstrated that hnRNP A2/

B1 directly binds to the $(CGG)_n$ [125] and overexpression of this protein and its two *Drosophila* homologues also suppresses the repeat RNA-induced eye phenotype [135]. In addition, Pur α, an RNA-BP expressed in neuronal cytoplasm, that is involved in dendritic mRNA transport, was also found to be associated with $(CGG)_n$ RNA and overexpression of Pur α suppressed the eye neurodegeneration phenotype. Pur α was also present in the inclusions in Drosophila [125], as well as in human FXTAS brain [124]. Thus, these findings strongly support the current pathogenesis model for FXTAS, namely that PM-sized $(CGG)_n$ sequester RNA-BPs, which leads to altered cellular function and ultimately neuronal cell death [125].

More evidence for the hypothesis that the levels of repeat RNA are important in inducing a neurodegenerative phenotype comes from the observation that simultaneous expression of a $(CGG)_n$ and a $(CCG)_n$, which is present in the 5'UTR of the *FMR2* gene, in *Drosophila* suppress their independent toxicity. The *FMR2* gene is located about 600 kb distal to the *FMR1* gene and is involved in non-syndromic X-linked mental retardation. Expression of only a $(CCG)_n$ of 90 CCGs also induced a neurodegenerative phenotype of the eye. The protective effect of co-expression was found to be dependent on the RNAi pathway; toxicity was reversed through a reduction of transcript levels, possibly through formation of double stranded RNA, which is then processed and cleaved. This is hopeful in light of potential therapeutical strategies involving complementary RNA molecules targeting mutant mRNAs carrying expanded trinucleotide repeats [136].

Mouse models for FXTAS | 1.10.2

Initial attempts to make transgenic mouse models expressing expanded $(CGG)_n$ tracts focused on investigating the mechanisms underlying $(CGG)_n$ instability, since FXTAS had not been discovered at the time. The first transgenic mouse model expressed a $(CGG)_{81}$, with two interruptions, but with a pure tract of 60 CGGs. Although in humans this repeat length is sufficient to cause instability upon transmission to the next generation, all transgenic mice showed exactly the same repeat length. Thus, the repeat was stably inherited in all animals tested [137]. In another transgenic mouse line, a randomly (autosomally) integrated *FMR1* PM allele ($(CGG)_{22}TGG(CGG)_{43}TGG(CGG)_{21}$) was also stably transmitted [138]. $(CGG)_n$ tracts usually have a few AGG interruptions. Expansion of the $(CGG)_n$ appears to be initiated upon loss of the interruptions of the otherwise pure $(CGG)_n$-tracts. Interruptions are predominantly lost at the 3' end of the repeat tract, causing a long pure $(CGG)_n$-tract [9,139]. Therefore, Lavedan and co-workers created new transgenic mouse lines with even longer uninterrupted $(CGG)_n$. In addition, because it was recognised that chromosomal context might be important for $(CGG)_n$ instability to take place, they situated the $(CGG)_n$ tract within the context of the first exon of the human *FMR1* gene and included the flanking CpG island. However, including nearby *cis*-acting elements in the transgene proved not to be sufficient to reproduce repeat instability as seen in humans, since only minor intergenerational instability was observed in these mice [140].

Another approach was taken with the use of yeast artificial chromosomes (YACs), which can contain large fragments of exogenous DNA. YACs in transgenic mice have been demonstrated to appropriately express the genes present on the YAC, as all splice isoforms and other regulatory elements can be included [141,142]. Several transgenic lines were created using YAC transgenes carrying $(CGG)_n$ of different lengths and with varying amounts of flanking sequences. Length-dependent intergenerational instability (small expansions and contractions) was seen. Parental origin of the repeat

allele did not influence the magnitude or direction of instability [143]. To investigate the effect of potential *cis*- and *trans*- factors promoting expansions, the transgenic mice described by Lavedan *et al*. (1998) were subjected to a low-folate or folate-free diet, or crossbred with mice deficient in genes involved in DNA replication (Wrn helicase) and repair (p53). Also, the potential effect of parental age was taken into consideration. Small changes in repeat length were seen in almost all allele transmissions. Large deletions occurred about 10% of transmissions. No large expansions were seen, neither somatic instability. The pattern of intergenerational instability was not affected by the absence of the proteins involved in DNA replication and repair, nor by folate levels or the parental age [144].

Since flanking of the $(CGG)_n$ with part of the *FMR1* gene proved not to be sufficient to recapitulate all aspects of repeat instability in humans, another approach was taken by Bontekoe and colleagues (2001) [145]. They generated a knock-in mouse model in which the endogenous murine $(CGG)_8$ was exchanged with a human $(CGG)_n$ of 98 trinucleotides. When cloning the human expanded repeat into the murine *FMR1* promoter, minimal changes were made. Comparison of the promoter sequence in mice and humans showed that all known regulatory elements are conserved. After the initial breedings, infrequent (15 out of 155 transmissions studied) and minor instabilities were observed, two of which were contractions, both of paternal origin. Expansions occurred upon transmission of either one of the parental alleles [145].

After FXTAS was first recognised and described, the aging expanded $(CGG)_n$ knock-in mouse (from now on called $(CGG)_n$ mouse) was analysed with respect to neurohistology, biochemistry and molecular aspects. The main findings were that *Fmr1* mRNA levels in brain were increased throughout life, as compared with wild type animals [146], which mimics the human situation [86,115]. Importantly, also intranuclear inclusions were found in many different areas throughout the brains of the $(CGG)_n$ mice (figure 1.2). Numbers and size of the inclusions increase with age; the first appearing around 30 weeks, with percentages up to 55% in certain brain regions at 72 weeks of age, which parallels with the progressive nature of the disorder in humans. In addition, a correlation was recognised between the presence of inclusions in certain brain areas and clinical features in patients with FXTAS. This clearly suggests a role for the formation of intranuclear inclusions in the development of the tremor/ataxia syndrome in elderly PM carriers. Interestingly, inclusions were only seen in neurons [146], while in humans also astrocytes bear inclusions [110]. Other neuropathological features observed in patients with FXTAS like neuronal loss, gliosis and Purkinje cell dropout have not been seen in the $(CGG)_n$ mice. Molecular chaperone Hsp40 and the 20S proteasome complex were found to be present in the inclusions. Among many other proteins, Fmrp, α-synuclein, poly-glutamine and tau were not present in the inclusions. Minor somatic repeat instability was seen in some organs at 52 weeks of age, as compared with in tail DNA taken 10 days postnatally [146].

The $(CGG)_n$ mice were subjected to several behavioural tests. No severely aberrant behavioural phenotype was seen, only mild age-dependent learning disturbances and decreased performance in a neuromotor task [147]. Thus, although the progressive character of the disorder is also seen in mice, the mouse appears to be less affected by the expression of the expanded $(CGG)_n$ than humans are, both with regard to neurodegeneration (neuronal cell loss) and the clinical and behavioural phenotype. The origin of these differences remains to be elucidated.

This first knock-in mouse model later turned out to be more promising in light of repeat instability than the earlier models with non-targeted autosomal $(CGG)_n$, when considerable repeat instability was reported. The $(CGG)_n$ in these mice has been expanding over generations since it was generated and now has reached lengths, which in humans represent the full mutation range. Despite $(CGG)_n$ lengths over 200 CGGs, no methylation of the promoter region has been detected in these mice thus far (chapter 3, 6, [148]). Furthermore, cell lines were derived from these mice expressing the expanded $(CGG)_n$, which were investigated further with regard to altered cellular morphology and function, as is described in chapter 4. Also, alterations in hypothalamo-pituitary-adrenal (HPA) axis physiology, among which increased glucocortoid levels, have been observed in this mouse model, which might explain the increased psychopathology seen in PM carriers, as is outlined in chapter 5 [149].

Another, similar knock-in mouse model was generated by Entezam and colleagues [150], which had an initial repeat tract of ~118 CGGs. Similar to what is described in chapter 3, these mice show high repeat instability with a bias towards expansions. They show an increase in *FMR1* mRNA levels with increasing $(CGG)_n$ length and an inverse correlation with Fmrp levels. Also, they reported area-specific decreases in Fmrp expression throughout the brain in mice with expanded $(CGG)_n$. For the first time, large expansions into the full mutation range were seen within a single generation in a mouse model, but the gene was not found to be methylated, nor silenced, as Fmrp was still present [150].

Thus, animal models have proven useful and will continue to prove useful in elucidating the molecular mechanisms underlying the clinical symptoms in FXTAS and thus might also provide potential therapeutical targets.

The overall aim of this PhD research was to further characterise the expanded $(CGG)_n$ knock-in mouse model and to study the role and formation of the intranuclear neuronal inclusions in FXTAS.

References |

1. Glass IA. 1991. X linked mental retardation. J Med Genet 28:361-363.
2. Lubs HA. 1969. A marker X-chromosome. Am J Hum Genet 21:231-244.
3. Turner G, Daniel A, Frost M. 1980. X-linked mental retardation, macro-orchidism, and the Xq27 fragile site. J Pediatr 96:837-841.
4. Sutherland GR. 1977. Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. Science 197:265-266.
5. Oberlé I, Rousseau F, Heitz D, Kretz C, Devys D *et al.* 1991. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. Science 252:1097-1102.
6. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP *et al.* 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905-914.
7. Richards RI, Holman K, Kozman H, Kremer E, Lynch M *et al.* 1991. Fragile X syndrome: genetic localisation by linkage mapping of two microsatellite repeats FRAXAC1 and FRAXAC2 which immediately flank the fragile site. J Med Genet 28:818-823.
8. Eichler EE, Richards S, Gibbs RA, Nelson DL. 1993. Fine structure of the human FMR1 gene. Hum Mol Genet 2:1147-1153.
9. Richards RI, Sutherland GR. 1992. Dynamic mutations: a new class of mutations causing human disease. Cell 70:709-712.
10. Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS *et al.* 1991. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67:1047-1058.
11. Dombrowski C, Levesque S, Morel ML, Rouillard P, Morgan K *et al.* 2002. Premutation and intermediate-size FMR1 alleles in 10 572 males from the general population: loss of an AGG interruption is a late event in the generation of fragile X syndrome alleles. Hum Mol Genet 11:371-378.
12. Zhong N, Ju WN, Pietrofesa J, Wang DW, Dobkin C *et al.* 1996. Fragile X "gray zone" alleles: AGG patterns, expansion risks, and associated haplotypes. Am J Med Genet 64:261-265.
13. Eichler EE, Holden J, Popovich BW, Reiss AL, Snow K *et al.* 1994. Length of uninterrupted CGG repeats determines instability in the FMR1 gene. Nature Genet 8:88-94.
14. Napierala M, Michalowski D, de Mezer M, Krzyzosiak WJ. 2005. Facile FMR1 mRNA structure regulation by interruptions in CGG repeats. Nucleic Acids Res 33:451-63.
15. Chen LS, Tassone F, Sahota P, Hagerman PJ. 2003. The (CGG)n repeat element within the 5'untranslated region of the FMR1 message provides both positive and negative cis effects on in vivo translation of a downstream reporter. Hum Mol Genet 12:3067-3074.
16. Maddalena A, Richards CS, McGinniss MJ, Brothman A, Desnick RJ *et al.* 2001. Technical standards and guidelines for fragile X: the first of a series of disease-specific supplements to the Standards and Guidelines for Clinical Genetics Laboratories of the American College of Medical Genetics. Quality Assurance Subcommittee of the Laboratory Practice Committee. Genet Med 3:200-205.
17. Nolin SL, Brown WT, Glicksman A, Houck Jr GE, Gargano AD *et al.* 2003. Expansion of the Fragile X CGG Repeat in Females with Premutation or Intermediate Alleles. Am J Hum Genet 72:454-464.
18. Bat O, Kimmel M, Axelrod DE. 1997. Computer simulation of expansions of DNA triplet repeats in the fragile X syndrome and Huntington's disease. Journal of Theoretical Biology 188:53-67.
19. Reyniers E, Vits L, De Boulle K, Van Roy B, Van Velzen D *et al.* 1993. The full mutation in the FMR-1 gene of male fragile X patients is absent in their sperm. Nature Genet 4:143-146.
20. Nolin SL, Lewis FA, Ye LL, Houck GE, Glicksman AE *et al.* 1996. Familial transmission of the FMR1 CGG repeat. Am J Hum Genet 59:1252-1261.
21. Rousseau F, Rouillard P, Morel ML, Khandjian EW, Morgan K. 1995. Prevalence of carriers of premutation-size alleles of the FMRI gene and implications for the population genetics of the fragile X syndrome. Am J Hum Genet 57:1006-1018.
22. Berkenstadt M, Ries-Levavi L, Cuckle H, Peleg L, Barkai G. 2007. Preconceptional and prenatal screening for fragile X syndrome: Experience with 40 000 tests. Prenat Diagn 27:991-994.
23. Tzeng CC, Tsai LP, Hwu WL, Lin SJ, Chao MC *et al.* 2005. Prevalence of the FMR1 mutation in Taiwan assessed by large-scale screening of newborn boys and analysis of DXS548-FRAXAC1 haplotype. Am J Med Genet A 133:37-43.
24. Pesso R, Berkenstadt M, Cuckle H, Gak E, Peleg L *et al.* 2000. Screening for fragile X syndrome in women of reproductive age. Prenat Diagn 20:611-614.
25. Toledano-Alhadef H, Basel-Vanagaite L, Magal N, Davidov B, Ehrlich S *et al.* 2001. Fragile-X Carrier Screening and the Prevalence of Premutation and Full-Mutation Carriers in Israel. Am J Hum Genet 69:351-360.
26. Bächner D, Manca A, Steinbach P, Wöhrle D, Just W *et al.* 1993. 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 2:2043-2050.
27. Bächner D, Steinbach P, Wöhrle D, Just W, Vogel W *et al.* 1993. Enhanced Fmr-1 expression in testis. Nature Genet 4:115-116.

28. Hinds HL, Ashley CT, Sutcliffe JS, Nelson DL, Warren ST *et al.* 1993. Tissue specific expression of FMR-1 provides evidence for a functional role in fragile X-Syndrome (Vol 3, Pg 36, 1993). Nature Genet 5:312.
29. Agulhon C, Blanchet P, Kobetz A, Marchant D, Faucon N *et al.* 1999. Expression of FMR1, FXR1, and FXR2 genes in human prenatal tissues. J Neuropathol Exp Neurol 58:867-880.
30. Abitbol M, Menini C, Delezoide AL, Rhyner T, Vekemans M *et al.* 1993. Nucleus basalis magnocellularis and hippocampus are the major sites of FMR-1 expression in the human fetal brain. Nature Genet 4:147-153.
31. Sutcliffe JS, Nelson DL, Zhang F, Pieretti M, Caskey CT *et al.* 1992. DNA methylation represses FMR-1 transcription in fragile X syndrome. Hum Mol Genet 1:397-400.
32. Verheij C, Bakker CE, de Graaff E, Keulemans J, Willemsen R *et al.* 1993. Characterization and localization of the FMR-1 gene product associated with fragile X syndrome. Nature 363:722-724.
33. Chiurazzi P, Pomponi MG, Pietrobono R, Bakker CE, Neri G *et al.* 1999. Synergistic effect of histone hyperacetylation and DNA demethylation in the reactivation of the FMR1 gene. Hum Mol Genet 8:2317-2323.
34. Coffee B, Zhang F, Ceman S, Warren ST, Reines D. 2002. Histone Modifications Depict an Aberrantly Heterochromatinized FMR1 Gene in Fragile X Syndrome. Am J Hum Genet 71:923-932.
35. Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU *et al.* 1998. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 19:187-191.
36. Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM *et al.* 1998. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393:386-389.
37. Razin A. 1998. CpG methylation, chromatin structure and gene silencing-a three-way connection. Embo J 17:4905-4908.
38. Coffee B, Zhang F, Warren ST, Reines D. 1999. Acetylated histones are associated with FMR1 in normal but not fragile X-syndrome cells. Nat Genet 22:98-101.
39. Chiurazzi P, Pomponi MG, Willemsen R, Oostra BA, Neri G. 1998. In vitro reactivation of the FMR1 gene involved in fragile X syndrome. Hum Mol Genet 7:109-113.
40. Tabolacci E, Pietrobono R, Moscato U, Oostra BA, Chiurazzi P *et al.* 2005. Differential epigenetic modifications in the FMR1 gene of the fragile X syndrome after reactivating pharmacological treatments. Eur J Hum Genet 13:641-646.
41. Turner BM. 2002. Cellular memory and the histone code. Cell 111:285-291.
42. Grewal SI, Moazed D. 2003. Heterochromatin and epigenetic control of gene expression. Science 301:798-802.
43. Lachner M, O'Sullivan RJ, Jenuwein T. 2003. An epigenetic road map for histone lysine methylation. J Cell Sci 116:2117-2124.
44. Cheutin T, McNairn AJ, Jenuwein T, Gilbert DM, Singh PB *et al.* 2003. Maintenance of stable heterochromatin domains by dynamic HP1 binding. Science 299:721-725.
45. Smeets H, Smits A, Verheij CE, Theelen J, Willemsen R *et al.* 1995. Normal phenotype in two brothers with a full FMR1 mutation. Hum Mol Genet 4:2103-2108.
46. Eiges R, Urbach A, Malcov M, Frumkin T, Schwartz T *et al.* 2007. Developmental Study of Fragile X Syndrome Using Human Embryonic Stem Cells Derived from Preimplantation Genetically Diagnosed Embryos. Cell Stem Cell 1:568-577.
47. Lock LF, Takagi N, Martin GR. 1987. Methylation of the Hprt gene on the inactive X occurs after chromosome inactivation. Cell 48:39-46.
48. Ashley C, Jr., Wilkinson KD, Reines D, Warren ST. 1993. FMR1 protein: conserved RNP family domains and selective RNA binding. Science 262:563-568.
49. Darnell JC, Jensen KB, Jin P, Brown V, Warren ST *et al.* 2001. Fragile X Mental Retardation Protein Targets G Quartet mRNAs Important for Neuronal Function. Cell 107:489-499.
50. Siomi H, Siomi MC, Nussbaum RL, Dreyfuss G. 1993. The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. Cell 74:291-298.
51. Eberhart DE, Malter HE, Feng Y, Warren ST. 1996. The fragile X mental retardation protein is a ribosonucleoprotein containing both nuclear localization and nuclear export signals. Hum Mol Genet 5:1083-1091.
52. Feng Y, Gutekunst CA, Eberhart DE, Yi H, Warren ST *et al.* 1997. Fragile X mental retardation protein: Nucleocytoplasmic shuttling and association with somatodendritic ribosomes. J Neurosci 17:1539-1547.
53. Fridell RA, Benson RE, Hua J, Bogerd HP, Cullen BR. 1996. A nuclear role for the fragile X mental retardation protein. EMBO J 15:5408-5414.
54. Sittler A, Devys D, Weber C, Mandel J-L. 1996. Alternative splicing of exon 14 determines nuclear or cytoplasmic localisation of FMR1 protein isoforms. Hum Mol Genet 5:95-102.
55. Feng Y, Absher D, Eberhart DE, Brown V, Malter HE *et al.* 1997. FMRP associates with polyribosomes as an mRNP, and the I304N mutation of severe fragile X syndrome abolishes this association. Mol Cell 1:109-118.

56. Willemsen R, Bontekoe C, Tamanini F, Galjaard H, Hoogeveen AT *et al.* 1996. Association of FMRP with ribosomal precursor particles in the nucleolus. Biochem Biophys Res Comm 225:27-33.
57. Bakker CE, de Diego Otero Y, Bontekoe C, Raghoe P, Luteijn T *et al.* 2000. Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse. Exp Cell Res 258:162-170.
58. De Diego Otero Y, Bakker CE, Raghoe P, Severijnen LWFM, Hoogeveen A *et al.* 2000. Immunocytochemical characterization of FMRP, FXR1P and FXR2P during embryonic development in the mouse. Gene Funct. Dis 1:28-37.
59. Devys D, Lutz Y, Rouyer N, Bellocq JP, Mandel JL. 1993. The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat Genet 4:335-340.
60. Tamanini F, Willemsen R, van Unen L, Bontekoe C, Galjaard H *et al.* 1997. Differential expression of FMR1, FXR1 and FXR2 proteins in human brain and testis. Hum Mol Genet 6:1315-1322.
61. Pacey LK, Doering LC. 2007. Developmental expression of FMRP in the astrocyte lineage: Implications for fragile X syndrome. Glia 55:1601-1609.
62. Wang H, Ku L, Osterhout DJ, Li W, Ahmadian A *et al.* 2004. Developmentally-programmed FMRP expression in oligodendrocytes: a potential role of FMRP in regulating translation in oligodendroglia progenitors. Hum Mol Genet 13:79-89.
63. Khandjian EW, Bardoni B, Corbin F, Sittler A, Giroux S *et al.* 1998. Novel isoforms of the fragile X related protein FXR1P are expressed during myogenesis. Hum Mol Genet 7:2121-2128.
64. Malter HE, Iber JC, Willemsen R, De Graaff E, Tarleton JC *et al.* 1997. Characterization of the full fragile X syndrome mutation in fetal gametes. Nature Genet 15:165-169.
65. Kiebler MA, DesGroseillers L. 2000. Molecular insights into mRNA transport and local translation in the mammalian nervous system. Neuron 25:19-28.
66. Steward O. 2002. mRNA at synapses, synaptic plasticity, and memory consolidation. Neuron 36:338-340.
67. Hinton VJ, Brown WT, Wisniewski K, Rudelli RD. 1991. Analysis of neocortex in three males with the fragile X syndrome. Am J Med Genet 41:289-294.
68. Rudelli RD, Brown WT, Wisniewski K, Jenkins EC, Laure-Kamionowska M *et al.* 1985. Adult fragile X syndrome. Clinico-neuropathologic findings. Acta Neuropathol 67:289-295.
69. Irwin SA, Patel B, Idupulapati M, Harris JB, Crisostomo RA *et al.* 2001. Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: A quantitative examination. Am J Med Genet 98:161-167.
70. Comery TA, Harris JB, Willems PJ, Oostra BA, Irwin SA *et al.* 1997. Abnormal dendritic spines in fragile X knockout mice: Maturation and pruning deficits. Proc Natl Acad Sci USA 94:5401-5404.
71. Nimchinsky EA, Oberlander AM, Svoboda K. 2001. Abnormal development of dendritic spines in fmr1 knock-out mice. J Neurosci 21:5139-46.
72. De Vrij FMS, Levenga J, Van der Linde HC, Koekkoek SK, De Zeeuw CI *et al.* 2008. Rescue of behavioral phenotype and neuronal protrusion morphology in FMR1 KO mice. Neurobiology of Disease 31:127-132.
73. Laggerbauer B, Ostareck D, Keidel EM, Ostareck-Lederer A, Fischer U. 2001. Evidence that fragile X mental retardation protein is a negative regulator of translation. Hum Mol Genet 10:329-338.
74. Brown V, Jin P, Ceman S, Darnell JC, O'Donnell WT *et al.* 2001. Microarray Identification of FMRP-Associated Brain mRNAs and Altered mRNA Translational Profiles in Fragile X Syndrome. Cell 107:477-487.
75. Willemsen R, Oostra BA, Bassell GJ, Dictenberg J. 2004. The fragile X syndrome: From molecular genetics to neurobiology. Ment Retard Dev Disabil Res Rev 10:60-67.
76. Hagerman RJ, Hagerman PJ. 2002. The fragile X premutation: into the phenotypic fold. Curr Opin Genet Dev 12:278-283.
77. Sherman SL. 2000. Premature Ovarian Failure among Fragile X Premutation Carriers: Parent-of-Origin Effect? Am J Hum Genet 67:11-13.
78. Welt CK, Smith PC, Taylor AE. 2004. Evidence of early ovarian aging in fragile x premutation carriers. J Clin Endocrinol Metab 89:4569-4574.
79. Rohr J, Allen EG, Charen K, Giles J, He W *et al.* 2008. Anti-Mullerian hormone indicates early ovarian decline in fragile X mental retardation (FMR1) premutation carriers: a preliminary study. Hum Reprod.23:1220-1225.
80. Sullivan AK, Marcus M, Epstein MP, Allen EG, Anido AE *et al.* 2004. Association of FMR1 repeat size with ovarian dysfunction. Hum Reprod.20:402-412.
81. Ennis S, Ward D, Murray A. 2005. Nonlinear association between CGG repeat number and age of menopause in FMR1 premutation carriers. Eur J Hum Genet.14:253-255.
82. Wittenberger MD, Hagerman RJ, Sherman SL, McConkie-Rosell A, Welt CK *et al.* 2007. The FMR1 premutation and reproduction. Fertil Steril 87:456-465.

83. Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R *et al.* 2001. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 57:127-130.
84. Jacquemont S, Hagerman RJ, Leehey M, Grigsby J, Zhang L *et al.* 2003. Fragile X Premutation Tremor/Ataxia Syndrome: Molecular, Clinical, and Neuroimaging Correlates. Am J Hum Genet 72:869-878.
85. Jacquemont S, Farzin F, Hall D, Leehey M, Tassone F *et al.* 2004. Aging in individuals with the FMR1 mutation. Am J Ment Retard 109:154-164.
86. Tassone F, Hagerman RJ, Taylor AK, Gane LW, Godfrey TE *et al.* 2000. Elevated levels of FMR1 mRNA in carrier males: A new mechanism of involvement in the Fragile-X syndrome. Am J Hum Genet 66:6-15.
87. Jacquemont S, Hagerman RJ, Leehey MA, Hall DA, Levine RA *et al.* 2004. Penetrance of the fragile x-associated tremor/ataxia syndrome in a premutation carrier population. JAMA 291:460-469.
88. Jacquemont S, Leehey MA, Hagerman RJ, Beckett LA, Hagerman PJ. 2006. Size bias of fragile X premutation alleles in late-onset movement disorders. J Med Genet.43:804-809.
89. Allen EG, Juncos J, Letz R, Rusin M, Hamilton D *et al.* 2008. Detection of early FXTAS motor symptoms using the CATSYS computerised neuromotor test battery. J Med Genet 45:290-297.
90. Hessl D, Tassone F, Loesch DZ, Berry-Kravis E, Leehey MA *et al.* 2005. Abnormal elevation of FMR1 mRNA is associated with psychological symptoms in individuals with the fragile X premutation. Am J Med Genet B Neuropsychiatr Genet 139B:115-121.
91. Grigsby J, Brega AG, Jacquemont S, Loesch DZ, Leehey MA *et al.* 2006. Impairment in the cognitive functioning of men with fragile X-associated tremor/ataxia syndrome (FXTAS). J Neurol Sci 248:227-233.
92. Bozeat S, Gregory CA, Ralph MA, Hodges JR. 2000. Which neuropsychiatric and behavioural features distinguish frontal and temporal variants of frontotemporal dementia from Alzheimer's disease? J Neurol Neurosurg Psychiatry 69:178-186.
93. Grigsby J, Brega AG, Leehey MA, Goodrich GK, Jacquemont S *et al.* 2007. Impairment of executive cognitive functioning in males with fragile X-associated tremor/ataxia syndrome. Mov Disord 22:645-650.
94. Hall DA, Berry-Kravis E, Jacquemont S, Rice CD, Cogswell J *et al.* 2005. Initial diagnoses given to persons with the fragile X associated tremor/ataxia syndrome (FXTAS). Neurology 65:299-301.
95. Greco CM, Berman RF, Martin RM, Tassone F, Schwartz PH *et al.* 2006. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). Brain 129:243-255.
96. Tassone F, Adams J, Berry-Kravis EM, Cohen SS, Brusco A *et al.* 2007. CGG repeat length correlates with age at onset of motor signs of the fragile X-associated tremor/ataxia syndrome (FXTAS). Am J Med Genet B Neuropsychiatr Genet 144B:566-569.
97. Tassone F, Hagerman RJ, Garcia-Arocena D, Khandjian EW, Greco CM *et al.* 2004. Intranuclear inclusions in neural cells with premutation alleles in fragile X associated tremor/ataxia syndrome. J Med Genet 41:E43.
98. Cohen S, Masyn K, Adams J, Hessl D, Rivera S *et al.* 2006. Molecular and imaging correlates of the fragile X-associated tremor/ataxia syndrome. Neurology 67:1426-1431.
99. Loesch DZ, Litewka L, Brotchie P, Huggins RM, Tassone F *et al.* 2005. Magnetic resonance imaging study in older fragile X premutation male carriers. Ann Neurol 58:326-330.
100. Bacalman S, Farzin F, Bourgeois JA, Cogswell J, Goodlin-Jones BL *et al.* 2006. Psychiatric Phenotype of the Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) in Males: Newly Described Fronto-Subcortical Dementia. J Clin Psychiatry 67:87-94.
101. Bourgeois JA, Farzin F, Brunberg JA, Tassone F, Hagerman P *et al.* 2006. Dementia with mood symptoms in a fragile x premutation carrier with the fragile x-associated tremor/ataxia syndrome: clinical intervention with donepezil and venlafaxine. J Neuropsychiatry Clin Neurosci 18:171-177.
102. Kogan CS, Turk J, Hagerman RJ, Cornish KM. 2007. Impact of the Fragile X mental retardation 1 (FMR1) gene premutation on neuropsychiatric functioning in adult males without fragile X-associated Tremor/Ataxia syndrome: A controlled study. Am J Med Genet B Neuropsychiatr Genet In press.
103. Roberts JE, Bailey DB, Jr., Mankowski J, Ford A, Sideris J *et al.* 2008. Mood and anxiety disorders in females with the FMR1 premutation. Am J Med Genet B Neuropsychiatr Genet In press.
104. Allen EG, Sullivan AK, Marcus M, Small C, Dominguez C *et al.* 2007. Examination of reproductive aging milestones among women who carry the FMR1 premutation. Hum Reprod 22:2142-2152.
105. Grigsby J, Brega AG, Engle K, Leehey MA, Hagerman RJ *et al.* 2008. Cognitive profile of fragile X premutation carriers with and without fragile X-associated tremor/ataxia syndrome. Neuropsychology 22:48-60.
106. Berry-Kravis E, Abrams L, Coffey SM, Hall DA, Greco C *et al.* 2007. Fragile X-associated tremor/ataxia syndrome: Clinical features, genetics, and testing guidelines. Mov Disord 14:2014-2030.
107. Hagerman RJ, Leavitt BR, Farzin F, Jacquemont S, Greco CM *et al.* 2004. Fragile-X-Associated Tremor/Ataxia Syndrome (FXTAS) in Females with the FMR1 Premutation. Am J Hum Genet 74:1051-1056.

108. Zuhlke C, Budnik A, Gehlken U, Dalski A, Purmann S *et al.* 2004. FMR1 premutation as a rare cause of late onset ataxia. Evidence for FXTAS in female carriers. J Neurol 251:1418-1419.
109. Berry-Kravis E, Potanos K, Weinberg D, Zhou L, Goetz CG. 2004. Fragile X-associated tremor/ataxia syndrome in sisters related to X-inactivation. Ann Neurol 57:144-147.
110. Greco CM, Hagerman RJ, Tassone F, Chudley AE, Del Bigio MR *et al.* 2002. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. Brain 125:1760-1771.
111. Brunberg JA, Jacquemont S, Hagerman RJ, Berry-Kravis EM, Grigsby J *et al.* 2002. Fragile X Premutation Carriers: Characteristic MR Imaging Findings of Adult Male Patients with Progressive Cerebellar and Cognitive Dysfunction. Am J Neuroradiol 23:1757-1766.
112. Hagerman PJ, Hagerman RJ. 2004. The Fragile-X Premutation: A Maturing Perspective. Am J Hum Genet 74:805-816.
113. Tassone F, Iwahashi C, Hagerman PJ. 2004. FMR1 RNA withinthe intranuclear inclusions of fragile X-associated Tremor/Ataxia syndrome (FXTAS). RNA biology 1:103-105.
114. Kenneson A, Zhang F, Hagedorn CH, Warren ST. 2001. Reduced FMRP and increased FMR1 transcription is proportionally associated with CGG repeat number in intermediate-length and premutation carriers. Hum Mol Genet 10:1449-1454.
115. Tassone F, Hagerman RJ, Chamberlain WD, Hagerman PJ. 2000. Transcription of the FMR1 gene in individuals with fragile X syndrome. Am J Med Genet 97:195-203.
116. Tassone F, Hagerman PJ. 2003. Expression of the FMR1 gene. Cytogenet Genome Res 100:124-128.
117. Allen EG, He W, Yadav-Shah M, Sherman SL. 2004. A study of the distributional characteristics of FMR1 transcript levels in 238 individuals. Hum Genet 114:439-447.
118. Beilina A, Tassone F, Schwartz PH, Sahota P, Hagerman PJ. 2004. Redistribution of transcription start sites within the FMR1 promoter region with expansion of the downstream CGG-repeat element. Hum Mol Genet 13:543-549.
119. Primerano B, Tassone F, Hagerman RJ, Hagerman P, Amaldi F *et al.* 2002. Reduced FMR1 mRNA translation efficiency in Fragile X patients with premutations. RNA 8:1-7.
120. Sabaratnam M. 2000. Pathological and neuropathological findings in two males with fragile-X syndrome. J Intellect Disabil Res 44:81-85.
121. Reiss AL, Lee J, Freund L. 1994. Neuroanatomy of fragile X syndrome: The temporal lobe. Neurology 44:1317-1324.
122. Reiss AL, Abrams MT, Greenlaw R, Freund L, Denckla MB. 1995. Neurodevelopmental effects of the FMR-1 full mutation in humans. Nature Med 1:159-167.
123. Schapiro MB, Murphy DGM, Hagerman RJ, Azari NP, Alexander GE *et al.* 1995. Adult fragile X syndrome: Neuropsychology, brain anatomy, and metabolism. Am J Med Genet 60:480-493.
124. Iwahashi CK, Yasui DH, An HJ, Greco CM, Tassone F *et al.* 2006. Protein composition of the intranuclear inclusions of FXTAS. Brain 129:256-271.
125. Jin P, Duan R, Qurashi A, Qin Y, Tian D *et al.* 2007. Pur alpha Binds to rCGG Repeats and Modulates Repeat-Mediated Neurodegeneration in a Drosophila Model of Fragile X Tremor/Ataxia Syndrome. Neuron 55:556-564.
126. Mankodi A, Takahashi MP, Jiang H, Beck CL, Bowers WJ *et al.* 2002. Expanded CUG repeats trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability of skeletal muscle in myotonic dystrophy. Mol Cell 10:35-44.
127. Ranum LP, Day JW. 2004. Pathogenic RNA repeats: an expanding role in genetic disease. Trends Genet 20:506-512.
128. Fardaei M, Larkin K, Brook JD, Hamshere MG. 2001. In vivo co-localisation of MBNL protein with DMPK expanded-repeat transcripts. Nucleic Acids Res 29:2766-2771.
129. Miller JW, Urbinati CR, Teng-Umnuay P, Stenberg MG, Byrne BJ *et al.* 2000. Recruitment of human muscleblind proteins to (CUG)(n) expansions associated with myotonic dystrophy. Embo J 19:4439-4448.
130. Seznec H, Lia-Baldini AS, Duros C, Fouquet C, Lacroix C *et al.* 2000. Transgenic mice carrying large human genomic sequences with expanded CTG repeat mimic closely the CM CTG repeat intergenerational and somatic instability. Hum Mol Genet 9:1185-94.
131. Conway GS, Payne NN, Webb J, Murray A, Jacobs PA. 1998. Fragile X premutation screening in women with premature ovarian failure. Hum Reprod 13:1184-1187.
132. Handa V, Goldwater D, Stiles D, Cam M, Poy G *et al.* 2005. Long CGG-repeat tracts are toxic to human cells: Implications for carriers of Fragile X premutation alleles. FEBS Lett 579:2702-8.
133. Arocena DG, Iwahashi CK, Won N, Beilina A, Ludwig AL *et al.* 2005. Induction of inclusion formation and disruption of lamin A/C structure by premutation CGG-repeat RNA in human cultured neural cells. Hum Mol Genet 14:3661-3671.
134. Jin P, Zarnescu DC, Zhang F, Pearson CE, Lucchesi JC *et al.* 2003. RNA-Mediated Neurodegeneration Caused by the Fragile X Premutation rCGG Repeats in Drosophila. Neuron 39:739-747.

135. Sofola OA, Jin P, Qin Y, Duan R, Liu H *et al.* 2007. RNA-Binding Proteins hnRNP A2/B1 and CUGBP1 Suppress Fragile X CGG Premutation Repeat-Induced Neurodegeneration in a Drosophila Model of FXTAS. Neuron 55:565-71.
136. Sofola OA, Jin P, Botas J, Nelson DL. 2007. Argonaute-2 dependent rescue of a Drosophila model of FXTAS by FRAXE premutation repeat. Hum Mol Genet 16:2326-32.
137. Bontekoe CJM, de Graaff E, Nieuwenhuizen IM, Willemsen R, Oostra BA. 1997. FMR1 premutation allele is stable in mice. Eur J Hum Genet 5:293-298.
138. Lavedan CN, Garrett L, Nussbaum RL. 1997. Trinucleotide repeats (CGG)22TGG(CGG)43TGG(CGG)21 from the fragile X gene remain stable in transgenic mice. Hum Genet 100:407-414.
139. Kunst CB, Warren ST. 1994. Cryptic and polar variation of the fragile X repeat could result in predisposing normal alleles. Cell 77:853-861.
140. Lavedan C, Grabczyk E, Usdin K, Nussbaum RL. 1998. Long uninterrupted CGG repeats within the first exon of the human FMR1 gene are not intrinsically unstable in transgenic mice. Genomics 50:229-240.
141. Gaensler KM, Kitamura M, Kan YW. 1993. Germ-line transmission and developmental regulation of a 150-kb yeast artificial chromosome containing the human beta-globin locus in transgenic mice. Proc Natl Acad Sci U S A 90:11381-11385.
142. Peterson KR, Clegg CH, Huxley C, Josephson BM, Haugen HS *et al.* 1993. Transgenic mice containing a 248-kb yeast artificial chromosome carrying the human beta-globin locus display proper developmental control of human globin genes. Proc Natl Acad Sci U S A 90:7593-7597.
143. Peier A, Nelson D. 2002. Instability of a premutation-sized CGG repeat in FMR1 YAC transgenic mice. Genomics 80:423-432.
144. Fleming K, Riser DK, Kumari D, Usdin K. 2003. Instability of the fragile X syndrome repeat in mice: the effect of age, diet and mutations in genes that affect DNA replication, recombination and repair proficiency. Cytogenet Genome Res 100:140-146.
145. Bontekoe CJ, Bakker CE, Nieuwenhuizen IM, van Der Linde H, Lans H *et al.* 2001. Instability of a (CGG)(98) repeat in the Fmr1 promoter. Hum Mol Genet 10:1693-1699.
146. Willemsen R, Hoogeveen-Westerveld M, Reis S, Holstege J, Severijnen LA *et al.* 2003. The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. Hum Mol Genet 12:949-959.
147. Van Dam D, Errijgers V, Kooy RF, Willemsen R, Mientjes E *et al.* 2005. Cognitive decline, neuromotor and behavioural disturbances in a mouse model for Fragile-X-associated tremor/ataxia syndrome (FXTAS). Behavioural Brain Research 162:233-239.
148. Brouwer JR, Mientjes EJ, Bakker CE, Nieuwenhuizen IM, Severijnen LA *et al.* 2007. Elevated Fmr1 mRNA levels and reduced protein expression in a mouse model with an unmethylated Fragile X full mutation. Exp Cell Res 313:244-253.
149. Brouwer JR, Severijnen E, de Jong FH, Hessl D, Hagerman RJ *et al.* 2008. Altered hypothalamus-pituitary-adrenal gland axis regulation in the expanded CGG-repeat mouse model for fragile X-associated tremor/ataxia syndrome. Psychoneuroendocrinology.
150. Entezam A, Biacsi R, Orrison B, Saha T, Hoffman GE *et al.* 2007. Regional FMRP deficits and large repeat expansions into the full mutation range in a new Fragile X premutation mouse model. Gene 395:125-34

CHAPTER 2

Microsatellite repeat instability and neurological disease

BioEssays. Adapted version in press.

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Abstract |

About twenty unstable microsatellite repeats have been identified as the cause of neurological disease in humans. The repeat nucleotide sequences, location within the genes, as well as the normal and disease-causing repeat length ranges differ, as much as the clinical outcomes. Unstable repeats can be located in the coding or the non-coding region of a gene. Different pathogenic mechanisms that are hypothesised to underlie the diseases are discussed, for which evidence is given both from studies in simple model systems, as well as from studies on human material and in animal models. Since somatic instability might affect the clinical outcome, this is briefly touched on. Available data and theories on the timing and mechanisms of the repeat instability itself are discussed, along with factors that have been observed to affect instability. Finally, the question of why the often harmful unstable repeats have been maintained throughout evolution is addressed.

In 1991, two different unstable and expanded trinucleotide repeats were identified as the genetic cause of two different neurological diseases. The $(CGG)_n$ in the *FMR1* gene was found to be responsible for fragile X syndrome [1-4]. Furthermore, an expanded polymorphic tandem $(CAG)_n$ in the coding region of the androgen receptor gene was strongly linked to X-linked spinal and bulbar muscle atrophy (SBMA: Kennedy's disease) [5]. These repeat expansions were recognised as a distinct class of mutations, based on their unusual properties. Since the repeat unit number is variable and the mutation rate depends on the repeat length, the risk of mutation of an allele in each subsequent generation is different from the risk of its predecessor. Therefore, this mechanism was termed 'dynamic mutation' [6]. Since instability increases with expanding repeat length, the risk of getting the disorder is greater in successive generations. In addition, age at onset is likely to be lower and severity of the disorder may be worse with increasing repeat length. This unusual genetic property of the unstable trinucleotide repeats has become known as 'anticipation'. In fragile X syndrome, this used to be referred to as the 'Sherman paradox' [7].

Since the initial discovery of the dynamic mutations involved in fragile X and SBMA, almost twenty other unstable repeats have been found to be associated with neurological disorders. This list is likely to grow in the near future. Although the vast majority of repeats consist of trinucleotides, also tetra- and pentanucleotide repeats can expand and cause disease, for example in myotonic dystrophy type 2 $(CCTG)_n$ and spinocerebellar ataxia 10 (SCA10: $(ATTCT)_n$). For clarity, all repeats will be referred to as trinucleotide repeats in this chapter, not meaning to exclude the tetranucleotide and pentanucleotide repeats. Clinical consequences range from congenital syndromes to late-onset neurodegenerative disorders.

Another group of disorders is caused by expression of polyalanine repeats, coded for by $(GCU)_n$, $(GCC)_n$, $(GCA)_n$ or $(GCG)_n$. Nine genes with expanded alanine tracts have been described thus far, most of which are transcription factors. These expansions predominantly cause congenital malformation syndromes and are not discussed in this chapter [8].

Most trinucleotide repeats are polymorphic and show a normal range, seen in healthy people, and a threshold length above which the repeat causes serious clinical problems. For instance in fragile X, individuals carrying up to 55 CGGs show no clear-cut clinical consequences [1], although in the range of 45 to 55 there is a slight risk for the repeat to become unstable, which has been termed 'grey zone' [1,9]. The fragile X locus also has an intermediate repeat length range that poses the carrier at risk of a distinct syndrome, namely fragile X-associated tremor/ataxia syndrome (FXTAS) or premature ovarian failure (POF) in carriers of alleles with between 55 and 200 CGGs. This repeat length range has been named the premutation (PM) [10-13].

Although this chapter mostly focuses on intergenerational repeat instability, somatic instability also occurs in some of the disorders, which might have clinical consequences. Thus, somatic instability will be touched on briefly. Pathogenic mechanisms through which different repeat tracts may cause disease will be discussed, as well as the timing of the events and the factors that are thought to play a role in instability of the repeat tracts.

Pathogenic mechanisms underlying disease | 2.1

Various pathogenic pathways can underlie trinucleotide repeat-induced disorders. Both loss-of-function and gain-of-function mechanisms have been recognised to result from expanded trinucleotides repeats. When a disease is associated with an mRNA or protein that has attained a new cellular function, it is referred to as a gain-of-function mechanism. A gain-of-function occurs at the RNA or at the protein level, depending on the location of the trinucleotide repeat within the gene. If an expanded repeat is translated, because it is within the coding sequence, it is likely to affect protein structure and thereby function (reviewed in: [14-17]). When a repeat is located in a non-coding region, it will not change protein structure or function directly. However, as the repeat is transcribed, mRNA function might be altered. All diseases and their proposed pathogenic pathways discussed in this chapter are summarised in figure 2.1 and table 2.1.

Loss-of-function at the protein level | 2.1.1

The most common syndrome in which protein function is lost, is fragile X syndrome (FRAXA), which occurs when the $(CGG)_n$ in the 5'UTR of the fragile X mental retardation 1 (*FMR1*) gene exceeds 200 CGGs (full mutation: FM) [2,18]. Beyond this length, the CpG island within the promoter region as well as the repeat become methylated and histone acetylation decreases, which leads to transcriptional silencing. The gene product FMRP is normally highly expressed in neurons of the brain [19,20] and is involved in mRNA translation in the dendrites. FMRP is able to regulate synaptic function by regulation of the translation of these mRNAs at the synapse. As a result, its absence leads to the mental retardation seen in fragile X syndrome [21].

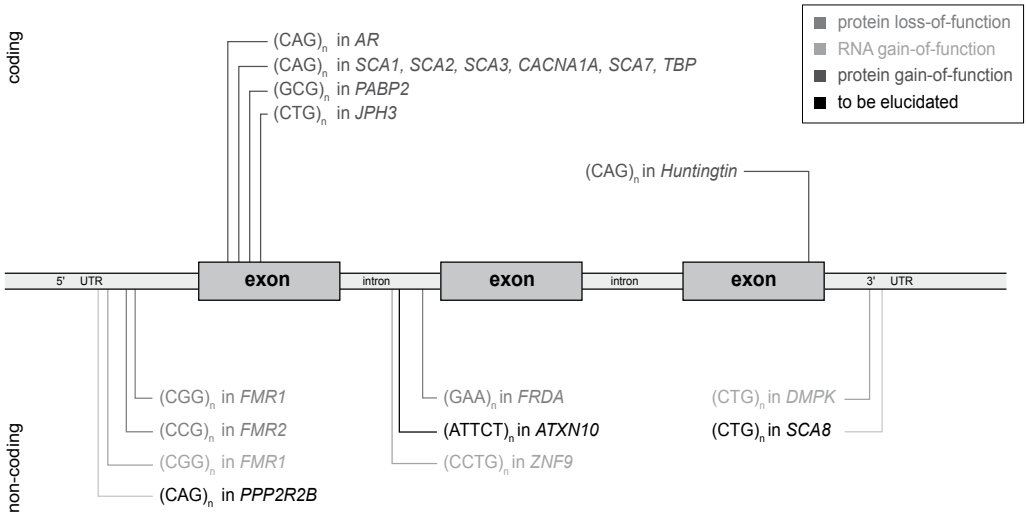

Figure 2.1 | Location of repeats within genes in relation to pathogenic mechanism. This schematic gene shows all microsatellite repeats described in this chapter, with their location within the gene. The greyscale indicates which pathogenic mechanism is thought to underlie the associated diseases. All repeats depicted above the gene are located in a coding sequence of the gene, while all repeats below are in non-coding regions. Please refer to the text and table 2.1 for further information regarding these repeats, genes and associated disorders. See page 163 onwards for colour version of this figure.

A closely related disease is due to a $(CCG)_n$ at the FRAXE locus, which lies just downstream of the FRAXA locus. Patients with a repeat length exceeding 200, suffer from mild mental retardation and developmental delay [22-24]. The expanded $(CCG)_n$ lies in the 5'UTR of the fragile X mental retardation 2 (*FMR2*) gene. Like in FRAXA, the region upstream of the repeat becomes methylated when the $(CCG)_n$ expands beyond 200 units, which silences the gene, thus protein function is lost [25]. The *FMR2* protein is a nuclear protein thought to act as a transcription factor [26], but its cellular function and the role in the disease remains to be elucidated.

Expansion of a $(GAA)_n$ in the first intron of the *FDRA* gene is the cause of Friedreich ataxia (FRDA) [27], which is the most common inherited form of ataxia [28]. FRDA is an autosomal recessive disorder, where repeats in the range of 66 to 1700 GAAs cause disease [29]. PM-sized (34-100 GAA) alleles are recognised, due to their propensity to expand to their up to ten-fold expanded disease-causing counterparts. It is largely unclear how this happens, since patients with FRDA generally inherit alleles that already have disease-causing lengths from their heterozygous parents [30]. In FRDA, the gene is not silenced. Instead, the $(GAA)_n$ expansion interferes with transcriptional elongation such that the level of the gene product, Frataxin, is greatly decreased [31]. Frataxin appears to control iron availability in mitochondria and to have a role in biogenesis of iron-sulfur clusters and haem. This decreases production of cellular energy and prevents the production of free radicals [32-34].

RNA gain-of-function | 2.1.2

An expanded repeat located in a non-coding region of the gene causes cellular toxicity in myotonic dystrophy (DM), FXTAS and likely in spinocerebellar ataxias type 8, 10 and 12 (SCA8, -10 and -12). Since the repeat is transcribed, but not translated, the proteins do not play a central role in the development of the disease.

Myotonic dystrophy

Two forms of DM exist: DM1 is caused by a $(CTG)_n$ in the 3'UTR of a gene encoding a protein kinase (*DMPK)*, while DM2 is the result of an expanded tetranucleotide repeat ($(CCTG)_n$) in intron 1 of the zinc finger protein gene *ZNF9*. Myotonic dystrophy is a multisystem disorder, in which patients not only suffer from myotonia (muscle hyperexcitability) and muscle degeneration, but also develop cataracts, cardiac conduction defects, insulin resistance, sleep disorders, testicular atrophy, hypogammaglobulinaemia and frontal balding [35,36]. The types are clinically similar, although DM2 is a milder disorder than DM1. Both DM1 and DM2 show childhood and adult onset, although most patients with DM2 have adult-onset disease. An important difference is that congenital forms only occur with DM1. Also, muscle weakness shows a distal pattern in DM1, while patients with DM2 suffer from proximal muscle weakness [36,37].

DMPK mRNA levels are decreased in adult DM1 tissue. Hence, DMPK deficiency was initially proposed to be the underlying pathogenic mechanism in DM1. The decreased levels were attributed to the retention in nuclear foci, because transcription of *DMPK* does not seem to be altered when an expanded $(CUG)_n$ is present [38-40]. Although initial studies of DMPK-deficient mice only showed muscle weakness [41,42], later it was suggested that *DMPK* haploinsufficiency causes skeletal and cardiac muscle abnormalities through changes in sodium and calcium channels [43,44]. However, reduced DMPK activity cannot account for all aspect of DM, thus other pathogenic mechanisms must also exist.

Although the two genes involved in DM1 and DM2 are functionally different, the syndromes share a toxic RNA gain-of function mechanism. The first evidence pointing in this direction was that almost all of the expanded repeat-containing mRNAs were retained in nuclear foci [38]. It is now known that the expanded RNA $(CUG)_n$ molecules can form secondary structures, like hairpins [45,46], which is thought to be the cause of their retention in nuclear foci. In these foci, they sequester RNA-binding proteins (RNA-BPs), which bind specifically to $(CUG)_n$ (CUG-BPs) [45,47,48]. Since mRNA processing, including splicing, is normally regulated by a dynamic complex of RNA-BPs, the function of these proteins in the presence of expanded $(CUG)_n$ was investigated. Splicing of the repeat-containing *DMPK* and *ZNF9* mRNAs occurs normally. However, expanded C(C)UG-repeats in DM were found to affect splicing of certain other mRNAs in a *trans* manner [17]. For instance, the expanded C(C)UG-repeats induce misregulation of alternative splicing of a muscle-specific chloride channel (*CLCN-1*) and the insulin receptor. This has clear clinical consequences, as DM1 patients have lower CLCN-1 levels and an insulin-resistant diabetes that is due to a skeletal muscle defect in insulin signalling. Thus, these two examples of splicing misregulation provide a strong molecular basis for the clinical symptoms [17].

CUG-BP1 and Muscleblind-like protein 1 (MBNL1) were found to bind $(CUG)_n$. CUG-BP1 is an hnRNP and a member of the CUG-BP/ETR-3-like (CELF) family of proteins that regulate alternative splicing, editing and translation [49,50]. Binding of MBNL1, but not CUG-BP1, is proportional to $(CUG)_n$ length. Timchenko and co-workers proposed a model in which $(CUG)_n$-BPs are sequestered to expanded *DMPK*-$(CUG)_n$, which then causes aberrant expression of other transcripts that need these proteins for normal gene expression [49]. MBNL1 is a specific $(CUG)_n$-BP, homologous to the *Drosophila muscleblind* proteins, which are essential in terminal differentiation of muscle and photoreceptor cells. The human homologue of muscleblind is also involved in mammalian myoblast differentiation. It accumulates in the nuclear foci in DM1 cells. Miller and colleagues expanded the protein sequestration hypothesis such that the mutant DM1 mRNA provides a competing alternative substrate for the MBNL1 protein. In this way it might be sequestered away from its normal RNA-binding sites during a critical period of cell differentiation [48].

MBNL1 and CUG-BP1 have been described as antagonistic splicing regulators of specific transcripts affected in DM [51-54]. Relative activities of CELF proteins and human Muscleblind control an important developmental switch in splice pattern. CELF proteins promote an embryonic splice pattern for certain transcripts, while an adult pattern is seen when MBNL1 activity predominates. In DM, the switch to an adult splice pattern does not occur. Thus developmentally programmed alternative splicing may be perturbed due to a loss-of-function mechanism for MBNL1, while a $(CUG)_n$-BP gain-of-function mechanism must be underlying the maintenance of the embryonic pattern (summarised in [55]).

Evidence supporting this hypothesis is now accumulating from murine models. For instance, deletion of *Mbnl1* exon 3, which prevents formation of $(CUG)_n$-binding isoforms, results in a DM-like phenotype, including splicing abnormalities similar to those seen in human DM. Kanadia *et al.* suggested that muscleblind-like proteins may play a direct role in splice-site selection [53]. The *Mbnl1* mutation did not reproduce all aspects of DM seen in humans, thus additional muscleblind proteins (Mbnl2 and Mbnl3), which are also recruited to nuclear RNA foci in DM [47], might also be involved. Alternatively, the repeat expansion may have other effects that contribute to the broad manifestation of the disease [53]. In

contrast, transgenic mice overexpressing CUG-BP1 in heart and skeletal muscle share disrupted tissue architecture with tissues in DM, as well as dysregulated splicing of transcripts known to be aberrantly regulated in DM. These results confirm the postulated gain-of-function activity for CUG-BP1 in the presence of expanded repeats, resulting in maintenance of the embryonic splice patterns, as well as a *trans*-dominant effect on specific pre-mRNA targets [51].

In addition, *Drosophila* models have provided evidence for the above outlined model. Flies expressing a non-coding expanded $(CUG)_n$ show several DM features, among which muscle wasting and altered CUG-bp1 and mbnl1 levels and distribution. Overexpression of human MBNL1 suppressed the $(CUG)_n$-induced toxicity, while higher levels of human CUG-BP1 worsens the phenotype [56]. It is, however, still unknown how expansion of the C(C)UG-repeats can alter the activities of CUG-BP1 and MBNL.

Fragile X-associated tremor/ataxia syndrome

In FXTAS, as described extensively in chapter 1, a $(CGG)_{55\text{-}200}$ is present in the 5'UTR of the *FMR1* gene [57]. Elevated *FMR1* mRNA levels and mildly reduced FMRP levels suggest that toxicity originates at the RNA level (reviewed in [58]). Also, the fact that no symptoms of FXTAS have been observed in elderly carriers with the full mutation (>200 CGGS, which silences *FMR1)*, indicates that a lack of FMRP is not likely the cause of the clinical symptomatology seen in FXTAS. *Post mortem* immunohistochemical studies on FXTAS brain revealed the presence of ubiquitin-positive intranuclear inclusions in neurons and astrocytes in many brain regions, which also contain *FMR1* mRNA [59-61].

FXTAS has been proposed to be the result of a pathogenic RNA gain-of-function mechanism similar to what happens in myotonic dystrophy [62]. Interestingly, MBNL1, among many other proteins, has been identified in the intranuclear inclusions in human FXTAS brain [63]. No downstream effects on splicing patterns like in DM have been described to occur in FXTAS yet. Although some signs of cellular toxicity (reduced viability, disrupted lamin A/C architecture) have been observed in cellular models [60,64], it remains to be elucidated how the expanded $(CGG)_n$ leads to altered cellular function and ultimately to clinical symptoms.

Studies are currently focussing on finding possible $(CGG)_n$-BPs that are depleted from their normal functions, thereby causing cellular dysfunction. Indeed, studies in *Drosophila* have identified hnRNP A2/B1 and pur α as $(CGG)_n$-BP. Expression of expanded $(CGG)_n$-containing constructs in flies induces a neurodegenerative eye phenotype, which could be suppressed when hnRNP A2/B1 or pur α was overexpressed. Also CUG-BP1 was found to interact with the $(CGG)_n$, via hnRNP A2/B1, and overexpression of CUG-BP1 suppressed the eye phenotype as well [65,66]. Thus, these findings support a pathogenesis model that predicts that PM-sized $(CGG)_n$ sequester RNA-BP, which leads to altered cellular function. However, a role for these proteins in humans remains to be confirmed, as well as the precise mechanism through which they cause the neuropathological features seen in FXTAS.

A clear difference in the pathogenesis of FXTAS and DM is that in FXTAS *FMR1* transcript levels are increased, while *DMPK* and *ZNF9* are normally expressed. The syndromes are, however, believed to result from similar downstream RNA-BP-mediated pathways. The explanation for this discrepancy might lie in the fact that disease-associated C(C)UG-repeats are much longer (DM1: ranging from 50 to

thousands of CUGs, DM2: between 75 and 11.000 CCTGs), as opposed to 55-200 CGGs in *FMR1*. MBNL1-binding was found to increase with repeat length [48]. The same toxic effect might, however, be exerted by shorter, but more abundant repeat tracts in FXTAS. In *Drosophila* models for FXTAS, the severity of a $(CGG)_n$-induced eye neurodegeneration phenotype was found to be dependent on both expression level and length of the $(CGG)_n$-containing transgene [67]. Interestingly, penetrance of FXTAS appears to be dependent on $(CGG)_n$ length [57]; a threshold might exist for the number of RNA-BPs bound to the expanded repeat, for cellular toxicity to become apparent.

Another difference lies in the fact that all carriers of expanded C(C)UG-repeats in *DMPK* and *ZNF9* will develop DM, while only a part of carriers of $(CGG)_{55-200}$ will suffer from FXTAS. It remains to be solved why some PM carriers are at higher risk of developing this syndrome than others, although additional aging factors might play a role.

mechanism	repeat sequence	gene	chromosomal localisation	position within gene	normal range	disease range	disease		reviewed in:
protein loss-of-function	$(CGG)_n$	*FMR1*	Xq27.3	5'UTR	5-55	>200	FXS	Fragile X syndrome	21
	$(GGC)_n$	*FMR2*	Xq28	5'UTR	6-25	>200	FRAXE	FRAXE associated mental retardation	26
	$(GAA)_n$	*FDRA*	9q13-21.1	intron 1	34-100	200-1700	FRDA	Friedreich ataxia	31, 32
RNA gain-of-function	$(CTG)_n$	*DMPK*	19q13	3'UTR	5-37	50-3000	DM1	Myotonic dystrophy type 1	35, 37
	$(CCTG)_n$	*ZNF9*	3q21	intron 1	<27	75-11000	DM2	Myotonic dystrophy type 2	35, 37
	$(CGG)_n$	*FMR1*	Xq27.3	5'UTR	5-55	55-200	FXTAS	Fragile X-associated tremor ataxia syndrome	58, 70
uncertain	$(CTG)_n$	*SCA8*	19q13	3'UTR	16-34	>100	SCA8	Spinocerebellar ataxia type 8	77
	$(ATTCT)_n$	*ATXN10*	22q13	intron 1	<29	>800	SCA10	Spinocerebellar ataxia type 10	78
	$(CAG)_n$	*PPP2ABb*	5q32	5'UTR	<32	51-78	SCA12	Spinocerebellar ataxia type 12	79, 80
protein gain-of-function	$(CAG)_n$	*AR*	Xq13-21	coding region	9-36	38-62	SBMA	Kennedy's disease / Spinobulbar muscular atrophy	93, 97
	$(CAG)_n$	*Huntingtin*	4p16.3	coding region	6-35	40-121	HD	Huntington's disease	104
	$(CAG)_n$	*SCA1*	6p23	coding region	6-44	39-82	SCA1	Spinocerebellar ataxia type 1	17
	$(CAG)_n$	*SCA2*	12q24.1	coding region	14-31	34-62	SCA2	Spinocerebellar ataxia type 2	17
	$(CAG)_n$	*SCA3*	14q32.1	coding region	12-~43	>60	SCA3	Spinocerebellar ataxia type 3/ Machado-Joseph disease	17
	$(CAG)_n$	*CACNA1A*	19p13	coding region	5-18	20-33	SCA6	Spinocerebellar ataxia type 6	17
	$(CAG)_n$	*SCA7*	3p12-21.2	coding region	7-34	>37	SCA7	Spinocerebellar ataxia type 7	17
	$(CAG)_n$	*TBP*	6q27	coding region	25-44	45-66	SCA17	Spinocerebellar ataxia type 17	17
	$(GCG)_n$	*PABP2*	5q32	coding region	6	8-13	OPMD	Oculopharyngeal myotonic dystrophy	108
	$(CTG)_n$	*JPH3*	16q24.3	coding region	6-28	40-58	HDL2	Huntington's disease-like 2	111

Table 2.1 | **Characteristics of all microsatellite repeats** described in this chapter and the diseases that they cause, categorised by the pathogenic mechanism thought to underlie these neurological trinucleotide repeat disorders.

Uncertain pathogenic mechanisms in the spinocerebellar ataxias

Expanding repeats furthermore cause a group of disorders referred to as the spinocerebellar ataxias (SCAs). Nine SCAs have been described to date, of which three (SCA8, -10 and -12) are caused by repeats in non-coding regions of different genes. SCA8 is the result of a $(CTG)_n$ in the 3'UTR of the gene coding for Ataxin-8, while SCA10 involves an $(ATTCT)_n$ in intron 1 of the *ATXN10* gene. SCA12 is caused by a $(CAG)_n$ in the 5'UTR of the gene for phosphatase PP2ABb. The other SCAs involve $(CAG)_n$ in coding regions of genes and will be discussed hereafter. Even though the repeats are non-coding, it is not yet certain that these SCAs results from an RNA gain-of-function mechanism. Many questions concerning these syndromes remain unanswered to date.

Patients with SCA8 usually present with a slowly progressive ataxia that largely spares brainstem and cerebral functions. Speech is affected and oculomotor problems are common, among other symptoms. $(CTG)_{>100}$ in the SCA8 locus is associated with the disease. However, some individuals show largely expanded alleles, while they are devoid of any clinical symptoms associated with SCA8. Thus, it seems likely that additional environmental or genetic modifiers influence disease penetrance. Based on observations in transgenic mice and flies, which show parallels to aspects of DM and FXTAS, an RNA gain-of-function mechanism has been proposed to underlie SCA8, although further studies will have to confirm this [69].

SCA10 is the only human disease known to be caused by an expansion of a pentanucleotide repeat. The clinical picture can range from pure cerebellar ataxia to a more complex syndrome also involving epilepsy, neuropsychological disturbances, sensory neuropathy and corticospinal tract signs [70]. Pentanucleotide repeats shorter than 29 units are present in normal alleles of the *ATXN10* gene. $(ATTCT)_{>800}$ are considered SCA10 mutations. It is currently uncertain what risks the intermediate repeat range pose on the carrier. The function of the *ATAXN10* protein product is unknown and the pathogenic mechanism that causes SCA10 remains to be elucidated. Since transcript levels are not reduced in patient cell lines, an RNA gain-of-function mechanism has been proposed [36].

SCA12 is a very rare disease. Normal alleles have up to 32 trinucleotides in the gene coding for phosphatase PP2ABb. The lower border of disease-causing repeat length is unclear, but clearly affected patients have alleles in the range of 51-78 CAGs. Early clinical signs are action tremor of arms and the head, which typically start in the third or fourth decade of life. Over the next 20-30 years the clinical pattern aggravates. Late stages of SCA12 may also involve cognitive decline. The gene *PPP2ABb* encodes one of the brain-specific regulatory subunits of the trimeric phosphatase PP2A. The repeat seems to affect transcriptional efficiency, as transcript levels are increased. Protein levels are also elevated. Many splice isoforms exist and it is conceivable that the SCA12 repeat expansion could disrupt splice regulation, thereby altering the function of PP2A. PP2A is an enzyme ubiquitously expressed and involved in many cellular processes, including apoptosis. The slow clinical course of SCA12 might suggest a gene dosage effect under the influence of modifying factors. A role for a toxic $(CAG)_n$-containing transcripts can, however, not be excluded in the development of SCA12 [71], neither a toxic polyQ-containing protein [72].

Gain-of-function at the protein level | 2.1.3

The most common trinucleotide repeat in coding regions causing disease by altering protein physiology is the $(CAG)_n$. When translated, this results in a polyglutamine (polyQ)-tract in the protein, obviously

affecting its structure and function. Although expansion sizes, structures, cellular localisation and functions of the resulting proteins differ, all $(CAG)_n$-induced diseases are neurodegenerative disorders, involving the central nervous system [78-80].

All disorders are associated with neuronal intranuclear aggregates that contain the disease-causing gene product. In some cases polyQ-containing aggregates may also form outside the nucleus [81-84]. *In vitro* studies demonstrated that polyQ-stretches have an inherent ability to aggregate. Apart from binding to many other proteins, glutamine also shows self-interaction, which may to a large extent account for the tendency of polyQ to aggregate [85]. Once polyQ-stretches exceed a certain length, they are no longer soluble and form aggregates in solution. The threshold length above which *in vitro* aggregation takes place, is strikingly similar to the threshold causing disease [86]. Nine expanded $(CAG)_n$ disease-associated genes have been identified. However, these are not the only polyQ-encoding sequences in the genome. Stretches above as well as below the threshold length for disease (~35-40 CAGs) are seen. Knowing the normal function of polyQ-tracts might facilitate understanding how expansion of the $(CAG)_n$-tracts and loss of the normal protein function causes disease, but to date not much is known about this [85].

In vivo protein aggregates also contain molecules other than the proteins containing the expanded polyQ-repeats. It is tempting to hold the aggregates responsible for the development of disease, some evidence exists however that large aggregates are not the primary cause of cell toxicity. Some disease models have indeed shown cell toxicity in the absence of aggregates, or toxicity was found to decrease as aggregate density increased [87].

Kennedy's disease (spinobulbar muscular atrophy)

Kennedy's disease (or spinobulbar muscular atrophy: SBMA) was the first disorder that was described to result from expansion of a polymorphic $(CAG)_n$ in the coding region of the androgen receptor gene located on the X-chromosome [5]. Patients have 38-62 CAGs as opposed to 9-36 in normal controls. Affected males suffer from muscle cramps and develop proximal muscle weakness from their forties onwards [88]. The androgen receptor is a nuclear receptor, which gets translocated to the nucleus upon androgen binding in the cytoplasm, in order to regulate transcription of hormone-responsive genes. The polyQ tract is located in the trans-activation domain [73,89]. Presence of an expanded $(CAG)_n$ results in decreased receptor expression and altered transcriptional activation [90-92]. This may account for the androgen insensitivity seen in SBMA patients, but it does not seem to explain motor neuron toxicity. Therefore it has been proposed that the mutant androgen receptor causes motor neuron dysfunction by a toxic gain-of-function [74].

Huntington's disease

George Hungtington described the clinical features of what would later be named Huntington's Disease (HD), including progressive involuntary movements (chorea), incoordination, general motor disability and several mental and personality problems already in 1872. Despite intensive efforts to find the gene for HD, identification of the coding $(CAG)_n$ in Kennedy's disease did not prompt the HD Collaborative Research Group to focus on this mechanism [93]. Only after myotonic dystrophy was recognised to result from an expanded trinucleotide repeat, such a repeat responsible for HD was searched for and

found. The expanded $(CAG)_n$ is located at the N-terminus of the gene that codes for a protein named Huntingtin [94]. The precise function of normal Huntingtin remains to be established, although it is known to be involved in several cellular processes such as vesicular transport, cytoskeletal anchoring and clathryn-mediated endocytosis [95]. Although loss-of-function of Huntingtin may account for some of the pathogenesis seen in HD, evidence points to a more prominent role for a toxic gain-of-function of the mutant Huntingtin protein. Inclusion bodies containing N-terminal Huntingtin and ubiquitin have been found in neuronal nuclei and neuronal processes of post-mortem brain material of HD patients. The clear correlation between the threshold of Huntingtin aggregation and the $(CAG)_n$ length which causes disease, suggests that aggregated $(CAG)_n$-containing Huntingtin is a major toxic mechanism underlying HD [96]. Abnormal protein interactions with mutant Huntingtin could take place, possibly leading to co-aggregation of other proteins. However, the precise mechanism by which inclusions lead to pathology, and whether they are a prerequisite for pathology remains to be elucidated [97,98]. Also, evidence exists that inclusions might exert a neuroprotective effect by sequestering more toxic forms of the aggregated protein [75,97]. However, transgenic mouse models have provided clear evidence that the presence of inclusions in the nucleus and neuronal processes is correlated to disease pathogenesis [99]. A conditional mouse model showed reversal of neuropathology and disappearance of inclusion bodies, when mutant Huntingtin expression was turned off [100].

Spinocerebellar ataxias

Expanded $(CAG)_n$ in coding regions are furthermore the cause of SCA1, 2, 3, 6, 7 and 17. These SCAs share many clinical and pathological features, which makes it difficult to distinguish them based on clinical symptoms, although some features may be more common in a specific SCA subtype. SCAs typically become evident around middle age, to progress to early death within 10 to 20 years. Cerebellar atrophy as seen in neuro-imaging studies is observed in all SCAs, as well as Purkinje cell loss. Loss of brain stem neurons, such as the inferior olive, is commonly seen in all SCAs, although milder in SCA6 and not in SCA3. SCA3 also seems to be relatively spared with regard to cerebellar neurodegeneration. In SCA3 basal ganglia, brain stem and spinal cord are most affected (summarised in [17]). Other than sharing a polyQ-tract, the gene products of the different SCA-genes bear no homology. SCA1-3 and -7 are caused by Ataxin-1 through -3 and -7 respectively, while SCA6 involves the α_1A-subunit of the voltage-dependent calcium channel (CACNA1A), and SCA17 is the result of an expanded polyQ-tract in the gene coding for TATA-binding protein (TBP). Each disease has different normal and disease-causing $(CAG)_n$ length ranges and different pathways through which they cause disease, but for all is true that the age at onset of symptoms gets earlier as the repeat tract gets longer [17].

All these $(CAG)_n$-induced diseases suggest that the pathogenic polyQ-containing proteins escape the otherwise strict protein quality control mechanisms. PolyQ-elements likely impair a protein to fold into its functional native conformation. Heat shock proteins (hsp) such as Hsp40 and Hsp70 act as chaperones by recognising misfolded proteins and facilitating refolding. When refolding is unsuccessful, a few ubiquitin monomers will be attached to the protein, which is the signal for degradation by the ubiquitin proteasome system (UPS). Formation of inclusions in many polyQ-associated diseases suggests that these cellular quality control mechanisms fail to deal appropriately with mutant polyQ-containing

proteins. Research focussing on the precise role for chaperones and the UPS in these diseases could lead to promising therapeutical strategies [96]. However, despite overlapping characteristics of the expanded polyQ diseases, it is clear that they all differ, depending on how the expanded $(CAG)_n$ affects the protein. It cannot be ruled out that in some cases loss-of-function of the polyQ-containing protein contributes to the pathogenesis [17]. Alternatively, one could wonder whether the repeat-containing RNA also contributes to pathogenesis in polyQ-mediated disease [101].

Other trinucleotide repeats in coding regions

Other coding repeats that cause neurological disease include a $(GCG)_n$, coding for a polyalanine-tract, in the poly(A)-binding protein 2 (PABP2), which causes oculopharyngeal myotonic dystrophy (OPMD). It is interesting to note that these $(GCG)_n$ are short, namely 8-13 repeat units in the OPMD families, as opposed to the normal allele which is 6 GCGs long. The $(GCG)_n$ represents the first short trinucleotide repeat discovered, which causes human disease. Furthermore, the $(GCG)_n$ does not show the features of a dynamic mutation. For instance, it is meiotically quite stable [76]. The polyalanine-tract was postulated to cause pathologically expanded PABP2 to accumulate as filament inclusions in nuclei, which are seen in OPMD patients [102].

Furthermore, an expanded $(CTG)_n$ in an alternatively spliced exon of the junctophilin-3 (*JPH3*) leads to Huntington disease-like 2 (HDL2). Two of three splice isoforms result in the presence of the repeat in the coding region, leading to a polyalanine or a polyleucine tract [103]. Intranuclear protein aggregates have been seen in brain, but the polyalanine and polyleucine tracts do not seem to play a major role in the formation of the aggregates, nor in the pathogenesis of HDL2 in general. It has also been postulated that HDL2 expansions can lead to formation of RNA inclusions, which might suggest that HDL2 pathogenesis might originate at the level of toxic transcripts [77].

Timing of repeat instability | 2.2

When trying to elucidate the mechanism of repeat instability, timing of the event is a major aspect that remains surrounded by question marks. The fact that repeat length changes between generations, tells us that instability occurs at some point between gametogenesis in the parent and early embryogenesis. Attempts have been made to gain insight into what happens around fertilisation, although availability of human material is limited. Some of the findings that shed light on when the fragile X dynamic mutation takes place will be discussed, as well as the timing of events in other trinucleotide repeat disorders.

Timing of expansion in the dynamic fragile X mutation | 2.2.1

Remarkable observations that expansions to a fragile X FM occur only upon maternal transmission of a PM allele, and that daughters of FM males are clinically and cytogenetically normal, were explained when it was observed that male fragile X patients only have PM alleles in their sperm [104]. Two models have been proposed to explain this finding.

First, the prezygotic model predicts that an expansion of PM to FM could occur during maternal meiosis (figure 2.2). This means that the FM must contract to a PM in gametes of male offspring. PM gametes might have some selectional advantage either due to the presence of FMRP, or against the presence of an expanded $(CGG)_n$. However, FMRP is not required for spermatogenesis [105,106]. FMRP

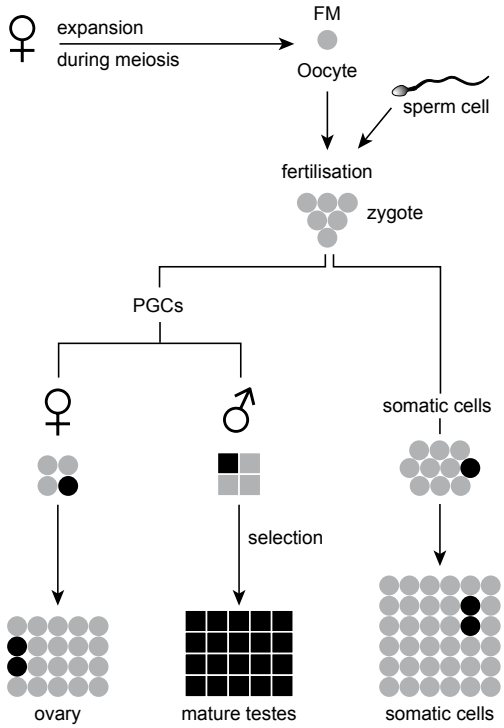

Figure 2.2 | The prezygotic model of expansion of the fragile X mutation. This model assumes that an expansion of a maternal PM to an FM takes place during meiosis. The fertilised oocyte carries an FM allele. After separation from the embryo proper, the primordial germ cells (PGCs) have an FM. Some alleles will contract to PMs. To explain why FMs are only transmitted through females, some selection must exist agains FMs in the male germ line during spermatogenesis. In the mature testes, PM alleles predominate. In somatic cells and the female germ line, this selection did not take place. Cells with a PM are shown in black and cells with an FM are depicted in grey. See page 163 onwards for colour version of this figure.

expression might still be beneficial to the spermatogonia as they mature. A selective advantage for cells expressing FMRP was indeed seen in cell culture studies [107].

In 13-week old FM foetuses, no PM alleles were detected in testes, while in 17-week old FM foetuses some primordial germ cells expressing FMRP were observed. This suggests that contraction from FM to PM occurs in the maturation process of the testis, after the initial replication event [108]. Paternally derived alleles have been shown to undergo rapid demethylation in murine zygotes, before the first round of replication, while CpG methylation is maintained in maternally derived alleles [109]. CpG methylation appears to stabilise the DNA. Hence, paternally derived alleles could be prone to deletions upon demethylation [110]. Furthermore, FMs are responsible for a delay in replication of the *FMR1* gene during the cell cycle [111,112]. The large number of cell cycles associated with spermatogenesis might enhance a selectional effect [113]. Thus, primordial germ cells with a PM might have a proliferative advantage, thereby overgrowing FM cells. However, if multiple contraction events can take place, it is puzzling why only one distinct PM band is mostly seen in sperm of FM men [104].

The other model considers a postzygotic expansion, after separation of the germ line. It assumes that the FM allele has never been present in male or female gametes [114,115]. However, oocytes of a foetal female FM carrier had only FM alleles [108]. It is however unknown whether oocytes of a female PM carrier bear PM or FM alleles. A postzygotic expansion mechanism occurring only for a PM allele on the maternal X-chromosome, would require an imprinting mechanism to distinguish it from a paternally derived PM, which would not expand [104,116].

Based on a simulation study and analysis of tissues of affected foetuses, Moutou and co-workers favour a prezygotic model, whereby a selection mechanism accounts for the sole presence of PM alleles in sperm of male offspring. However, they cannot exclude a postzygotic model in which a transition occurs at the early morula stage [117]. Naturally, a final conclusion can only be drawn after analysis of oocytes in ovaries from an embryo carrying a PM allele. Unless the embryo suffers from a different detrimental defect, this material is not available for obvious reasons. Also, oocytes from an adult PM carrier would provide useful information. However, the most likely reason for an ovarium to become available is when a female PM carrier suffers from POF. In this case, naturally, almost no oocytes are present anymore. Expanded $(CGG)_n$ knock-in mouse models have been developed [118,119], that show intergenerational repeat instability [119,120]. These models will be valuable in answering remaining questions.

Timing of expansion in other trinucleotide repeat disorders | 2.2.2

Also for other trinucleotide repeat disorders, the exact timing of expansion is still under debate. Intergenerational expansion in DM1 was suggested to result from initial expansion, followed by somatic instability [121]. This initial instability was first suggested to occur between week 13 and 16 of gestation [122-125]. However, the youngest foetuses that were investigated were obtained at 10-12 weeks of gestation and they already showed major expansion as compared to the parental allele [121,122,124]. Furthermore, studies in tissues of monozygotic twin pairs showed identical expansion patterns [121,126,127]. Monozygotic twinning occurs at the latest on day 10.5 of embryonic development [128], thus this first wave of instability is likely to happen in very early embryonic stages. Interestingly enough, analysis of expanded *DMPK* alleles has been done in oocytes and embryos of mothers with expanded $(CTG)_n$, who underwent *in vitro* fertilisation (IVF) with pre-implantation diagnostics for DM1. This study revealed that expansions are already detectable in oocytes, including an immature oocyte (germinal vesicle stage) and embryos, as early as the three-cell stage [129]. An earlier study had also observed that enlargement of a $(CTG)_n$ had occurred at the germinal vesicle stage, that is, either during premeiotic proliferation of oogonia, or during prophase I of meiosis I [130]. Similar to the fragile X dynamic mutation, although to a lesser extent, a selection mechanism against further expansion of long alleles in sperm has also been proposed for DM1 [121].

$(CAG)_n$ expansions have also been detected in oocytes in a transgenic mouse model for SCA1, thus after meiotic DNA replication and prior to fertilisation [131]. Thus, oogenesis seems the most likely moment of $(CTG)_n$ expansion [129,130]. However, an asymptomatic male carrying a somatically stable $(CTG)_{52}$, showed major instability in the germ line, with a bias towards expansion. The mean repeat length in this man's sperm was $(CTG)_{238}$, which was consistent with the large alleles inherited by two of his offspring [132].

Transmission of normal alleles was also investigated in donated supernumerary IVF embryos. In individuals who carried both a $(CTG)_{5-18}$ and a $(CTG)_{19-37}$ allele, the $(CTG)_{19-37}$ allele was transmitted to the offspring in 59% of cases. Of these transmissions, 7% was unstable when the $(CTG)_{19-37}$ allele was of paternal origin, whereas no instability of maternal alleles was seen. Thus, instability of normal *DMPK* alleles can occur during male gametogenesis or early embryogenesis. It remains to be elucidated whether expansions in the normal range are the result of the same mechanism as that which yields

larger expansions. Also, further studies will need to clarify whether $(CTG)_n$ expansion continues after fertilisation, during embryonic cell division [129].

The magnitude of intergenerational $(CAG)_n$ expansions increases greatly with the age of the transmitting father [133]. The frequency of $(CAG)_n$ expansions in sperm was found to increase with length of the inherited repeat allele [134,135]. Both increased magnitude and frequency with age are mimicked in mouse models for DM [136,137]. Accumulation of expansions in sperm explains the positive correlation between age of the transmitting father and the repeat length in the offspring [137,138]. However, another study in a DM mouse model did not show significant differences between spermatozoa and spermatogonia, implicating that germinal expansions are already present very early in spermatogenesis [136]. Furthermore, a recent study did not find a correlation between $(CAG)_n$ length variation in sperm and age of HD patients [139].

Instability of repeats in coding regions has been less well characterised. Expansions of the $(CAG)_n$ have been observed in sperm of HD patients [134,135], as well as transgenic mouse models for HD [140]. In the mouse model the $(CAG)_n$ expansions in the male germ line seemed to occur in late spermatogenesis, in post-mitotic and post-meiotic cells (transition from round to elongated spermatids) [140]. Analysis of testicular germ cells of two men who died from HD showed that repeat expansions were already present before the end of the first meiotic division, and in some cells even before the start of meiosis. However, the majority of the larger expansions was found in the postmeiotic cell population, suggesting that instability processes may continue after meiosis [141].

Study of a possible age-effect of transmitting females has led to opposing results [142]. A study in superovulated female transgenic SCA1 mice showed an age-dependent contraction-biased repeat instability in unfertilised mature oocytes. In oocytes harvested from mice of 7-weeks of age, very little repeat size heterogeneity was seen, which suggests that instability might occur during germ cell cycle arrest in meiosis I [131]. Thus, different mechanisms appear to underlie the instability occurring in male and female gametogenesis.

Somatic repeat instability | 2.3

Despite the intergenerational repeat instability that links all diseases described in this chapter, the extent of somatic instability varies. In fragile X syndrome, for instance, many patients show a smear of FM repeats on Southern blot, indicating a range of repeat lengths [2,143]. A subset of patients also has a PM allele (mosaisics) [144-146]. Repeat patterns of monozygotic twins of 9- and 30-year old showed extraordinary similarity [114]. Thus very little, if any, somatic $(CGG)_n$ instability occurs over the course of life. The proposal that CpG methylation stabilises the repeat [110] might explain the lack of somatic instability in fragile X patients. Cell culture experiments with FM fibroblast lines showed remarkably stable transmission of the repeat to progeny cells. Thus, this contradicts that $(CGG)_n$ are mitotically unstable. Indeed, if they were, one would expect more random patterns of repeat lengths among different tissues [115]. However, foetal female fragile X fibroblasts, which were cultured for many passages, showed dramatic instability, while male adult fragile X fibroblasts did not. Thus, further repeat expansion was observed during cell proliferation in female fibroblasts, outside the proposed time window of early embryonic development [147].

Minor somatic instability was observed in the expanded $(CGG)_n$ mouse model, when comparing tissues at 52 weeks of age with tail DNA obtained at 10 days postnatally. Increase in repeat length was never larger than 10 triplets, with kidney and testis showing more instability than brain. Somatic instability might contribute to tissue specificity and disease progression. Thus, it was concluded that somatic instability in the brain does not play a major role in this mouse model, although the influence of instability on individual neurons cannot be excluded [148].

Other trinucleotide repeat disorders show substantial somatic instability. In particular, the $(CTG)_n$ in DM1 is highly unstable in many tissues. Studies set up to investigate intergenerational instability, already revealed tissue heterogeneity of repeat length [122-125]. $(CTG)_n$ length indeed appears to depend on the type of cell lineage [123,125,149-153]. As it was found that the $(CTG)_n$ length in lymphocytes does not correlate well with the severity of the phenotype [154-158], somatic mosaicism was suggested as a possible explanation [123]. Observations that muscle cells consistently show longer repeats than peripheral blood leukocytes, point in this direction [149-152]. To date no studies have been published that correlate repeat length in several tissues from adult DM1 to the severity of the phenotype. However, it has been suggested that ongoing expansions in somatic cells may contribute to the progressive and tissue-specific nature of DM [153,159].

Somatic $(CTG)_n$ instability is repeat-size and age-dependent. Thus, older patients show a longer average repeat size [151,153]. Since somatic expansion occurs in both post-mitotic and proliferating tissues, it must be a process independent of cell division [121]. In a knock-in mouse model for DM1, which reproduces somatic instability of the $(CTG)_n$, it was observed that once alleles had expanded, they continued to increase in length. In contrast, the portion of cells that did not show instability, remained constant. The researchers observed a bimodal profile for repeat length (i.e. two major repeat lengths), which became more pronounced with age. They considered aging and terminal differentiation to play a role [160]. In liver cells, polyploidisation marks late differentiation [161]. It was therefore investigated whether acquiring a greater than diploid DNA content affects hypermutability. Indeed, about 50% of liver cells became tetraploid-octaploid, and 80% of cells were binuclear. These changes were accompanied by an overall increase in expansion frequency, as compared with diploid, mononuclear cells. It is unknown how these factors affect somatic $(CTG)_n$ stability. These findings also serve as evidence for expansion to be a post-replicative event [160]. To date, this has not been described for other mouse models for DM1, neither for other trinucleotide repeat disorders. The mouse model for FXTAS [118,120,148] does not show increased somatic instability in polyploidic or multinuclear hepatocytes (unpublished results).

Mouse models for DM also show somatic instability throughout life [151,153]. It is interesting to note that different individual mice of the same mouse line presented the same pattern of mosaicism, that is, more instability in some tissues and less in others. This means that somatic instability is not a random process, but that some, as yet unknown, deterministic processes underlie these tissue differences (reviewed in [142]).

The observation that the $(CAG)_n$ length heterogeneity observed in HD offspring does not correspond to that in the transmitting parent, suggests somatic instability [134]. Early studies demonstrated $(CAG)_n$ length heterogeneity within and between tissues, with the largest degree of mosaicism seen in brain and sperm [162,163]. Interestingly, region-specific repeat length analysis revealed dramatic increases

in repeat length in the striatum, which is the brain region that is primarily affected in HD, both in a mouse model for HD [164] and in early-stage HD patients [165].

In a mouse model for HD, a gender effect for direction of instability was seen in offspring from identical fathers, which shows that repeat length can change in the early embryo [166,167]. No further somatic instability was seen in these animals, when testing several tissues and at later stages in life [167].

Higher levels of $(CAG)_n$ length variation had been observed in buccal cell DNA as compared with DNA obtained from blood in patients with SCA7 [168]. Analysis of buccal cell DNA of HD patients showed that $(CAG)_n$ length variability depends on the size of the inherited allele, where larger repeats lead to a higher degree of somatic mosaicism. No large length gains were seen in buccal cells, as opposed to the previously detected large expansions in brain. No effect of age on repeat instability in buccal cells was seen [169]. Somatic $(CAG)_n$ instability has also been seen in SCA1, SCA3, SCA7, SBMA and FRDA [142].

Molecular mechanisms of repeat instability | 2.4

It remains to be solved whether intergenerational and somatic instability arise from the same mechanisms. Most studies that attempt to elucidate the molecular mechanisms of repeat instability deal with intergenerational instability. Evidence from these studies will be described.

One of the first proposed mechanisms involved in repeat instability at the molecular level was slippage of the replication fork during replication. Unpaired bases form loops, which result in expansions or contractions in a next round of replication, depending on whether the looped repeats are in the newly synthesised or template strand (figure 2.3, [170,171]). However, slippage cannot explain all aspects of repeat expansions, for example, it would predict only limited expansions, rather than large-scale expansions.

New insights came from the realisation that the expandable repeats have unusual structural properties. Disease-causing trinucleotide repeats are almost exclusively formed of $(CNG)_n$ triplets. Single-stranded $(CNG)_n$ can form hairpin-like structures that can have both Watson-Crick and mismatched base pairs [172]. The leading and lagging strand have different tendencies to form hairpins, due to their different sequences. This makes the slipped-stranded DNA asymmetrical, which has important biological consequences. Hairpin formation on one strand might cause the slip-out on the other strand to be in random-coil state. The structure of the slip-outs is likely to affect recognition and subsequent repair or recombination of this structure [173]. Most prominent instability occurs when the structure-prone strand serves as the lagging strand template [16].

An alternative model is based on *in vitro* observations that unusual structures formed by expanded repeats stall DNA polymerases [174-176]. *In vivo* studies showed that the orientation of the repeat relative to an origin of replication largely determines the stability of the repeat [177-180], which underscores the biological meaning of the asymmetrical conformation of the slipped-stranded DNA. The orientation-dependence of instability was hypothesised to be a result of temporary existence of single-stranded DNA during lagging strand synthesis. This could allow formation of hairpins. The tendency to form hairpins is dependent on the exact sequence of the repeat of the lagging strand template. However, evidence that does not support this model exists. Thus, it is unclear why hairpins form in an

orientation-dependent manner, but most models agree that replication can generate small insertions and deletions [181]. An origin of replication was recently identified adjacent to the *FMR1* promoter. The position of this origin of replication with respect to the $(CGG)_n$ is such that it favours contraction. This is because the CGG sequence, which is more prone to form stable hairpins than is $(CCG)_n$, is in the lagging strand template [182]. However, usage of origins of replication might be different during early embryonic development. Use of an alternative origin of replication downstream of the repeat would cause the $(CGG)_n$ to be in the nascent Okazaki fragment, which could lead to expansions [101].

Evidence for replication-based mechanisms came from studies in yeast replication mutants, which showed a marked increase in frequency of repeat instability. Proteins involved in lagging strand synthesis, coordination between synthesis of the leading and the lagging strand and restarting of the replication fork play a role in the stability of microsatellite repeats (reviewed in [16]). A model based on replication fork stalling and restarting was proposed [183], which can explain both contraction and expansion. Unusual secondary structures in the lagging strand template causes stalling of the DNA polymerase situated on this strand. DNA replication could deal with this obstacle in two ways, resulting in either contraction or expansion. In short, contraction would result from resuming lagging strand synthesis after skipping one Okazaki fragment. The gap between adjacent pieces of nascent DNA strands

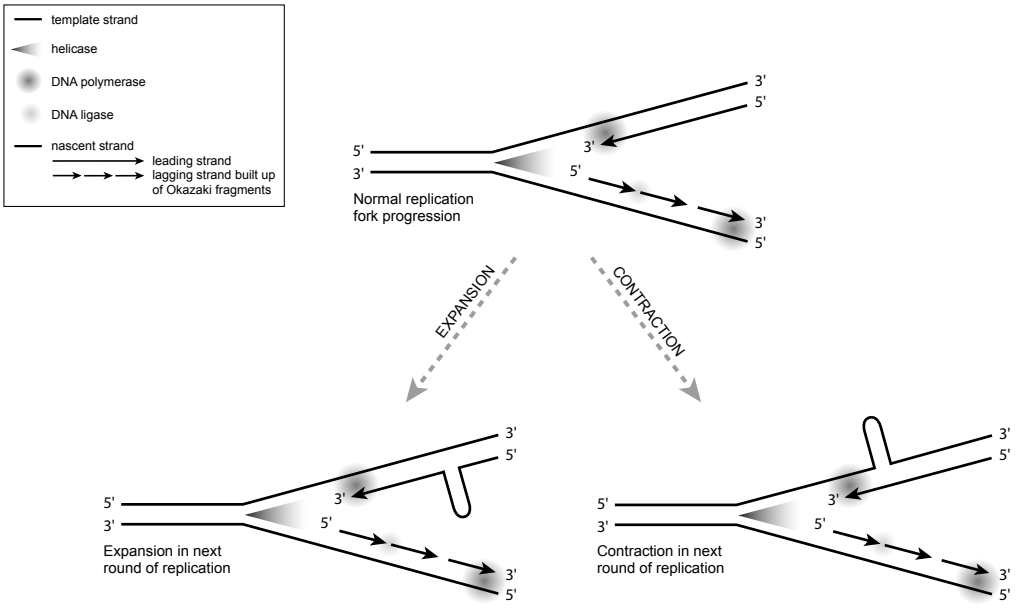

Figure 2.3 | Replication fork progression and repeat instability. During normal replication, helicases break the hydrogen bonds that keep the two DNA strands together, which yields the replication fork. DNA polymerase can only synthesise a new strand in a 5′ to 3′ direction. Hence, on one strand (leading strand) DNA polymerase reads the DNA and adds nucleotides to the nascent strand in a continuous matter. On the other strand (lagging strand) the complementary strand is synthesised in short segments (Okazaki fragments) at a time, that are later joined together by DNA ligase. Formation of secondary structures, such as hairpins can form in one of the strands. This can impair normal replication fork processing. The sequence of the strands, together with the position of the origin of replication with respect to the repeat sequence, determine which strand is more prone to form hairpins. Whether a hairpin is present in the template or nascent strand, in turn determines whether contraction or expansion results in the next round of replication. See page 163 onwards for colour version of this figure.

will be repaired, resulting in repeat contraction. The alternative route could lead to fork reversal. Briefly, this refers to a complex 'chicken-foot' structure, which contains a single-stranded repetitive extension in the nascent leading strand, which could cause extra repeats to be added when the reversed fork restarts replication (discussed in more detail in [183] and [184]). This is the only model to date that can explain the observations on trinucleotide instability on a molecular level. For instance, the phenomenon of anticipation could be the result of progressively increased instability due to consecutive replication stalls and restarts within longer repeat tracts. Also, the threshold-length of the repeat tract for formation of secondary structures coincides with the length of the average eukaryotic Okazaki initiation zone (~200 bases), which might explain why various repeats show similar thresholds for expansion. Lastly, varying degrees of instability observed in different model organisms could be explained by different probabilities of fork reversal and fork bypass to occur [16]

Modifiers of repeat instability | 2.5

Initial studies into the dynamics of triplet repeat expansion were conducted in simple model systems such as *Escherichia coli* and *Saccharomyces Cerevisiae*. These studies have shed light on some factors that play a role in repeat dynamics. However, they are of limited value when considering that trinucleotide repeats have a tendency to contract in bacteria and yeast, while almost all repeats in humans show a bias towards expansions. In addition, germ line and somatic repeat instability cannot be studied in these simple model systems. Therefore, transgenic and knock-in mouse models have been generated to mimic human repeat dynamics more closely. The repetitive nature of the sequence of a trinucleotide repeat is a primary determinant of its unstable nature. However, given the fact that different repeats behave differently, and that the same repeat might act differently among individuals, other factors must also play a role. A commonly used categorisation of factors that influence repeat instability, is based on *cis*- and *trans*-acting factors, where *cis*-acting refers to factors that are directly associated with the repeat, whereas elements that interact with the repeat can be considered *trans*-acting elements. Many of the results obtained in the different model systems will be discussed below.

Cis-acting factors | 2.5.1

The most obvious factor involved in repeat instability, is the length of the repeat tract. Most trinucleotides repeat disorders have distinct repeat categories for normal alleles, which are generally stable upon transmission to the next generation, and longer unstable alleles [142]. Transgenic mouse models also show that a threshold repeat length exists, above which repeats become unstable across generations. In some mouse models, instability increases with longer repeat tracts [119,138,185].

Repeat sequence

Apart from the length of the repeat tract, also the precise sequence plays a role. When a different trinucleotide interrupts a repeat tract, it is more stable than a pure repeat. Interruptions determine the chances of folding into hairpins and their stability [186,187]. For example, AGG interruptions greatly stabilise expanded $(CGG)_n$ tracts [186,188]. Also the position of the AGG interruption influences stability, as most $(CGG)_n$ length variations are seen at the 3' side of the repeat in the *FMR1* gene [189]. In fragile X, FM $(CGG)_n$ tracts were found to have fewer AGG interruptions and longer pure $(CGG)_n$ stretches, when compared

with normal length *FMR1* alleles [190]. A similar stabilising effect of interruptions on $(CAG)_n$ has been described in men [191,192] and mice [131]. Thus, when generating a mouse model to study intergenerational repeat instability, it is wise to introduce a repeat as long and pure as possible.

A sequence-specific effect can also be concluded from observations that $(CTA)_n$ and $(TAG)_n$ did not show detectable expansions in a yeast model that did show $(CAG)_n$ and $(CTG)_n$ instability, possibly related to the impossibility of forming secondary structures for the former repeat sequences. Also, $(CAG)_{25}$ was found to be much more stable than $(CTG)_{25}$, which was explained as that $(CAG)_n$ are unlikely to form hairpins in this assay [180].

Flanking sequences

It has become evident that factors outside the repeat sequence itself also have an influence on repeat instability. Much of this insight has been obtained through animal studies. The first attempts to mimic intergenerational repeat instability in mouse models expressing expanded trinucleotide repeats failed. Transgenic models expressing $(CGG)_n$ of lengths that in humans cause instability, showed stable transmission to next generations [193,194].

Database analysis of sequences of C/G-rich trinucleotide repeat-associated genes revealed that in almost all cases studied a CpG island was located close to the repeat. This was interpreted as a clue that flanking regions and chromatin structure near the triplets might play a role in repeat instability [195]. The relative expandability of a repeat was found to be associated with the GC-content of the flanking region. GC-rich chromatin might affect the flexibility of the trinucleotide repeat and the stability of secondary structures that are formed by the repeat. This might have an effect on DNA processes [196]. The observation that a fragile site can be induced at the FRAXA locus might also hint at an abnormal chromatin structure [197]. It should be noted, however, that $(CAG)_n$ and $(CTG)_n$ expansions have not been associated with fragile sites.

The importance of the flanking region also became evident when a transgenic mouse model was generated by introducing a 45 kb genomic segment with a $(CTG)_{55}$ [185]. In contrast to other mouse models, expressing $(CAG)_n$ in a cDNA context [198-200], these mice showed moderate intergenerational and somatic repeat instability. No large expansions were seen. As the threshold for large increases in humans is thought to lie between 40 and 50 repeats, it was suggested that this threshold is higher in transgenic mice [185]. Subsequent studies focused on differences in instability between transgenic mice expressing a large human genomic fragment (45 kb) with either a normal ($(CTG)_{20}$), slightly expanded ($(CTG)_{55}$) or a large CTG-repeat ($(CTG)_{>300}$). As expected, the normal allele showed no instability at all. Mice expressing the long repeat exhibited dramatically increased instability, as compared to the $(CTG)_{55}$-line, again confirming that instability strongly depends on repeat length [138,201].

Also in the case of $(CGG)_n$ instability, transgenic expression of a yeast artificial chromosome (YAC), which contained an expanded $(CGG)_n$ tract as well as some flanking sequences, resulted in moderate instability [202]. But only when a knock-in mouse model was generated by substituting the endogenous murine $(CGG)_8$ repeat in the *Fmr1* gene with a human $(CGG)_{PM}$ tract, thus preserving the genomic context [193], substantial repeat instability was observed [119,120]. Thus, not only the presence of a certain degree of flanking region, but also the insertion site is important for the occurrence of repeat instability. In the process of generating transgenic mice expressing expanded $(CTG)_n$ repeats, a few

similar mouse lines were obtained that expressed the same repeat tract, albeit integrated in different regions in the genome. The different integration sites resulted in slight variation in intergenerational, as well as somatic repeat instability [185,203,204].

Orientation of the repeat with respect to the replication fork

As mentioned above, the molecular basis of repeat instability is thought to lie at the replication fork machinery [16]. Thus, it is not surprising that factors associated with this machinery influence instability. Studies on $(CTG)_n$ instability in *E.coli* and yeast [178,180] already showed that the direction of replication determines the frequency of expansions and deletions. When the $(CTG)_n$ template is on the leading strand, expansions predominate, whereas $(CTG)_n$ oriented the other way cause mostly deletions.

2.5.2 | *Trans*-acting factors

Not only characteristics of the repeat itself or the direct vicinity thereof play a role, but also other factors such as DNA metabolism or function of other genes have been described to affect trinucleotide repeat stability.

Parental effects

Different trinucleotide repeat disorders have different patterns of transmission. A paternal bias for transmission of expansions is seen in HD, while expansions into the fragile X-range occur solely with alleles of maternal origin [204]. In DM patients, a very specific transmission pattern is observed: the change from asymptomatic (PM: 38-50 CTGs) to clear DM1 often happens upon paternal transmission of an allele that expanded into the disease-causing range, whereas children with the severe congenital form are almost exclusively born if a mutated allele is transmitted by the mother [156]. Paternal transmissions are usually smaller (not exceeding $(CTG)_{1000}$), since size of parental $(CTG)_n$ is inversely correlated with the intergenerational expansion only upon paternal transmission [122]. Despite the differences in the different repeat disorders, it is clear that there is a parental gender effect. Not all mouse models mimic the clear parental gender effects. Nevertheless, the observation that the intergenerational mutability of some repeats depends on the sex of the parent, suggests that sex-specific factors might induce mosaicism in the male and female germ line. This could be due to sex-specific differences in DNA metabolism or other factors, for instance the number of rounds of replication, during gametogenesis (summarised in [141]). In females, it has been postulated that instability occurs when oocytes are arrested in meiosis I, after meiotic DNA replication, which gives a large time window of opportunity for DNA damage to occur [130]. Cells at this stage have only undergone a limited number of cell divisions, which would point to a role for DNA repair in quiescent cells [129,205].

When considering the number of cell cycles, it is not surprising that age of the transmitting parent also appears to have an effect on instability. Magnitude of $(CAG)_n$ expansions has been shown to correlate with the age of the transmitting father [132]. Expansions can accumulate in sperm over time [137,136]. A transgenic mouse model for DM expressing $(CTG)_{>300}$ showed a higher number of expansions with increasing age of the transmitting father, but not the transmitting mother [137].

DNA replication and repair

Despite the abundant presence of repetitive DNA in the genome, no general microsatellite instability occurs in the diseases described. Thus, repeat instability is limited to the disease locus. It was therefore

concluded that expansions are not resulting from aberrant *trans*-acting factors involved in DNA replication, repair and recombination. However, evidence is currently accumulating that factors involved in DNA repair and replication are in fact influencing trinucleotide instability. Some of this evidence will be discussed.

Cell culture studies using foetal DM1 fibroblasts showed expansion of the $(CTG)_{216}$ allele only in proliferating cells but not in quiescent cells. Addition of agents that interfere with DNA metabolism revealed that $(CTG)_n$ expansion was seen specifically at the disease locus when lagging strand synthesis alone, or simultaneously with leading strand synthesis, was blocked. This suggests that disturbed replication fork dynamics might play a role in $(CTG)_n$ instability [207]. Also observations that DNA polymerases *in vitro* [208], and replication forks in *E.coli* [209] and yeast [210] have difficulty processing trinucleotide repeats that have a high G/C content, pointed towards a role for these events in the origin of instability. Flap endonuclease 1 (FEN1) has endonuclease activities acting specifically on 5' flaps that are created by strand displacement during Okazaki fragment maturation [211]. Studies in yeast [212-214] and mice [215] showed that Fen1 deficiency led to increased (intergenerational) instability of $(CAG)_n/(CTG)_n$. However, another study in a mouse model for DM1 did not show an effect of *Fen1* heterozygosity on $(CTG)_n$ instability [216]. Later these different outcomes could be explained by a repeat length dependent effect of Fen1 on instability, such that long $(CAG)_n/(CTG)_n$ tracts need more Fen1 protein to ensure stability of the repeat [217].

Mosaicism has been seen in both proliferating and quiescent tissues, suggesting a replication-independent mechanism [121,123,149,150,152,206,218,219]. Thus, if intergenerational instability is the result of the same mechanism as somatic instability, it cannot solely depend on replication-dependent mechanisms.

DNA repair pathways ought to maintain genome integrity and stability, thus they are likely candidates for modifying trinucleotide repeat instability. Impaired mismatch repair (MMR) activity is highly correlated to genetic instability of simple DNA repeat sequences in hereditary nonpolyposis colorectal cancer (HNPCC). This suggests that aberrant or absent components of MMR systems may cause repeats to become unstable [220]. In MMR, heterodimers are formed of MSH2 with either MSH3 or MSH6, which can then recognise single-base mismatches or short unpaired regions, such as small insertions and deletions. This results in recruitment of other factors involved in DNA repair [221]. Studies in *Msh2*-deficient yeast strains did not show altered $(CTG)_n$ expansion rates, as compared with *Msh2*-wt strains. Neither was an effect on instability seen for the recombination gene *Rad52* [180].

In an attempt to gain more insight into the possible role of DNA repair in repeat instability *in vivo*, transgenic mice expressing $(CTG)_{>300}$ were crossed with mice knock out for *Msh2*. Lack of Msh2 changed the direction of instability, with only contractions taking place, as opposed to mostly expansions in a wt *Msh2* background. This was true for both intergenerational and somatic instability [222]. Mouse models for HD have also shown increased contraction frequencies in an *Msh2*-deficient background [223-226]. *Msh3* deficiency also decreased the expansion frequency in a knock-in DM1 mouse model, which carries $(CTG)_{80}$ in the 3' end of the *Dmpk* gene [227]. Another study confirmed the necessity of Msh3 in the expansion of $(CTG)_n$. Heterozygous animals revealed that Msh3 is rate-limiting in the process, since this decreased the number of expansions. Msh6, a partner of Msh2 in MMR, did

not play a key role [228]. Msh3 expression might provide a permissive environment for $(CAG)_n$-instability, since in a mouse model for HD, Msh3 was almost exclusively expressed in neuronal cells, which showed clear somatic instability over time, as opposed to non-neuronal cells [219]. It is unclear whether the same mechanism is responsible for expansions in *Msh2*-wt and contractions in *Msh2*-deficient animals, whereby the presence or absence of Msh2 determines the direction of instability. Another possibility is that, in the absence of Msh2, a distinct process takes place which leads to contractions [136].

Since $(CTG)_n$ have been shown to induce length-dependent breakage in yeast, the influence of two genes involved in double strand break repair by homologous recombination (*Rad52* and *Rad54*) were also investigated. These did not show a substantial effect on $(CTG)_n$ instability [222]. The influence of genes involved in replication and repair on instability of the $(CGG)_n$ repeat in the *FMR1* gene has also been investigated in a mouse model for FXTAS. Neither a helicase (*Wrn)*, which facilitates replication of the repeat, nor a protein (p53) with a function in DNA damage surveillance and repair affected instability [229]. The same lab later investigated the effect of ATR deficiency on $(CGG)_n$ instability in their expanded $(CGG)_n$ mouse model [230]. ATR is the ataxia-telangiectasia and rad3-related kinase, which responds to stalled replication forks and bulky DNA adducts [231]. Parental *ATR* heterozygosity led to an increased frequency of expansion of alleles only of maternal origin, in offspring wt for *ATR*. This suggests that expansion can occur prior to fertilisation of the oocyte. However, a higher prevalence of repeat expansion was seen in offspring heterozygous for ATR, in comparison with offspring wt of *ATR*, for alleles derived from mothers heterozygous for *ATR*. The most likely origin of the additional expansions in *ATR* heterozygous offspring is in the haploid oocyte [230]. The authors propose that two mechanisms exist that might explain intergenerational instability: one that causes a higher expansion frequency in males, which is not so sensitive to ATR haploinsufficiency and a second that is affected by ATR haploinsufficiency, which occurs mostly in females. The authors point out, however, that ATR insufficiency might not be a prerequisite in humans, but that it might allow events to become visible in the lifespan of mice, which would have years to accumulate in humans [230].

Mutant screens have been conducted in yeast to search for genes that have a modulating effect on $(CAG)_n$ instability. This revealed Mrc1 as a mediator of instability. Mrc1, and associated proteins, have a role in preventing development of too long stretches of single stranded DNA (ssDNA), which are likely to form aberrant secondary structures, which impair replication. Mrc1 also has a function as a checkpoint protein after secondary structure formation, aiming at prevention of establishing the mutagenic event. Thus, at least two Mrc1-dependent mechanisms seem to act in an effort to reduce $(CAG)_n$ instability [232].

Taken together, it is clear that disturbed replication and repair processes increase the frequency of repeat instability events. It is noteworthy that these two processes can be difficult to distinguish, as replication can be part of normal mitosis, or repair-dependent. It remains to be elucidated whether expansion in human disease is the result of normal or repair-induced replication [181].

Combined pathogenic pathways

A *Drosophila* model for SCA3, expressing a polyQ-stretch, showed little repeat instability. However, when the transgene was expressed in the germ cells, substantial instability was seen in the progeny. This model was also used to investigate the influence of DNA repair pathways on $(CAG)_n$ instability.

The Mus201 protein is a fly orthologue of human Rad2/XP6, which is crucial for nucleotide excision repair (NER) and transcription-coupled repair function. In a *Mus201* deficient background, flies showed a dramatic reduction in instability. Furthermore, as cyclic adenosine 3'5'-monophosphate (cAMP) response element binding protein (CREB)-binding protein (CBP) is found in polyQ-associated inclusions, which reduces soluble CBP levels, the effect of lower CBP levels on repeat instability was investigated. Toxic polyQ-containing protein might increase CBP-degradation, further decreasing soluble levels. It may, however, also inhibit CBP/p300 histone acetyltransferase (HAT) activity. Increased instability was seen when CBP levels were decreased, which was accompanied with increased polyQ toxicity. Instability levels could be reduced with CBP overexpression [233]. Treating fly or mouse models of polyQ disease with histone deacetylases (HDAC) inhibitors normally protects against the toxic effects of the mutant protein [234]. Treating flies expressing the polyQ protein in germ cells with HDAC inhibitors revealed that normalisation of acetylated histones also reduced repeat instability, most likely (at least partially) due to compensation for decreased dCBP and/or HAT activity. Thus, these studies suggest that repeat instability is not only dependent on what happens at the DNA level, but it might be affected by downstream effects of polyQ toxicity [233]. Naturally, similar processes with several factors operating at different levels, all contributing to repeat instability could turn out to play a role in other trinucleotide repeat disorders.

The studies in flies [233] also pointed toward a role for transcription of repeats in the origin of instability. These results together with studies in human cells expressing $(CAG)_n$, suggest that transcription of repeats might promote formation of secondary structures in the repeat tracts, which then attract various DNA repair systems [233,235,236]. This is in line with the stabilising effect of CpG methylation that is known for the $(CGG)_n$ in the FRAXA locus, since the methylation causes transcriptional silencing [104,237-239]. Also other epigenetic changes may occur in the vicinity of long $(CTG)_n$, which can trigger formation and spreading of heterochromatin, affecting transcription. Thus, the effect of epigenetic changes on $(CAG)_n$ was investigated in a mouse model for SCA1. Heterozygosity for the maintenance DNA methyltransferase Dnmt1, which preserves the patterns of CpG methylation, indeed promoted intergenerational $(CAG)_n$ expansion [240].

A transgenic mouse model expressing a construct containing an origin of replication of SV40, followed by a $(CGG)_{26}$, showed large intergenerational expansions, into the FRAXA FM range [241]. SV40 origin of replication is known to exclude nucleosomes, which is likely the cause of the large instability seen in these mice, since expanded $(CGG)_n$ have also been shown to exclude nucleosomes in *in vitro* experiments. This also hints at chromatin characteristics having an influence on repeat instability [242].

Concluding remarks | 2.6

Since this chapter has dealt solely with the negative consequences of the presence of expanded trinucleotide repeats, it is interesting to wonder why these repetitive elements have evolved, and even more striking, been maintained. Transcriptome-wide database analysis has identified several hundreds of transcripts containing triplet repeat tracts (> 6 repeat units). The vast majority (67%) of the repeats are located in open reading frames, while 24% are situated in the 5'UTR and 9% in the 3'UTR. However, when considering the median length of these regions in human mRNAs, repeats are overrepresented

in 5'UTR and underrepresented in 3'UTR. 5'UTRs often play a role in regulation of translation, and 3'UTR are involved in mRNA stability, transport and cellular localisation. The preferential colocalisation of the repeats within the mRNA might hint at their biological role. Of the repeat-containing mRNAs with a known biological function, indeed a large amount is involved in intracellular and extracellular signalling or transcription and translation. The function of transcripts might be compromised due to the presence of an expanded repeat [243]. This study only mainly found and analysed repeats of up to twenty triplets. It is conceivable that longer repeats impair the normal function to a greater extent.

This does however not explain why these repeats have been maintained over the course of evolution. Concerning polyQ-tracts, it is noteworthy that glutamine is encoded for by both CAG and CAA. Therefore, if there were some selectional advantage for a polyQ-tract, it would not need a pure CAG tract, as is generally seen. Evidence exists that both $(CTG)_n$ and $(CAG)_n$repeats form a functional component of an insulator element, thereby having a role in the regulation of gene expression. Replacement of repeats with non-repeat sequence was shown to decrease insulator activity. $(CTG)_n$ and $(CAG)_n$ repeats have been found to act as strong nucleosome positioning elements *in vitro* [244-247] as well as $(CGG)_n$ [242].

Thus, it seems that trinucleotide repeats do not only sort negative effects. However, negative effects might prevail once they expand beyond a certain threshold. Future studies might shed light on beneficial characteristics, which will explain why they have been maintained over the course of evolution. Naturally, future studies should also focus on how instability, as well as the negative effects of expansion, can be prevented.

References |

1. Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS *et al.* 1991. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67:1047-1058.
2. Oberlé I, Rousseau F, Heitz D, Kretz C, Devys D *et al.* 1991. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. Science 252:1097-1102.
3. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP *et al.* 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905-914.
4. Yu S, Pritchard M, Kremer E, Lynch M, Nancarrow J *et al.* 1991. Fragile X genotype characterized by an unstable region of DNA. Science 252:1179-1181.
5. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. 1991. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352:77-79.
6. Richards RI, Sutherland GR. 1992. Heritable unstable DNA sequences. Nature Genet 1:7-9.
7. Sutherland GR, Haan EA, Kremer E, Lynch M, Pritchard M *et al.* 1991. Hereditary unstable DNA: a new explanation for some old genetic questions? Lancet 338:289-292.
8. Albrecht A, Mundlos S. 2005. The other trinucleotide repeat: polyalanine expansion disorders. Curr Opin Genet Dev 15:285-293.
9. Zhong N, Ju WN, Pietrofesa J, Wang DW, Dobkin C *et al.* 1996. Fragile X "gray zone" alleles: AGG patterns, expansion risks, and associated haplotypes. Am J Med Genet 64:261-265.
10. Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R *et al.* 2001. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 57:127-310.
11. Jacquemont S, Hagerman RJ, Leehey M, Grigsby J, Zhang L *et al.* 2003. Fragile X Premutation Tremor/Ataxia Syndrome: Molecular, Clinical, and Neuroimaging Correlates. Am J Hum Genet 72:869-878.
12. Jacquemont S, Hagerman RJ, Leehey MA, Hall DA, Levine RA *et al.* 2004. Penetrance of the fragile x-associated tremor/ataxia syndrome in a premutation carrier population. JAMA 291:460-469.
13. Sherman SL. 2000. Premature ovarian failure in the fragile X syndrome. Am J Med Genet 97:189-194.
14. Gatchel JR, Zoghbi HY. 2005. Diseases of unstable repeat expansion: mechanisms and common principles. Nat Rev Genet 6:743-755.
15. Lutz RE. 2007. Trinucleotide repeat disorders. Semin Pediatr Neurol 14:26-33.
16. Mirkin SM. 2007. Expandable DNA repeats and human disease. Nature 447:932-940.
17. Orr HT, Zoghbi HY. 2007. Trinucleotide repeat disorders. Annu Rev Neurosci 30:575-621.
18. Sutcliffe JS, Nelson DL, Zhang F, Pieretti M, Caskey CT *et al.* 1992. DNA methylation represses FMR-1 transcription in fragile X syndrome. Hum Mol Genet 1:397-400.
19. Verheij C, Bakker CE, de Graaff E, Keulemans J, Willemsen R *et al.* 1993. Characterization and localization of the FMR-1 gene product associated with fragile X syndrome. Nature 363:722-724.
20. Devys D, Lutz Y, Rouyer N, Bellocq JP, Mandel JL. 1993. The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat Genet 4:335-340.
21. Willemsen R, Oostra BA, Bassell GJ, Dictenberg J. 2004. The fragile X syndrome: From molecular genetics to neurobiology. Ment Retard Dev Disabil Res Rev 10:60-67.
22. Mulley JC, Yu S, Loesch DZ, Hay DA, Donnelly A *et al.* 1995. FRAXE and mental retardation. J Med Genet 32:162-169.
23. Knight SJ, Flannery AV, Hirst MC, Campbell L, Christodoulou Z *et al.* 1993. Trinucleotide repeat amplification and hypermethylation of a CpG island in FRAXE mental retardation. Cell 74:127-134.
24. Hamel BC, Smits AP, de Graaff E, Smeets DF, Schoute F *et al.* 1994. Segregation of FRAXE in a large family: clinical, psychometric, cytogenetic, and molecular data. Am J Hum Genet 55:923-31.
25. Gecz J, Oostra BA, Hockey A, Carbonell P, Turner G *et al.* 1997. FMR2 expression in families with FRAXE mental retardation. Hum Mol Genet 6:435-441.
26. Gecz J, Bielby S, Sutherland GR, Mulley JC. 1997. Gene structure and subcellular localization of FMR2, a member of a new family of putative transcription activators. Genomics 44:201-213.
27. Campuzano V, Montermini L, Molto MD, Pianese L, Cossee M *et al.* 1996. Friedreich's ataxia: Autosomal recessive disease caused by an intronic GAA triplet repeat expansion. Science 271:1423-1427.
28. Cossee M, Schmitt M, Campuzano V, Reutenauer L, Moutou C *et al.* 1997. Evolution of the Friedreich's ataxia trinucleotide repeat expansion: founder effect and premutations. Proc Natl Acad Sci U S A 94:7452-457.

29. Durr A, Cossee M, Agid Y, Campuzano V, Mignard C *et al.* 1996. Clinical and genetic abnormalities in patients with Friedreich's ataxia. N Engl J Med 335:1169-1175.
30. De Biase I, Rasmussen A, Bidichandani SI. 2006. Evolution and instability of the GAA triplet-repeat sequence in Friedreich's Ataxia. In: Wells RD, Ashizawa T. ed. Genetic instabilities and neurological diseases; p 305-319.
31. Campuzano V, Montermini L, Lutz Y, Cova L, Hindelang C *et al.* 1997. Frataxin is reduced in Friedreich ataxia patients and is associated with mitochondrial membranes. Hum Mol Genet 6:1771-1780.
32. Karthikeyan G, Santos JH, Graziewicz MA, Copeland WC, Isaya G *et al.* 2003. Reduction in frataxin causes progressive accumulation of mitochondrial damage. Hum Mol Genet 12:3331-3342.
33. Yoon T, Cowan JA. 2003. Iron-sulfur cluster biosynthesis. Characterization of frataxin as an iron donor for assembly of [2Fe-2S] clusters in ISU-type proteins. J Am Chem Soc 125:6078-6084.
34. Lesuisse E, Santos R, Matzanke BF, Knight SA, Camadro JM *et al.* 2003. Iron use for haeme synthesis is under control of the yeast frataxin homologue (Yfh1). Hum Mol Genet 12:879-889.
35. Machuca-Tzili L, Brook D, Hilton-Jones D. 2005. Clinical and molecular aspects of the myotonic dystrophies: a review. Muscle Nerve 32:1-18.
36. Ashizawa T, Harper P. 2006. Myotonic dystrophies: an overview. In: Wells R, Ashizawa T. ed. Genetic instabilities and neurological diseases: Academic Press; p 21-36.
37. Cho DH, Tapscott SJ. 2007. Myotonic dystrophy: emerging mechanisms for DM1 and DM2. Biochim Biophys Acta 1772:195-204.
38. Davis BM, McCurrach ME, Taneja KL, Singer RH, Housman DE. 1997. Expansion of a CUG trinucleotide repeat in the 3' untranslated region of myotonic dystrophy protein kinase transcripts results in nuclear retention of transcripts. Proc Natl Acad Sci U S A 94:7388-7393.
39. Furling D, Lemieux D, Taneja K, Puymirat J. 2001. Decreased levels of myotonic dystrophy protein kinase (DMPK) and delayed differentiation in human myotonic dystrophy myoblasts. Neuromuscul Disord 11:728-735.
40. Krahe R, Ashizawa T, Abbruzzese C, Roeder E, Carango P *et al.* 1995. Effect of myotonic dystrophy trinucleotide repeat expansion on DMPK transcription and processing. Genomics 28:1-14.
41. Jansen G, Groenen PJTA, Bachner D, Jap PHK, Coerwinkel M, *et al.* 1996. Abnormal myotonic dystrophy protein kinase levels produce only mild myopathy in mice. Nature Genet 13:316-324.
42. Reddy S, Smith DBJ, Rich MM, Leferovich JM, Reilly P *et al.* 1996. Mice lacking the myotonic dystrophy protein kinase develop a late onset progressive myopathy. Nature Genet 13:325-335.
43. Mounsey JP, Mistry DJ, Ai CW, Reddy S, Moorman JR. 2000. Skeletal muscle sodium channel gating in mice deficient in myotonic dystrophy protein kinase. Hum Mol Genet 9:2313-2320.
44. Reddy S, Mistry DJ, Wang QC, Geddis LM, Kutchai HC *et al.* 2002. Effects of age and gene dose on skeletal muscle sodium channel gating in mice deficient in myotonic dystrophy protein kinase. Muscle Nerve 25:850-857.
45. Michalowski S, Miller JW, Urbinati CR, Paliouras M, Swanson MS *et al.* 1999. Visualization of double-stranded RNAs from the myotonic dystrophy protein kinase gene and interactions with CUG-binding protein. Nucleic Acids Res 27:3534-3542.
46. Napierala M, Krzyzosiak WJ. 1997. CUG repeats present in myotonin kinase RNA form metastable "slippery" hairpins. J Biol Chem 272:31079-1085.
47. Fardaei M, Larkin K, Brook JD, Hamshere MG. 2001. In vivo co-localisation of MBNL protein with DMPK expanded-repeat transcripts. Nucleic Acids Res 29:2766-2771.
48. Miller JW, Urbinati CR, Teng-Umnuay P, Stenberg MG, Byrne BJ *et al.* 2000. Recruitment of human muscleblind proteins to (CUG)(n) expansions associated with myotonic dystrophy. Embo J 19:4439-4448.
49. Timchenko LT, Miller JW, Timchenko NA, DeVore DR, Datar KV *et al.* 1996. Identification of a (CUG)n triplet repeat RNA-binding protein and its expression in myotonic dystrophy. Nucleic Acids Res 24:4407-4414.
50. Timchenko LT, Timchenko NA, Caskey CT, Robert R. 1996. Novel proteins with binding specificity for DNA CTG repeats and RNA CUG repeats: implications for myotonic dystrophy. Hum Mol Genet 5:115-121.
51. Ho TH, Bundman D, Armstrong DL, Cooper TA. 2005. Transgenic mice expressing CUG-BP1 reproduce splicing mis-regulation observed in myotonic dystrophy. Hum Mol Genet 14:1539-1547.
52. Ho TH, Charlet BN, Poulos MG, Singh G, Swanson MS *et al.* 2004. Muscleblind proteins regulate alternative splicing. Embo J 23:3103-3112.
53. Kanadia RN, Johnstone KA, Mankodi A, Lungu C, Thornton CA *et al.* 2003. A muscleblind knockout model for myotonic dystrophy. Science 302:1978-1980.
54. Ladd AN, Stenberg MG, Swanson MS, Cooper TA. 2005. Dynamic balance between activation and repression regulates pre-mRNA alternative splicing during heart development. Dev Dyn 233:783-793.

55. Pascual M, Vicente M, Monferrer L, Artero R. 2006. The Muscleblind family of proteins: an emerging class of regulators of developmentally programmed alternative splicing. Differentiation 74:65-80.
56. de Haro M, Al-Ramahi I, De Gouyon B, Ukani L, Rosa A *et al.* 2006. MBNL1 and CUGBP1 modify expanded CUG-induced toxicity in a Drosophila model of myotonic dystrophy type 1. Hum Mol Genet 15:2138-2145.
57. Jacquemont S, Leehey MA, Hagerman RJ, Beckett LA, Hagerman PJ. 2006. Size bias of fragile X premutation alleles in late-onset movement disorders. J Med Genet 43:804-809.
58. Berry-Kravis E, Abrams L, Coffey SM, Hall DA, Greco C *et al.* 2007. Fragile X-associated tremor/ataxia syndrome: Clinical features, genetics, and testing guidelines. Mov Disord 14:2014-2030.
59. Greco CM, Hagerman RJ, Tassone F, Chudley AE, Del Bigio MR *et al.* 2002. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. Brain 125:1760-1771.
60. Arocena DG, Iwahashi CK, Won N, Beilina A, Ludwig AL *et al.* 2005. Induction of inclusion formation and disruption of lamin A/C structure by premutation CGG-repeat RNA in human cultured neural cells. Hum Mol Genet 14:3661-3671.
61. Tassone F, Iwahashi C, Hagerman PJ. 2004. FMR1 RNA withinthe intranuclear inclusions of fragile X-associated Tremor/Ataxia syndrome (FXTAS). RNA biology 1:103-105.
62. Hagerman PJ, Hagerman RJ. 2004. The Fragile-X Premutation: A Maturing Perspective. Am J Hum Genet 74:805-816.
63. Iwahashi CK, Yasui DH, An HJ, Greco CM, Tassone F *et al.* 2006. Protein composition of the intranuclear inclusions of FXTAS. Brain 129:256-271.
64. Handa V, Goldwater D, Stiles D, Cam M, Poy G *et al.* 2005. Long CGG-repeat tracts are toxic to human cells: Implications for carriers of Fragile X premutation alleles. FEBS Lett 579:2702-2708.
65. Jin P, Duan R, Qurashi A, Qin Y, Tian D *et al.* 2007. Pur alpha Binds to rCGG Repeats and Modulates Repeat-Mediated Neurodegeneration in a Drosophila Model of Fragile X Tremor/Ataxia Syndrome. Neuron 55:556-564.
66. Sofola OA, Jin P, Qin Y, Duan R, Liu H *et al.* 2007. RNA-Binding Proteins hnRNP A2/B1 and CUGBP1 Suppress Fragile X CGG Premutation Repeat-Induced Neurodegeneration in a Drosophila Model of FXTAS. Neuron 55:565-571.
67. Jin P, Zarnescu DC, Zhang F, Pearson CE, Lucchesi JC *et al.* 2003. RNA-Mediated Neurodegeneration Caused by the Fragile X Premutation rCGG Repeats in Drosophila. Neuron 39:739-747.
68. Oostra BA, Willemsen R. 2003. A fragile balance: FMR1 expression levels. Hum Mol Genet 12 Suppl 2:R249-257.
69. Ikeda Y, Dick KA, Day JW, Ranum LPW. 2006. Molecular genetics of spinocerebellar ataxia type 8. In: Wells RD, Ashizawa T. ed. Genetic instabilities and neurological disease: Academic Press; p 417-431.
70. Rasmussen A, Matsuura T, Ruano L, Yescas P, Ochoa A *et al.* 2001. Clinical and genetic analysis of four Mexican families with spinocerebellar ataxia type 10. Ann Neurol 50:234-239.
71. Holmes SE, O'Hearn E, Cortez-Apreza N, Hwang HS, Ross CA *et al.* 2006. Spinocerebellar ataxia type 12. In: Wells RD, Ashizawa T. ed. Genetic instabilities and neurological diseases: Academic Press; p 461-473.
72. Guyenet SJ, La Spada AR. 2008. Triplet repeat diseases. In: Meyers RA. ed. Neurobiology. From molecular basis to disease. Weinheim: Wiley-VCH Verlag GmbH & Co; p 589-647.
73. Poletti A. 2004. The polyglutamine tract of androgen receptor: from functions to dysfunctions in motor neurons. Front Neuroendocrinol 25:1-26.
74. Chen CJ, Fischbeck KH. 2006. Clinical features and molecular biology of Kennedy's disease. In: Wells R, Ashizawa T. ed. Genetic instabilities and neurological disease: Academic Press; p 211-220.
75. Kopito RR. 2000. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol 10:524-530.
76. Brais B, Bouchard JP, Xie YG, Rochefort DL, Chretien N *et al.* 1998. Short GCG expansions in the PABP2 gene cause oculopharyngeal muscular dystrophy. Nat Genet 18:164-167.
77. Margolis RL, Holmes SE, Rudnicki D, O'Hearn E, Ross CA *et al.* 2006. Huntington's disease-like 2. In: Wells R, Ashizawa T. ed. Genetic instabilities and neurological diseases: Academic Press; p 261-273.
78. Andrew SE, Goldberg YP, Hayden MR. 1997. Rethinking genotype and phenotype correlations in polyglutamine expansion disorders. Hum Mol Genet 6:2005-2010.
79. Nance MA. 1997. Clinical aspects of CAG repeat diseases. Brain Pathol 7:881-900.
80. Duyao M, Ambrose C, Myers R, Novelletto A, Persichetti F *et al.* 1993. Trinucleotide repeat length instability and age at onset in Huntington's disease. Nature Genet 4:387-392.
81. DiFiglia M, Sapp E, Chase KO, Davies SW, Bates GP *et al.* 1997. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277:1990-1993.
82. Davies SW, Turmaine M, Cozens BA, DiFiglia M, Sharp AH *et al.* 1997. Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90:537-548.

83. Paulson HL, Perez MK, Trottier Y, Trojanowski JQ, Subramony SH *et al.* 1997. Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. Neuron 19:333-344.
84. Skinner PJ, Koshy BT, Cummings CJ, Klement IA, Helin K *et al.* 1997. Ataxin-1 with an expanded glutamine tract alters nuclear matrix-associated structures. Nature 389:971-974.
85. Wetzel R. 2006. Chemical and physical properties of polyglutamine repeat sequences. In: Wells R, Ashizawa T. ed. Genetic instabilities and neurological disease: Academic Press; p 517-534.
86. Scherzinger E, Sittler A, Schweiger K, Heiser V, Lurz R *et al.* 1999. Self-assembly of polyglutamine-containing huntingtin fragments into amyloid-like fibrils: implications for Huntington's disease pathology. Proc Natl Acad Sci U S A 96:4604-4609.
87. Ross CA, Wood JD, Peters MF, Schilling G, Nucifora FC *et al.* 2001. Polyglutamine pathogenesis, potential role of protein interactions, proteolytic processing and nuclear localization. In: Harper P, Perutz M. ed. Glutamine repeats and neurodegenerative diseases: molecular aspects. New York Oxford University Press, Inc, ; p 93-112.
88. Arbizu T, Santamaria J, Gomez JM, Quilez A, Serra JP. 1983. A family with adult spinal and bulbar muscular atrophy, X-linked inheritance and associated testicular failure. J Neurol Sci 59:371-382.
89. MacLean HE, Warne GL, Zajac JD. 1997. Localization of functional domains in the androgen receptor. J Steroid Biochem Mol Biol 62:233-242.
90. Brooks BP, Paulson HL, Merry DE, Salazar-Grueso EF, Brinkmann AO *et al.* 1997. Characterization of an expanded glutamine repeat androgen receptor in a neuronal cell culture system. Neurobiol Dis 3:313-323.
91. Lieberman AP, Harmison G, Strand AD, Olson JM, Fischbeck KH. 2002. Altered transcriptional regulation in cells expressing the expanded polyglutamine androgen receptor. Hum Mol Genet 11:1967-1976.
92. Mhatre AN, Trifiro MA, Kaufman M, Kazemi-Esfarjani P, Figlewicz D *et al.* 1993. Reduced transcriptional regulatory competence of the androgen receptor in X-linked spinal and bulbar muscular atrophy. Nat Genet 5:184-188.
93. Harper P. 2001. Huntington's disease: a clinical, genetic and molecular model for polyglutamine repeat disorders. In: Harper P, Perutz M. ed. Glutamine repeats and neurodegenerative diseases: molecular aspects. New York: Oxford University Press Inc.; p 1-9.
94. Group HsDCR. 1993. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. . Cell 72:971-983.
95. Li SH, Li XJ. 2004. Huntingtin-protein interactions and the pathogenesis of Huntington's disease. Trends Genet 20:146-154.
96. Bett JS, Bates GP, Hockly E. 2006. Molecular pathogenesis and therapeutic targets in Huntington's disease. In: Wells R, Ashizawa T. ed. Genetic instabilities and neurological disease: Academic Press; p 223-249.
97. Arrasate M, Mitra S, Schweitzer ES, Segal MR, Finkbeiner S. 2004. Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature 431:805-810.
98. Saudou F, Finkbeiner S, Devys D, Greenberg ME. 1998. Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell 95:55-66.
99. Li H, Li SH, Cheng AL, Mangiarini L, Bates GP *et al.* 1999. Ultrastructural localization and progressive formation of neuropil aggregates in Huntington's disease transgenic mice. Hum Mol Genet 8:1227-1236.
100. Yamamoto A, Lucas JJ, Hen R. 2000. Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. Cell 101:57-66.
101. Mirkin SM, Smirnova EV. 2002. Positioned to expand. Nat Genet 31:5-6.
102. Tome FM, Fardeau M. 1980. Nuclear inclusions in oculopharyngeal dystrophy. Acta Neuropathol 49:85-87.
103. Holmes SE, O'Hearn E, Rosenblatt A, Callahan C, Hwang HS *et al.* 2001. A repeat expansion in the gene encoding junctophilin-3 is associated with Huntington disease-like 2. Nat Genet 29:377-378.
104. Reyniers E, Vits L, De Boulle K, Van Roy B, Van Velzen D *et al.* 1993. The full mutation in the FMR-1 gene of male fragile X patients is absent in their sperm. Nature Genet 4:143-146.
105. Bakker CE, Verheij C, Willemsen R, Vanderhelm R, Oerlemans F *et al.* 1994. Fmr1 knockout mice: A model to study fragile X mental retardation. Cell 78:23-33.
106. Meijer H, De Graaff E, Merckx DML, Jongbloed RJE, De Die-Smulders CEM *et al.* 1994. 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 3:615-620.
107. Rousseau F, Heitz D, Oberlé I, Mandel JL. 1991. Selection in blood cells from female carriers of the fragile X syndrome: inverse correlation between age and proportion of active X chromosomes carrying the full mutation. J Med Genet 28:830-836.
108. Malter HE, Iber JC, Willemsen R, De Graaff E, Tarleton JC *et al.* 1997. Characterization of the full fragile X syndrome mutation in fetal gametes. Nature Genet 15:165-169.
109. Reik W, Santos F, Mitsuya K, Morgan H, Dean W. 2003. Epigenetic asymmetry in the mammalian zygote and early embryo: relationship to lineage commitment? Philos Trans R Soc Lond B Biol Sci 358:1403-1409.

110. Nichol Edamura K, Pearson CE. 2005. DNA Methylation and Replication: Implications for the "Deletion Hotspot" Region of FMR1. Hum Genet 118:301-304.
111. Hansen RS, Canfield TK, Lamb MM, Gartler SM, Laird CD. 1993. Association of fragile X syndrome with delayed replication of the FMR1 gene. Cell 73:1403-1409.
112. Samadashwily GM, Dayn A, Mirkin SM. 1993. Suicidal nucleotide sequences for DNA polymerization. Embo J 12:4975-4983.
113. Ashley AE, Sherman SL. 1995. Population dynamics of a meiotic/mitotic expansion model for the fragile X syndrome. Am J Hum Genet 57:1414-1425.
114. Devys D, Biancalana V, Rousseau F, Boue J, Mandel JL *et al.* 1992. 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 43:208-216.
115. Wöhrle D, Hennig I, Vogel W, Steinbach P. 1993. Mitotic stability of fragile X mutations in differentiated cells indicates early post-conceptional trinucleotide repeat expansion. Nature Genet 4:140-142.
116. Trottier Y, Devys D, Mandel JL. 1993. An expanding story. Curr Biol 3:783-86.
117. Moutou C, Vincent MC, Biancalana V, Mandel JL. 1997. Transition from premutation to full mutation in fragile X syndrome is likely to be prezygotic. Hum Mol Genet 6:971-979.
118. Bontekoe CJ, Bakker CE, Nieuwenhuizen IM, van Der Linde H, Lans H *et al.* 2001. Instability of a (CGG)(98) repeat in the Fmr1 promoter. Hum Mol Genet 10:1693-1699.
119. Entezam A, Biacsi R, Orrison B, Saha T, Hoffman GE *et al.* 2007. Regional FMRP deficits and large repeat expansions into the full mutation range in a new Fragile X premutation mouse model. Gene 395:125-134.
120. Brouwer JR, Mientjes EJ, Bakker CE, Nieuwenhuizen IM, Severijnen LA *et al.* 2007. Elevated Fmr1 mRNA levels and reduced protein expression in a mouse model with an unmethylated Fragile X full mutation. Exp Cell Res 313:244-253.
121. Jansen G, Willems P, Coerwinkel M, Nillesen W, Smeets H *et al.* 1993. Gonosomal mosaicism in myotonic dystrophy patients: involvement of mitotic events in (CTG)n repeat variation and selection against extreme expansion in sperm. Am J Hum Genet 54:575-585.
122. Hecht BK, Donnelly A, Gedeon AK, Byard RW, Haan EA *et al.* 1993. Direct molecular diagnosis of myotonic dystrophy. Clin Genet 43:276-285.
123. Lavedan C, Hofmann-Radvanyi H, Shelbourne P, Rabes JP, Duros C *et al.* 1993. Myotonic dystrophy: size- and sex-dependent dynamics of CTG meiotic instability, and somatic mosaicism. Am J Hum Genet 52:875-883.
124. Martorell L, Johnson K, Boucher CA, Baiget M. 1997. Somatic instability of the myotonic dystrophy (CTG)n repeat during human fetal development. Hum Mol Genet 6:877-880.
125. Wöhrle D, Kennerknecht I, Wolf M, Enders H, Schwemmle S *et al.* 1995. Heterogeneity of DM kinase repeat expansion in different fetal tissues and further expansion during cell proliferation in vitro: evidence for a causal involvement of methyl-directed DNA mismatch repair in triplet repeat stability. Hum Mol Genet 4:1147-1153.
126. Dubel JR, Armstrong RM, Perryman MB, Epstein HF, Ashizawa T. 1992. Phenotypic expression of the myotonic dystrophy gene in monozygotic twins. Neurology 42:1815-1817.
127. Lopez de Munain A, Cobo AM, Huguet E, Marti Masso JF, Johnson K *et al.* 1994. CTG trinucleotide repeat variability in identical twins with myotonic dystrophy. Ann Neurol 35:374-375.
128. Phillips DI. 1993. Twin studies in medical research: can they tell us whether diseases are genetically determined? Lancet 341:1008-1009.
129. Dean NL, Tan SL, Ao A. 2006. Instability in the transmission of the myotonic dystrophy CTG repeat in human oocytes and preimplantation embryos. Fertil Steril 86:98-105.
130. De Temmerman N, Sermon K, Seneca S, De Rycke M, Hilven P *et al.* 2004. Intergenerational instability of the expanded CTG repeat in the DMPK gene: studies in human gametes and preimplantation embryos. Am J Hum Genet 75:325-329.
131. Kaytor MD, Burright EN, Duvick LA, Zoghbi HY, Orr HT. 1997. Increased trinucleotide repeat instability with advanced maternal age. Hum Mol Genet 6:2135-2139.
132. Abbruzzese C, Costanzi Porrini S, Mariani B, Gould FK, McAbney JP *et al.* 2002. Instability of a premutation allele in homozygous patients with myotonic dystrophy type 1. Ann Neurol 52:435-441.
133. Sato T, Oyake M, Nakamura K, Nakao K, Fukusima Y *et al.* 1999. Transgenic mice harboring a full-length human mutant DRPLA gene exhibit age-dependent intergenerational and somatic instabilities of CAG repeats comparable with those in DRPLA patients. Hum Mol Genet 8:99-106.
134. Leeflang EP, Arnheim N. 1995. A novel repeat structure at the myotonic dystrophy locus in a 37 repeat allele with unexpectedly high stability. Hum Mol Genet 4:135-136.

135. Leeflang EP, Tavare S, Marjoram P, Neal CO, Srinidhi J *et al.* 1999. Analysis of germline mutation spectra at the Huntington's disease locus supports a mitotic mutation mechanism. Hum Mol Genet 8:173-183.
136. Savouret C, Garcia-Cordier C, Megret J, te Riele H, Junien C *et al.* 2004. MSH2-dependent germinal CTG repeat expansions are produced continuously in spermatogonia from DM1 transgenic mice. Mol Cell Biol 24:629-637.
137. Zhang Y, Monckton DG, Siciliano MJ, Connor TH, Meistrich ML. 2002. Age and insertion site dependence of repeat number instability of a human DM1 transgene in individual mouse sperm. Hum Mol Genet 11:791-798.
138. Seznec H, Lia-Baldini AS, Duros C, Fouquet C, Lacroix C *et al.* 2000. Transgenic mice carrying large human genomic sequences with expanded CTG repeat mimic closely the CM CTG repeat intergenerational and somatic instability. Hum Mol Genet 9:1185-1194.
139. Wheeler VC, Persichetti F, McNeil SM, Mysore JS, Mysore SS *et al.* 2007. Factors associated with HD CAG repeat instability in Huntington disease. J Med Genet 44:695-701.
140. Kovtun IV, McMurray CT. 2001. Trinucleotide expansion in haploid germ cells by gap repair. Nat Genet 27:407-411.
141. Yoon SR, Dubeau L, de Young M, Wexler NS, Arnheim N. 2003. Huntington disease expansion mutations in humans can occur before meiosis is completed. Proc Natl Acad Sci U S A 100:8834-8838.
142. Gomes-Pereira M, Foiry L, Gourdon G. 2006. Transgenic mouse models of unstable trinucleotide repeats: toward an understanding of disease-associated repeat size mutation. In: Wells R, Ashizawa T. ed. Genetic instabilities and neurological diseases: Academic press; p 563-583.
143. Rousseau F, Heitz D, Biancalana V, Blumenfeld S, Kretz C *et al.* 1991. Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. N Engl J Med 325:1673-1681.
144. Nolin SL, Glicksman A, Houck GE, Brown WT, Dobkin CS. 1994. Mosaicism in fragile X affected males. Am J Med Genet 51:509-512.
145. Pieretti M, Zhang FP, Fu YH, Warren ST, Oostra BA *et al.* 1991. Absence of expression of the FMR-1 gene in fragile X syndrome. Cell 66:817-822.
146. de Graaff E, Willemsen R, Zhong N, de Die-Smulders CE, Brown WT *et al.* 1995. Instability of the CGG repeat and expression of the FMR1 protein in a male fragile X patient with a lung tumor. Am J Hum Genet 57:609-618.
147. Sun YJ, Han X. 2004. Dynamic behavior of fragile X full mutations in cultured female fetal fibroblasts. Acta Pharmacol Sin 25:973-976.
148. Willemsen R, Hoogeveen-Westerveld M, Reis S, Holstege J, Severijnen L *et al.* 2003. The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. Hum Mol Genet 12:949-959.
149. Anvret M, Ahlberg G, Grandell U, Hedberg B, Johnson K *et al.* 1993. Larger expansions of the CTG repeat in muscle compared to lymphocytes from patients with myotonic dystrophy. Hum Mol Genet 2:1397-1400.
150. Ashizawa T, Dubel JR, Harati Y. 1993. Somatic instability of CTG repeat in myotonic dystrophy. Neurology 43:2674-26748.
151. Monckton DG, Wong LJ, Ashizawa T, Caskey CT. 1995. Somatic mosaicism, germline expansions, germline reversions and intergenerational reductions in myotonic dystrophy males: small pool PCR analyses. Hum Mol Genet 4:1-8.
152. Thornton CA, Griggs RC, Moxley Rr. 1994. Myotonic dystrophy with no trinucleotide repeat expansion. Ann Neurol 35:269-272.
153. Wong L-JC, Ashizawa T, Monckton DG, Caskey CT, Richards CS. 1995. Somatic heterogeneity of the CTG repeat in myotonic dystrophy is age and size dependent. Am J Hum Genet 56:114-122.
154. Fu YH, Pizzuti A, Fenwick RJ, King J, Rajnarayan S *et al.* 1992. An unstable triplet repeat in a gene related to myotonic muscular dystrophy. Science 255:1256-1258.
155. Harley HG, Brook JD, Rundle SA, al. e. 1992. Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. Nature 355:545-546.
156. Mahadevan M, Tsilfidis C, Saborin L, Shutler G, Amemiya C *et al.* 1992. Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene. Science 255:1253-1255.
157. Tsilfidis C, MacKenzie AE, Mettler G, Barcelo J, Korneluk RG. 1992. Correlation between CTG trinucleotide repeat length and frequency of severe congenital myotonic dystrophy. Nature Genet 1:192-195.
158. Yamagata H, Miki T, Ogihara T, Nakagawa M, Higuchi I *et al.* 1992. Expansion of unstable DNA region in Japanese myotonic dystrophy patients. Lancet 339:692.
159. Martorell L, Monckton DG, Gamez J, Johnson KJ, Gich I *et al.* 1998. Progression of somatic CTG repeat length heterogeneity in the blood cells of myotonic dystrophy patients. Hum Mol Genet 7:307-312.
160. van den Broek WJ, Wansink DG, Wieringa B. 2007. Somatic CTG*CAG repeat instability in a mouse model for myotonic dystrophy type 1 is associated with changes in cell nuclearity and DNA ploidy. BMC Mol Biol 8:61.
161. Guidotti JE, Bregerie O, Robert A, Debey P, Brechot C *et al.* 2003. Liver cell polyploidization: a pivotal role for binuclear hepatocytes. J Biol Chem 278:19095-19101.

162. De Rooij KE, De Koning Gans PA, Roos RA, Van Ommen GJ, Den Dunnen JT. 1995. Somatic expansion of the (CAG)n repeat in Huntington disease brains. Hum Genet 95:270-274.
163. Telenius H, Kremer B, Goldberg YP, Theilmann J, Andrew SE *et al.* 1994. Somatic and gonadal mosaicism of the Huntington disease gene CAG repeat in brain and sperm. Nature Genet 6:409-414.
164. Kennedy L, Shelbourne PF. 2000. Dramatic mutation instability in HD mouse striatum: does polyglutamine load contribute to cell-specific vulnerability in Huntington's disease? Human Molecular Genetics 9:2539-2544.
165. Kennedy L, Evans E, Chen CM, Craven L, Detloff PJ *et al.* 2003. Dramatic tissue-specific mutation length increases are an early molecular event in Huntington disease pathogenesis. Hum Mol Genet 12:3359-3367.
166. Kovtun IV, Therneau TM, McMurray CT. 2000. Gender of the embryo contributes to CAG instability in transgenic mice containing a Huntington's disease gene. Hum Mol Genet 9:2767-2775.
167. Kovtun IV, Thornhill AR, McMurray CT. 2004. Somatic deletion events occur during early embryonic development and modify the extent of CAG expansion in subsequent generations. Hum Mol Genet 13:3057-6308.
168. Monckton DG, Cayuela ML, Gould FK, Brock GJ, Silva R *et al.* 1999. Very large (CAG)(n) DNA repeat expansions in the sperm of two spinocerebellar ataxia type 7 males. Hum Mol Genet 8:2473-2478.
169. Veitch NJ, Ennis M, McAbney JP, Shelbourne PF, Monckton DG. 2007. Inherited CAG.CTG allele length is a major modifier of somatic mutation length variability in Huntington disease. DNA Repair (Amst) 6:789-796.
170. Kunkel TA. 1993. Nucleotide repeats. Slippery DNA and diseases. Nature 365:207-208.
171. Strand M, Prolla TA, Liskay RM, Petes TD. 1993. Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. Nature 365:274-276.
172. Gacy AM, Goellner G, Juranic N, Macura S, McMurray CT. 1995. Trinucleotide repeats that expand in human disease form hairpin structures *in vitro*. Cell 81:533-540.
173. Pearson CE, Tam M, Wang YH, Montgomery SE, Dar AC *et al.* 2002. Slipped-strand DNAs formed by long (CAG)*(CTG) repeats: slipped-out repeats and slip-out junctions. Nucleic Acids Res 30:4534-4547.
174. Usdin K, Woodford KJ. 1995. CGG repeats associated with DNA instability and chromosome fragility form structures that block DNA synthesis in vitro. Nucleic Acids Res 23:4202-4209.
175. Gacy AM, Goellner GM, Spiro C, Chen X, Gupta G *et al.* 1998. GAA instability in Friedreich's Ataxia shares a common, DNA-directed and intraallelic mechanism with other trinucleotide diseases. Mol Cell 1:583-593.
176. Ohshima K, Wells RD. 1997. Hairpin formation during DNA synthesis primer realignment in vitro in triplet repeat sequences from human hereditary disease genes. J Biol Chem 272:16798-16806.
177. Cleary JD, Nichol K, Wang YH, Pearson CE. 2002. Evidence of cis-acting factors in replication-mediated trinucleotide repeat instability in primate cells. Nat Genet 31:37-46.
178. Freudenreich CH, Stavenhagen JB, Zakian VA. 1997. Stability of a CTG/CAG trinucleotide repeat in yeast is dependent on its orientation in the genome. Mol Cell Biol 17:2090-2098.
179. Kang S, Jaworski A, Ohshima K, Wells RD. 1995. Expansion and deletion of CTG repeats from human disease genes are determined by the direction of replication in *E. coli*. Nature Genet 10:213-218.
180. Miret JJ, Pessoa-Brandao L, Lahue RS. 1998. Orientation-dependent and sequence-specific expansions of CTG/CAG trinucleotide repeats in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 95:12438-12443.
181. Kovtun IV, McMurray CT. 2008. Features of trinucleotide repeat instability in vivo. Cell Res 18:198-213.
182. Gray SJ, Gerhardt J, Doerfler W, Small LE, Fanning E. 2007. An Origin of DNA Replication in the Promoter Region of the Human Fragile X Mental Retardation (FMR1) Gene. Mol Cell Biol 27:426437.
183. Mirkin SM. 2006. DNA structures, repeat expansions and human hereditary disorders. Curr Opin Struct Biol 16:351-358
184. Fouche N, Ozgur S, Roy D, Griffith JD. 2006. Replication fork regression in repetitive DNAs. Nucleic Acids Res 34:6044-6050.
185. Gourdon G, Radvanyi F, Lia AS, Duros C, Blanche M *et al.* 1997. Moderate intergenerational and somatic instability of a 55-CTG repeat in transgenic mice. Nature Genet 15:190-192.
186. Eichler EE, Holden J, Popovich BW, Reiss AL, Snow K *et al.* 1994. Length of uninterrupted CGG repeats determines instability in the FMR1 gene. Nature Genet 8:88-94.
187. Napierala M, Michalowski D, de Mezer M, Krzyzosiak WJ. 2005. Facile FMR1 mRNA structure regulation by interruptions in CGG repeats. Nucleic Acids Res 33:451-463.
188. Dombrowski C, Levesque S, Morel ML, Rouillard P, Morgan K *et al.* 2002. Premutation and intermediate-size FMR1 alleles in 10 572 males from the general population: loss of an AGG interruption is a late event in the generation of fragile X syndrome alleles. Hum Mol Genet 11:371-378.

189. Kunst CB, Warren ST. 1994. Cryptic and polar variation of the fragile X repeat could result in predisposing normal alleles. Cell 77:853-861.
190. Poon PM, Chen QL, Zhong N, Lam ST, Lai KY *et al.* 2006. AGG interspersion analysis of the FMR1 CGG repeats in mental retardation of unspecific cause. Clin Biochem 39:244-248.
191. Chung M-Y, Ranum LPW, Duvick LA, Servadio A, Zoghbi HY. 1993. Evidence for a mechanism predisposing to intergenerational CAG repeat instability in spinocerrebellar ataxia type I. Nature Genet 5:254-258.
192. Jodice C, Malaspina P, Persichetti F, Novelletto A, Spadaro M *et al.* 1994. Effect of trinucleotide repeat length and parental sex on phenotypic variation in spinocerebellar ataxia I. Am J Hum Genet 54:959-965.
193. Bontekoe CJM, de Graaff E, Nieuwenhuizen IM, Willemsen R, Oostra BA. 1997. FMR1 premutation allele is stable in mice. Eur J Hum Genet 5:293-298.
194. Lavedan CN, Garrett L, Nussbaum RL. 1997. Trinucleotide repeats (CGG)22TGG(CGG)43TGG(CGG)21 from the fragile X gene remain stable in transgenic mice. Hum Genet 100:407-414.
195. Gourdon G, Dessen P, Lia AS, Junien C, Hofmann-Radvanyi H. 1997. Intriguing association between disease associated unstable trinucleotide repeat and CpG island. Ann Genet 40:73-77.
196. Brock GJ, Anderson NH, Monckton DG. 1999. Cis-acting modifiers of expanded CAG/CTG triplet repeat expandability: associations with flanking GC content and proximity to CpG islands. Hum Mol Genet 8:1061-1067.
197. Sutherland GR. 1977. Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. Science 197:265-266.
198. Bingham PM, Scott MO, Wang S, McPhaul MJ, Wilson EM *et al.* 1995. Stability of an expanded trinucleotide repeat in the androgen receptor gene in transgenic mice. Nature Genet 9:191-196.
199. Burright EN, Clark HB, Servadio A, Matilla T, Feddersen RM *et al.* 1995. *SCA1* transgenic mice: A model for neurodegeneration caused by an expanded CAG trinucleotide repeat. Cell 82:937-948.
200. Goldberg YP, Kalchman MA, Metzler M, Nasir J, Zeisler J *et al.* 1996. Absence of disease phenotype and intergenerational stability of the CAG repeat in transgenic mice expressing the human Huntington disease transcript. Hum Mol Genet 5:177-185.
201. Seeburg PH, Osten P. 2003. Neurobiology: a thorny issue. Nature 424:627-628.
202. Peier A, Nelson D. 2002. Instability of a premutation-sized CGG repeat in FMR1 YAC transgenic mice. Genomics 80:423-432.
203. Monckton DG, Coolbaugh MI, Ashizawa KT, Siciliano MJ, Caskey CT. 1997. Hypermutable myotonic dystrophy CTG repeats in transgenic mice. Nature Genet 15:193-196.
204. Fortune MT, Vassilopous C, Coolbaugh MI, Siciliano MJ, Monckton DG. 2000. Dramatic, expansion-biased, age-dependent, tissue-specific somatic mosaicism in a transgenic mouse model of triplet repeat instability. Hum Mol Genet 9:439-445.
205. Cleary JD, Pearson CE. 2003. The contribution of cis-elements to disease-associated repeat instability: clinical and experimental evidence. Cytogenet Genome Res 100:25-55.
206. Pearson CE. 2003. Slipping while sleeping? Trinucleotide repeat expansions in germ cells. Trends Mol Med 9:490-495.
207. Yang Z, Lau R, Marcadier JL, Chitayat D, Pearson CE. 2003. Replication inhibitors modulate instability of an expanded trinucleotide repeat at the myotonic dystrophy type 1 disease locus in human cells. Am J Hum Genet 73:1092-1105.
208. Petruska J, Hartenstine MJ, Goodman MF. 1998. Analysis of strand slippage in DNA polymerase expansions of CAG/CTG triplet repeats associated with neurodegenerative disease. J Biol Chem 273:5204-5210.
209. Samadashwily GM, Raca G, Mirkin SM. 1997. Trinucleotide repeats affect DNA replication in vivo. Nat Genet 17:298-304.
210. Pelletier R, Krasilnikova MM, Samadashwily GM, Lahue R, Mirkin SM. 2003. Replication and expansion of trinucleotide repeats in yeast. Mol Cell Biol 23:1349-1357.
211. Rossi ML, Purohit V, Brandt PD, Bambara RA. 2006. Lagging strand replication proteins in genome stability and DNA repair. Chem Rev 106:453-473.
212. Freudenreich CH, Kantrow SM, Zakian VA. 1998. Expansion and length-dependent fragility of CTG repeats in yeast. Science 279:853-856.
213. Schweitzer JK, Livingston DM. 1998. Expansions of CAG repeat tracts are frequent in a yeast mutant defective in Okazaki fragment maturation. Hum Mol Genet 7:69-74.
214. Spiro C, Pelletier R, Rolfsmeier ML, Dixon MJ, Lahue RS *et al.* 1999. Inhibition of FEN-1 processing by DNA Secundary structure at trinucleotide repeats. Molecular Cell 4:1079-1085.
215. Spiro C, McMurray CT. 2003. Nuclease-deficient FEN-1 blocks Rad51/BRCA1-mediated repair and causes trinucleotide repeat instability. Mol Cell Biol 23:6063-6074.
216. van den Broek WJ, Nelen MR, van der Heijden GW, Wansink DG, Wieringa B. 2006. Fen1 does not control somatic hypermutability of the (CTG)(n)*(CAG)(n) repeat in a knock-in mouse model for DM1. FEBS Lett 580:5208-5214.

217. Yang J, Freudenreich CH. 2007. Haploinsufficiency of yeast FEN1 causes instability of expanded CAG/CTG tracts in a length-dependent manner. Gene 393:110-115.
218. Lahue RS, Slater DL. 2003. DNA repair and trinucleotide repeat instability. Front Biosci 8:s653-665.
219. Gonitel R, Moffitt H, Sathasivam K, Woodman B, Detloff PJ *et al.* 2008. DNA instability in postmitotic neurons. Proc Natl Acad Sci U S A 105:3467-3472.
220. Peltomaki P. 2001. Deficient DNA mismatch repair: a common etiologic factor for colon cancer. Hum Mol Genet 10:735-740.
221. Marti TM, Kunz C, Fleck O. 2002. DNA mismatch repair and mutation avoidance pathways. J Cell Physiol 191:28-41.
222. Savouret C, Brisson E, Essers J, Kanaar R, Pastink A *et al.* 2003. CTG repeat instability and size variation timing in DNA repair-deficient mice. Embo J 22:2264-2273.
223. Mangiarini L, Sathasivam K, Mahal A, Mott R, Seller M *et al.* 1997. Instability of highly expanded CAG repeats in mice transgenic for the Huntington's disease mutation. Nat Genet 15:197-200.
224. Manley K, Shirley TL, Flaherty I, Messer A. 1999. Msh2 deficiency prevents in vivo somatic instability of the CAG repeat in Huntington disease transgenic mice. Nature Genetics 23:471-473.
225. Kovtun IV, Spiro C, McMurray CT. 2004. Triplet repeats and DNA repair: germ cell and somatic cell instability in transgenic mice. Methods Mol Biol 277:309-320.
226. Wheeler VC, Lebel LA, Vrbanac V, Teed A, te Riele H *et al.* 2003. Mismatch repair gene Msh2 modifies the timing of early disease in Hdh(Q111) striatum. Hum Mol Genet 12:273-281.
227. van den Broek WJ, Nelen MR, Wansink DG, Coerwinkel MM, te Riele H *et al.* 2002. Somatic expansion behaviour of the (CTG)n repeat in myotonic dystrophy knock-in mice is differentially affected by Msh3 and Msh6 mismatch-repair proteins. Hum Mol Genet 11:191-198.
228. Foiry L, Dong L, Savouret C, Hubert L, te Riele H *et al.* 2006. Msh3 is a limiting factor in the formation of intergenerational CTG expansions in DM1 transgenic mice. Hum Genet 119:520-526.
229. Fleming K, Riser DK, Kumari D, Usdin K. 2003. Instability of the fragile X syndrome repeat in mice: the effect of age, diet and mutations in genes that affect DNA replication, recombination and repair proficiency. Cytogenet Genome Res 100:140-146.
230. Entezam A, Usdin K. 2007. ATR protects the genome against CGG*CGG-repeat expansion in Fragile X premutation mice. Nucleic Acids Res 36:1050-1056
231. Jiang G, Sancar A. 2006. Recruitment of DNA damage checkpoint proteins to damage in transcribed and nontranscribed sequences. Mol Cell Biol 26:39-49.
232. Razidlo DF, Lahue RS. 2008. Mrc1, Tof1 and Csm3 inhibit CAG.CTG repeat instability by at least two mechanisms. DNA Repair (Amst) 7:633-640.
233. Jung J, Bonini N. 2007. CREB-binding protein modulates repeat instability in a Drosophila model for polyQ disease. Science 315:1857-1859.
234. Steffan JS, Bodai L, Pallos J, Poelman M, McCampbell A *et al.* 2001. Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. Nature 413:739-743.
235. Lin Y, Dion V, Wilson JH. 2006. Transcription promotes contraction of CAG repeat tracts in human cells. Nat Struct Mol Biol 13:179-180.
236. Lin Y, Wilson JH. 2007. Transcription-induced CAG repeat contraction in human cells is mediated in part by transcription-coupled nucleotide excision repair. Mol Cell Biol 27:6209-6217.
237. Nichol K, Pearson CE. 2002. CpG methylation modifies the genetic stability of cloned repeat sequences. Genome Res 12:1246-1256.
238. Taylor AK, Tassone F, Dyer PN, Hersch SM, Harris JB *et al.* 1999. Tissue heterogeneity of the FMR1 mutation in a high-functioning male with fragile X syndrome. Am J Med Genet 84:233-239.
239. Wohrle D, Salat U, Glaser D, Mucke J, Meisel-Stosiek M *et al.* 1998. Unusual mutations in high functioning fragile X males: apparent instability of expanded unmethylated CGG repeats. J Med Genet 35:103-111.
240. Dion V, Lin Y, Hubert L, Jr., Waterland RA, Wilson JH. 2008. Dnmt1 Deficiency Promotes CAG Repeat Expansion in the Mouse Germline. Hum Mol Genet 17:1306-1317
241. Baskaran S, Datta S, Mandal A, Gulati N, Totey S *et al.* 2002. Instability of CGG Repeats in Transgenic Mice. Genomics 80:151.
242. Wang YH, Griffith J. 1996. Methylation of expanded CCG triplet repeat DNA from fragile X syndrome patients enhances nucleosome exclusion. J Biol Chem 271:22937-22940.
243. Jasinska A, Michlewski G, de Mezer M, Sobczak K, Kozlowski P *et al.* 2003. Structures of trinucleotide repeats in human transcripts and their functional implications. Nucleic Acids Res 31:5463-5468.

244. Filippova GN, Thienes CP, Penn BH, Cho DH, Hu YJ *et al.* 2001. CTCF-binding sites flank CTG/CAG repeats and form a methylation-sensitive insulator at the DM1 locus. Nat Genet 28:335-343.
245. Godde JS, Kass SU, Hirst MC, Wolffe AP. 1996. Nucleosome assembly on methylated CGG triplet repeats in the Fragile X Mental Retardation gene 1 promoter. J Biol Chem 271:24325-24328.
246. Wang YH, Amirhaeri S, Kang S, Wells RD, Griffith JD. 1994. Preferential nucleosome assembly at DNA triplet repeats from the myotonic dystrophy gene. Science 265:669-671.
247. Wang YH, Griffith J. 1995. Expanded CTG triplet blocks from the myotonic dystrophy gene create the strongest known natural nucleosome positioning elements. Genomics 25:570-573.

CHAPTER 3

Elevated *Fmr1* mRNA levels and reduced protein expression in a mouse model with an unmethylated Fragile X full mutation

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Abstract |

The human *FMR1* gene contains a CGG repeat ($(CGG)_n$) in its 5' untranslated region. The repeat length in the normal population is polymorphic ($(CGG)_{5-55}$). Lengths beyond 200 CGGs (full mutation) result in the absence of the *FMR1* gene product, FMRP, through abnormal methylation and gene silencing. This causes Fragile X Syndrome, the most common inherited form of mental retardation. Elderly carriers of the premutation, defined as a repeat length between 55-200 CGGs can develop a progressive neurodegenerative syndrome: Fragile X-associated tremor/ataxia syndrome (FXTAS). In FXTAS, *FMR1* mRNA levels are elevated and it has been hypothesised that FXTAS is caused by a pathogenic RNA gain-of-function mechanism. We have developed a knock-in mouse model carrying an expanded ($(CGG)_n$ (98 repeats), which shows repeat instability and displays biochemical, phenotypic and neuropathological characteristics of FXTAS. Here, we report further repeat instability, up to 230 CGGs. An expansion bias was observed, with the largest expansion being 43 CGG units and the largest contraction 80 CGGs. In humans, this length would be considered a full mutation and would be expected to result in gene silencing. Mice carrying long repeats (~230 CGGs) display elevated mRNA levels and decreased FMRP levels, but absence of abnormal methylation, suggesting that modelling the Fragile X full mutation in mice require additional repeats, or other genetic manipulation.

Introduction | 3.1

The Fragile X mental retardation gene 1 (*FMR1*), involved in fragile X syndrome, contains a $(CGG)_n$ in its 5'-untranslated region (5'UTR). Depending on the length of this trinucleotide repeat, different clinical outcomes are possible. Repeats of normal individuals are within the range of 5 to 55 CGGs [1]. Repeat lengths greater than 200 CGGs (full mutation: FM) typically leads to methylation of both the $(CGG)_n$ and the *FMR1* promoter resulting in transcriptional silencing of the gene. The consequent absence of FMRP in neurons is the cause of the mental retardation in Fragile X patients. Fragile X Syndrome is the most common genetic disorder associated with mental retardation [2].

The premutation (PM), comprising ~55 to ~200 unmethylated CGGs, was long thought to be associated only with a high risk of expansion to a full mutation upon maternal transmission. However, elevated *FMR1* mRNA levels and normal or slightly reduced *FMR1* protein (FMRP) were reported [3,4,5]. Additionally, 20% of female PM carriers are at risk of developing premature ovarian failure [6]. In 2001 the first evidence of a new neurological syndrome (Fragile X Associated Tremor/Ataxia Syndrome: FXTAS) was published by Hagerman and colleagues, observed in five elderly male PM carriers. Patients presented with progressive intention tremor, leading to executive function deficits and generalised brain atrophy on MRI scans. Since this neurological syndrome is restricted to the PM range, reduced levels of FMRP are unlikely to be the underlying cause [7]. However, since elevation of *FMR1* mRNA levels seems to be correlated to the length of the $(CGG)_n$ [5,8,9], and cognitive and functional impairment increases with the number of $(CGG)_n$ [10], an RNA-gain-of-function effect has been proposed, in which elevated levels of *FMR1* mRNA containing an expanded $(CGG)_n$ lead to progressive neurodegeneration [7].

Little is known about when and how $(CGG)_n$ instability takes place. In order to be able to elucidate the timing and mechanism of $(CGG)_n$ instability and methylation of the *FMR1* gene, a mouse model was generated by Bontekoe and colleagues [11]. In this model, the endogenous mouse $(CGG)_n$ (8 trinucleotides) of a wild type mouse was exchanged with a human $(CGG)_{98}$, which is in the PM range in humans. This 'knock in' CGG triplet mouse shows moderate repeat instability upon both maternal and paternal transmission and displays biochemical, phenotypic and neuropathological characteristics of FXTAS [11,12].

In this paper, we report further repeat instability, up to lengths above 200 CGG units. In humans, this would implicate a full mutation, thus silencing of the gene. Mice carrying these long repeats (~230 CGGs) display elevated mRNA levels and decreased FMRP levels, but absence of CpG methylation, which would mean that in mice a full mutation has not occurred as yet. However, additional mice with long $(CGG)_n$ will be necessary to fully evaluate this issue.

Materials and Methods | 3.2

Mice

Both the knock in CGG triplet mice and wild type (wt) mice (parent of the knock in mouse, with a mouse endogenous $(CGG)_8$ repeat) were housed in standard conditions. All experiments were carried out with permission of the local ethical committee. Repeat lengths were determined for the whole mouse colony but only male mice were used for the experiments.

Isolation of DNA from mouse tails

DNA was extracted from mouse tails by incubating with 0.2 mg/ml Proteinase K (Roche Diagnostics) in 335 µl lysis buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% SDS) overnight at 55°C. After adding 100 µl saturated NaCl solution the next day, the suspension was centrifuged. Two volumes of 100% ethanol were added and gently mixed. The appearing DNA cloud was fished out with a plastic pipet tip and subsequently washed and centrifuged in 500 µl 70% ethanol. The DNA was then dissolved in 100 µl milliQ-H_2O.

Repeat length determination

Following a method kindly provided by K. Usdin (personal communication), $(CGG)_n$ lengths were measured using the Expand High Fidelity PCR System (Roche Diagnostics). Approximately 300 ng of tail DNA was added to a PCR mixture with a total volume of 50 µl, containing 0.2 µM of each primer, 200 µM of each dNTP (Invitrogen), 2% DMSO (Sigma), 2.5 M Betaine (Sigma), 5U Expand High Fidelity Plus PCR System Enzyme and 10 µl of the 5x Expand HF buffer with Mg (7.5 µM). As forward primer 5'- CGGAGGCGCCGCTGCCAGG-3' was used and 5'-TGCGGGCGCTCGAGGCCCAG-3' as reverse. PCR conditions were 10 minutes initial denaturation at 95°C, followed by 35 cycles of denaturation for 1 minute at 95°C, annealing for 1 minute at 65°C and elongation for 5 minutes at 75°C, with a final elongation step of 10 minutes at 75°C. 25 µl of PCR product was mixed with an equal volume of loading dye (95% formamide, 0.1% brome phenol blue, 0.1% xylene cyanol) and loaded onto a denaturing 6% poly acrylamide (PA)(19:1) gel, which was run in 0.6x TBE at 18 Watts. Separated DNA was visualised on a UV-lamp after soaking the gel in 0.6x TBE with ethidium bromide. Approximate repeat lengths were determined using a standard curve, based on DNA samples of which repeat lengths were determined with an ABI-based Fragile X size polomorphism assay in the past [11].

As these primers are specific to the knock-in allele and do not detect wild type alleles, a separate PCR is necessary to distinguish females heterozygous or homozygous for the repeat. TaKaRa LA Taq was used with GC buffer II according to manufacturer's instructions (Takara Bio Inc), with 5'-gctcagctccgtttcggtttcacttccggt-3' as forward primer and 5'-agccccgcacttccaccaccagctcctcca-3' as reverse primer.

Transmission pattern of repeat alleles

The mode of transmission of the repeat allele was investigated, by comparison of the repeat lengths present in the tails of the breeding couple with those of their offspring. Since the ability of the PA gels to resolve lengths of PCR products in this range is limited, relatively large bin sizes were chosen to describe repeat length. Comparisons between parents and offspring were made using PCR products run on the same gel. Frequencies of contractions, stable transmissions and expansions were tested with a one-sample T-test. Magnitude and direction (contraction, stable transmission or expansion) of the repeat instability, gender of the offspring and parental origin of the repeat allele were taken into account. Transmission groups were tested for differences in magnitude of contractions and expansion separately, using one-way ANOVA. Effect of gender of offspring, as well as effect of parental allele of origin was investigated with independent samples T-tests for both contractions and expansions. Because of small expected frequencies, Fisher's Exact Test was performed to test for possible differences in frequencies of contractions, stable transmissions or expansions, among the different transmission groups. The same test was used to determine the significance of differences in frequencies of an allele fitting into the categories distinguishing magnitude of instability (categories shown in figure 3.2). A difference in absolute magnitude of contractions vs. expansions was investigated using an independent samples T-test.

RNA isolation, RT and Q-PCR analysis

Mouse brains were homogenised on ice in buffer (PBS with 1% NP-40, Complete protease inhibitor cocktail tablets (Roche Diagnostics), 3 mM DTT (Invitrogen) and 20U RNAsin (Promega). 1 ml RNA bee (Tel-Test) was added to 100 µl brain homogenate. 200 µl chloroform was then added and the mixture was then spun at 4°C for phase separation. 1 Volume of isopropanol was added to the aqueous phase to precipitate the RNA. The pellet was washed with 80% ethanol and dissolved in DEPC-treated MilliQ-H2O. RNA concentration and purity was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). RT-PCR was performed on 2 µg RNA using iScript cDNA Synthesis Kit (BioRad) according to manufacturer's instructions.

For Q-PCR two primer sets for *Fmr1* (transition exon 5/6 (5'-AGATCAAGCTGGAGGTGCCA-3' as forward primer and 5'-CAGAGAAGGCACCAACTGCC-3' as reverse) and transition exon 16/17 (5'-CCGAACAGATAATCGTCCACG-3' as forward primer and 5'-ACGCTGTCTGGCTTTTCCTTC-3' as reverse) and two primer sets for two distinct internal controls (*Gapdh*: forward: 5'-CCTGGAGAAACCTGCCAAGTAT-3' and reverse: 5'-CCCTCAGATGCCTGCTTCA-3') and *Rpl13a*: forward: 5'-TCTCAAGGTTGTTCGGCTGA-3' and reverse: 5'-GTGGCTGTCACTGCCTGGTA-3' were used. Since efficiencies of the different primer sets were not identical when tested, it was necessary to make a standard curve for each primer pair in every run. Input quantities were calculated by subjecting Ct values to the formula of the appropriate standard curves. A ratio of *Fmr1* mRNA to the reference gene was then calculated for each mouse. This *Fmr1*/reference-ratio was standardised to 1 for wild type (wt) mice. *Fmr1*/reference-ratios were compared with the ratio in wt mice of the same age. 1/10 and 1/100 dilutions of RT product were used as input material. All dilutions were performed in duplicate.

Western blotting

Half brains (saggital) were homogenised in 500 µl HEPES-buffer (10 mM HEPES, 300 mM KCl, 3 mM $MgCl_2$, 100 µM $CaCl_2$, 0.45% Triton X-100 and 0.05% Tween-20, pH 7.6, with Complete protease inhibitor cocktail (Roche Diagnostics), while kept on ice. After incubating the homogenates on ice for 30 minutes, they were sonicated twice for 20 seconds. Cell debris was spun down and the supernatant was collected. Loading mix was added to 100 µg of protein, heated at 95°C for 5 minutes and loaded onto a 10% SDS-PAGE gel. After electroblotting the gel onto a nitrocellulose membrane, the membrane was incubated overnight at 4°C with the monoclonal 2F5-1 antibody specific for FMRP [13], in PBS-T with 5% milk powder. The lower part of the membrane was incubated, under the same conditions, with an antibody for synaptophysin. The next day the membrane was incubated with a horseradish peroxidase conjugated secondary antibody rabbit-α-mouse (DAKO), allowing chemiluminescence detection with an ECL KIT (Amersham).

Methylation status

DNA was checked for the presence of methylated CpGs in the promoter region in two ways. A quick screen consists of overnight digestion of 1 µg DNA with the methylation-sensitive enzyme BssHII (after pre-digestion with EcoRI to facilitate complete digestion with BssHII). The digested DNA is then subjected to PCR over the promoter region containing the BssHII-site (forward primer: 5'-AAATCTACCCAATGCCCCTCC-3', reverser primer: 5'-CCTGGGTACCTGCCTCAGG-3'). The PCR mixture contained 10x PCR buffer, W-1, 1 unit Taq Polymerase (all Invitrogen) and 50 mM Spermidine (Fluka BioChemika). The PCR program was as follows: 5 minutes denaturation at 95°C, 35 cycles of 30 seconds at 95°C, 30 seconds annealing at 56°C and 1.5 minutes at 72°C, followed by a final elongation step of 10 minutes at 72°C. A non-methylated BssHII site can be cut by BssHII, and hence will not produce a PCR product. Methylated DNA prevents BssHII digestion, thus a PCR product can be produced.

The other method consists of subjecting 1.5 µg of DNA to bisulphite conversion, using the Qiagen Epitect kit, according to manufacturer's instructions. 1.5 µl of converted DNA was then used for a PCR using forward primers 5'-GAAGTGAAATCGAAACGTTTTTTAGCGTTT-3' and 5'-GAAGTGAAATTGAAATGTTTTTTAGTGTTT-3' and reverse primer 5'-ATTTATCACTTTTTTTTTTACTTCCCTCC-3'. In order to be able to amplify a fragment with unknown methylation status, it is necessary to use a combination of primers, one of which matches the sequence after conversion in the case where the DNA was methylated and another primer in case it was unmethylated. The PCR mixture was prepared

as described above for the PCR after methylation-sensitive digestion, except for that 50 mM $MgCl_2$ was added. The PCR program was the same as described above with the exception of an annealing temperature of 63.5°C. 5 µl of PCR product was then used for a semi-nested re-PCR, since no PCR product was seen on a 2% agarose gel initially. Primers used in the second PCR reaction were forward: 5′-TTTTGTAAATAGTTAAATGATGTTATGTGATTTG-3′ and 5′-TTTTGTAAATAGTTAAACGATGTTACGTGATTCG-3′ and reverse: 5′-TTTTTTACTTCCCTCCCACCAC-3′ and 5′-TTTTTTACTTCCCTCCCGCCGC-3′. PCR products were visualised and cut out of a 2% agarose gel and the bisulphite converted PCR product was purified using an Illustra GFX PCR DNA and gel band purification kit (GE Healthcare). The resulting converted DNA was then sequenced as follows: amplification reactions were performed in 22 µl containing 1x Invitrogen PCR buffer, 1.5mM $MgCl_2$, 0.01% W-1, 250 µM of each dNTP, 1 µM forward primer,1 µM reverse primer (primers were the same as the ones used in the second PCR reaction), 0.75 units of Platinum Taq DNA polymerase (Invitrogen) and 25 ng genomic DNA; DNA Cycle conditions: 7′30″ 95°C; 10 cycles of 30″ denaturation 94°C, annnealing 68°C minus 1°C per cycle, 1′ extension 72°C followed by 25 cycles of 30″ denaturation 94°C , annealing 58°C , 1′ extension 72°C; final extension 5′ 72°C. Templates for the Direct Sequencing reactions were cleaned from dNTP's and primers using 1 ul ExoSAP-IT (USB) during 30 minutes at 37°C followed by a 15 minutes inactivation step at 80°C. Direct sequencing of both strands was performed using Big Dye Terminator chemistry ver.3.1 (Applied Biosystems) as recommended by the manufacturer. Fragments were loaded on an ABI3100 automated sequencer and analyzed with DNA Sequencing Analysis (ver.3.7) and SeqScape (ver.2.1) software (Applied Biosystems).

Immunocytochemistry

Mice were sacrificed by cervical dislocation, brains were dissected immediately and fixed overnight in 3% paraformaldehyde. The brains were embedded in paraffin according to standard protocols. Sections (7 µm) were deparaffinised, followed by antigen retrieval using microwave treatment in 0.01 M sodium citrate solution. Endogenous peroxidase activity blocking and immunoincubation was performed as described before using monoclonal antibodies against FMRP (1C3) [14].

Results | 3.3

The original knock in construct introduced $(CGG)_{98}$ into the mouse *Fmr1* locus and has been used as a model for the human premutation disorder, FXTAS [11,12,15]. This line was initially developed to study repeat instability of long $(CGG)_n$ in the mouse *Fmr1* locus and has been maintained for several years now. In the course of breeding, animals with longer repeats were born and selected for breeding in an attempt to further increase the size of the CGG array, with the hypothesis that repeat instability will be greater as the repeat tract gets longer. We previously reported expansions of this tract to sizes of 110 CGG units [15]. $(CGG)_n$ lengths in the mouse model have now expanded to above the threshold of human *FMR1* full mutations. This study focuses on the characteristics of these mice, which are summarised in table 3.1.

Mouse	Repeat length	Western		Q-PCR		Immunohistochemistry α-Fmrp
		Id	%Fmrp of wt	category	fold change vs. wt	
1	>230	C	75	>230	5x	↓ Fmrp: see figure 3.5
2	~230	D	45	>200	2x	↓ Fmrp: see figure 3.5
3	~230	E	40	-		-
4	~215	-		>200	2x	-

Table 3.1 | Main characteristics of the CGG-repeat knock-in mice described in this study.

Repeat instability

In order to investigate the mode of transmission of $(CGG)_n$ in the expanded $(CGG)_n$ mouse model, mice were bred and repeat lengths in the offspring were compared to repeat lengths in the breeding couple. The $(CGG)_n$ shows instability upon transmission to the next generation (figure 3.1). Both expansions and contractions occur in addition to stable transmissions. Predominantly small expansions are found along with stable transmissions. Both germ line and somatic mutations can be observed. In figure 3.1, for example, animal 2 is a mosaic for repeat length, showing two long repeat alleles (approx. 190 and 200 CGGs) as well as a shorter allele (approx. 60 CGGs), which must represent a mitotic contraction, which was transmitted to one of her male offspring (animal 3). Figure 3.1 also shows the mouse with the longest $(CGG)_n$ length (>230: animal d in figure 3.1) observed thus far, as well as the controls used for repeat length determination.

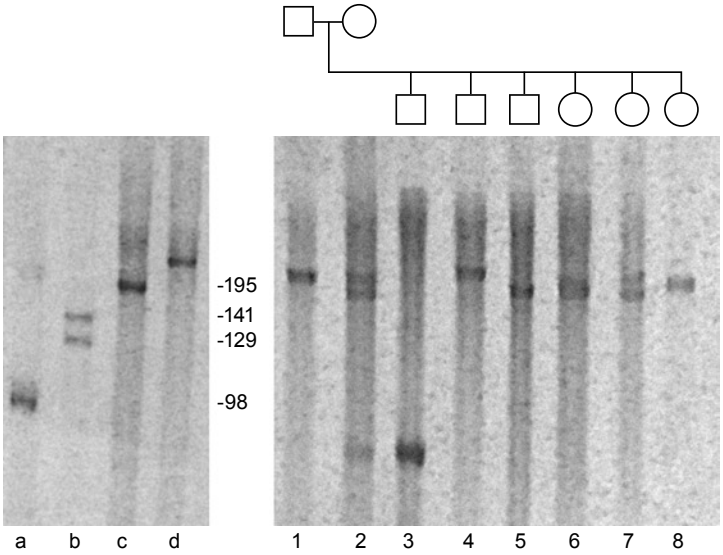

Figure 3.1 | PA gel with PCR products of an expanded $(CGG)_n$ litter and the breeding couple. The $(CGG)_n$ shows both expansion and contraction upon transmission to the next generation. A-d represent DNA samples with previously determined repeat lengths, which are used as a standard curve for approximation of repeat lengths of other samples.

Transmission pattern of repeat alleles

Mode of transmission of $(CGG)_n$ alleles was investigated by comparison of repeat alleles of breeding couples with those of their offspring and subjected to statistical analysis. Figure 3.2 shows instability of repeat alleles in three different transmission groups, based on gender of offspring and origin of the allele. Seventy-nine alleles, of which the parental allele of origin was known, were subjected to statistical analysis. Among all transmissions, 15 contractions (19%), 16 stable transmissions (20%) and 48 expansions (61%) were observed. A one-sample T-test revealed that these frequencies were statistically significantly different from expected frequencies (adjusted p-value for contractions=0.006,

for stable transmissions: p_{adj}=0.006 and for expansions p_{adj}<0.001). The largest contraction was of 80 CGGs and the biggest expansion was 43 trinucleotide units. Comparing mean magnitude of instability between contractions (n=15, mean=15.93, sd=21.0) and expansions (n=48, mean=12.8, sd=10.5), among all alleles considered, with an independent samples T-test revealed no statistical significance (t=0.55, p=0.59).

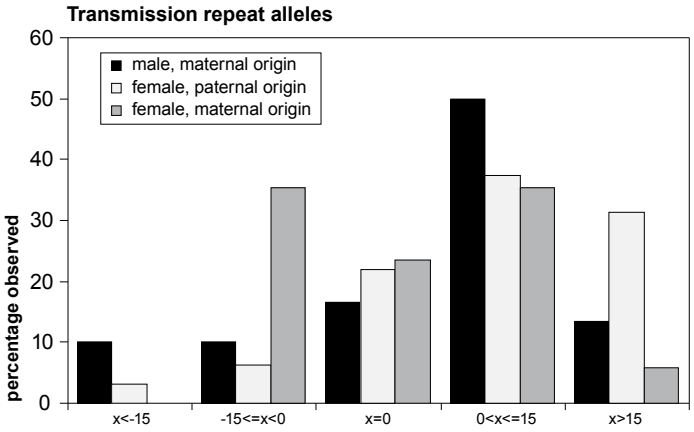

Figure 3.2 | Histogram summarising the observed instability of $(CGG)_n$ alleles upon transmission to the next generation. Bars represent the percentage of times a transmitted allele fits in an instability category (x=change in repeat length), within a transmission group (male offspring, maternal origin, or female offspring with either maternal or paternal origin). Fisher's Exact Test revealed no statistically significant differences in frequencies of instability categories amongst the transmission groups (P=12.86, sd=0.09).

Table 3.2 summarises these data. One-way ANOVA revealed no statistically significant difference in magnitude of instability of contractions (F=3.28, p=0.07) between the different transmission groups, namely male offspring with a repeat allele of maternal origin (MM)(mean=-30.7, standard deviation (sd)= 11.3), female offspring with an allele of paternal origin (FP)(mean=-7.67, sd=4.7) and female offspring with a maternally derived repeat allele (FM)(mean=-5.33, sd=0.42). Neither was a difference found in magnitude of expansions (F=1.36, P=0.27) between MM (mean=11.32, sd=2.4), FP (mean=15.36, sd=2.5) and FM (mean=8.86, sd=1.9). When looking at the factors that determine the transmission groups, gender of offspring and parental allele of origin, separately, for both contractions and expansions, no specific transmission pattern is revealed with independent samples T-tests.

Type of instability	n	Largest instability	Mean Magnitude (sd)
Contractions	15	-80	16 (21,0)
Stable transmissions	16	-	-
Expansions	48	+43	13 (10,5)
Statistically significant?	SS	NSS	NSS

Table 3.2 | Summary of the observations on direction and magnitude of instability. SS: statistically significant, NSS: not statistically significant (see text for test statistics and p-values).

Considering contractions, no effect (t=-2.15, p=0.08) for gender of offspring (male: n=6, mean=-30.7, sd=27.8, female: n=9, mean=-6.1, sd=4.3). It must be noted that numbers are small, so that the largest contraction, which occurred in a male mouse, has a relatively big influence on the outcome. There was no difference (t=-7.96, p=0.43) between magnitude of mean male expansion (11.32, sd=10.4, n=19) and mean female expansion (13.79, sd=10.6, n=29). Testing for an effect of parental allele of origin revealed no statistically significant outcome for contractions (t=0.75, p=0.47) nor expansions (t=1.6, p=0.12) (contractions: mean(paternal origin)=-7.7, sd=8.4, n=3, mean(maternal origin)=-18.0, n=12, sd=22.9; expansions: mean(paternal origin)=15.4, sd=11.5, n=22, mean(maternal origin)=10.7, sd=9.2, n=26). Fisher's Exact Test revealed no statistically significant differences in observed frequencies of contractions, stable transmissions and expansions (P=5.70, p=0.33) between the transmission groups. Neither were there statistically significant differences in frequencies of instability categories amongst the transmission groups (P=12.86, sd=0.09). Means of the magnitude of repeat instability are shown in table 2.3 for the different transmission groups.

	Instability category					
Transmission groups	**Δrep < -15**	**-15<Δrep <0**	**Δrep=0**	**0<Δrep<15**	**Δrep>15**	**total**
Male, maternal	-51 (25.9), n=3	-11 (7.5), n=3	n=5	7 (3.9), n=15	28 (10.5), n=4	1 (21.9), n=30
Female, paternal	-17, n=1	-3 (1.4), n=2	n=7	7 (4.0), n=12	26 (8.5), n=10	10 (12.9), n=32
Female, maternal	n=0	-5 (1.0), n=6	n=4	8 (4.4), n=6	16, n=1	2 (7.2), n=17
total	-42 (27.0), n=4	-6 (4.5), n=11	n=16	7 (3.9), n=33	26 (8.9), n=15	5 (16.5), n=79

Table 3.3 | Summary of the mean repeat instability observed in mice specified for the different transmission groups. Displayed are the mean magnitude of instability (sd) and number of observations. See text for the outcome of statistical analyses.

Q-PCR analysis

Since human carriers of the premutation have elevated Fmr1 mRNA levels 3-5, we investigated mRNA levels of mice with different repeat lengths, to determine whether repeat length has an influence on mRNA abundance. mRNA ratios proved to be independent of the amount of input RNA, tested by using two dilutions of input material (data not shown). First we investigated wt mice of different ages and found a two-fold increase of *Fmr1* mRNA in 100-week old wt mice, as compared with 25-week old wt mice. Next we tested *Fmr1* mRNA levels in $(CGG)_n$ mice of different repeat lengths, with reference to age-matched wt mice. 100-week old mice with a $(CGG)_n$ of 110 and 130 trinucleotide units showed a two-fold increase of *Fmr1* mRNA, with reference to age-matched wt mice (data not shown). Figure 3.3 shows *Fmr1* mRNA levels for mice around 25 weeks of age, with reference to 25-week old wt mice. Bars represent two mice in each category, with an exception of category >230, which represents a single mouse. Four combinations of two primer pairs within the *Fmr1* gene and two primer pairs for two distinct reference genes, *Gapdh* and *Rpl13a*, were measured and calculated. Figure 3.3 shows comparison of *Fmr1* mRNA levels as measured with a primer pair amplifying the transition of exon

16 to exon 17 and *Gapdh* as a reference gene (the ratio in wt mice is standardised to 1). This graph is representative for all combinations of primer pairs. Both *Gapdh* and *Rpl13a* showed stable Ct values throughout all CGG length categories, thus proved to be suitable reference genes in this assay. The range of Ct values obtained for the different genes were 26 to 28 for Fmr1, 20 to 22 for *Gapdh* and 19 to 21 for *Rpl13a*. Throughout all repeat length categories an approximately two-fold increase of the *Fmr1* mRNA is observed with reference to the age-matched wt, with exception of the single observation of the mouse in category >230, which shows a five-fold increase.

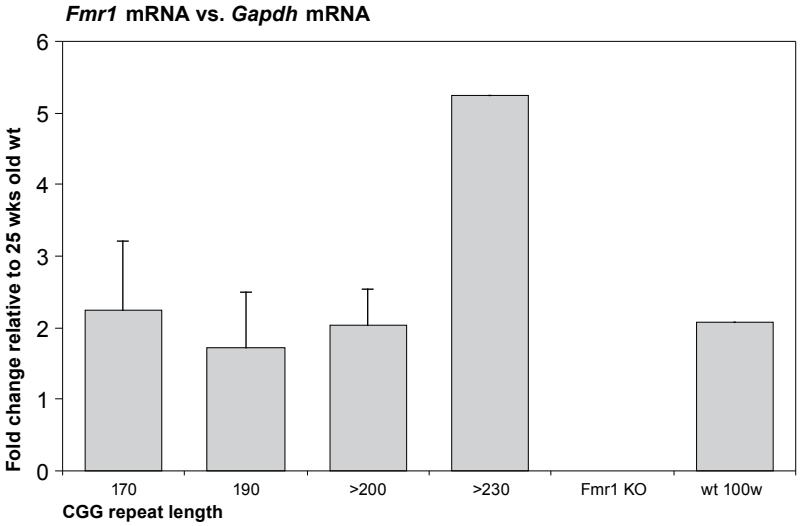

Figure 3.3 | *Fmr1* mRNA levels are shown relative to 25-week old mice. Bars represent two mice of roughly the same repeat length (SEM were 0.9, 0.8 and 0.5 respectively for 170, 190 and >200 CGGs), with exception of >230, which only represents one mouse.

Protein levels

After finding elevated *Fmr1* mRNA levels in a mouse with over 230 CGGs, we were interested in *Fmr1* protein levels in these mice. We performed Western blotting on whole brain lysates and found significantly reduced Fmrp levels (75%, 45% and 40% of wt levels respectively (corrected for input material)) in three mice with approximately 230 CGGs, one of which (C), with the longest repeat tract, is the mouse that showed five-fold *Fmr1* mRNA levels (figure 3.4).

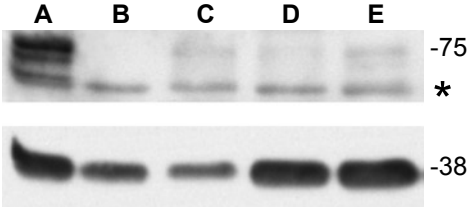

Figure 3.4 | Western blot showing Fmrp isoforms (at 70-80kD) of wt (A), *Fmr1* KO mouse (B) and mice with approximately 230 CGGs (C, D and E). Mouse C,D and E show clearly reduced Fmrp levels (75%, 45% and 40% for C, D and E respectively). The asterisk marks a background band. Synapthophysin (at 38 kD) was used as a loading control.

Immunohistochemistry

It was of interest to investigate the decrease in Fmrp expression *in vivo*, using immunohistochemistry on brains of two of the three mice tested by Western blotting. Mice with approximately $(CGG)_{\sim 230}$ show significantly diminished Fmrp expression throughout the brain, although detectable Fmrp remained (figure 3.5). Strikingly, the CA2 and CA3 region of the hippocampus showed the highest levels of FMRP expression in the ~230 CGG mice. This region, however, is also highly immunoreactive for Fmrp in wt mice.

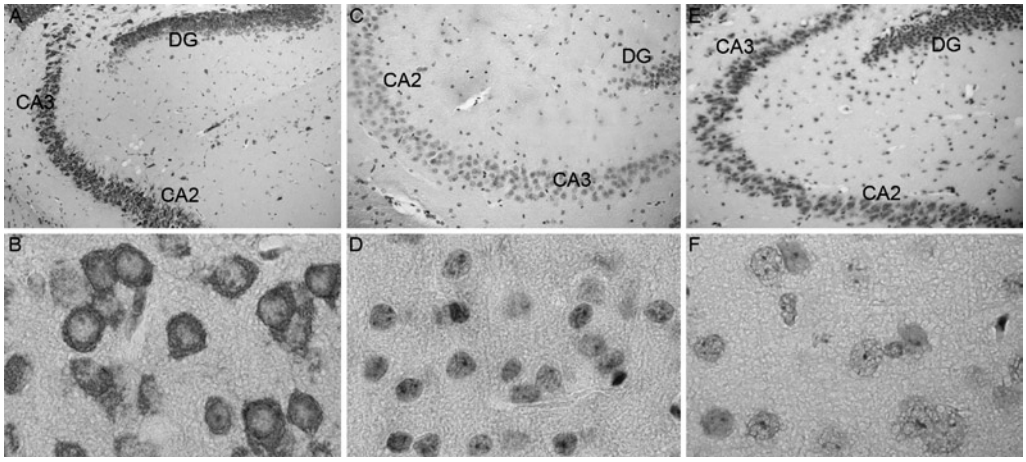

Figure 3.5 | Mouse brains from wt (**A** and **B**), Fmr1 KO (**C** and **D**) and the mouse with >230 CGGs (**E** and **F**) were stained with antibodies against Fmrp. Photos **A**,**C** and **E** show hippocampus and **B**,**D**, and **F** show cortex tissue. DG: dentate gyrus. See page 163 onwards for colour version of this figure.

Methylation status

The decrease in Fmrp expression led to the question of whether the *Fmr1* promoter and $(CGG)_n$ are methylated in mice carrying ~230 repeats. To obtain a general idea thereof, a methylation sensitive digestion was performed, followed by PCR over the promoter region. No PCR products were seen after methylation-sensitive digestion of tail DNA of all male mice tested thus far, suggesting that the restriction site (BssHII) was not methylated (data not shown). We confirmed the unmethylated status of the *Fmr1* promoter by applying bisulphite conversion followed by sequencing of PCR products of the converted DNA. We have successfully applied the bisulphite-conversion method in the past [16,17]. Sequencing revealed complete bisulphite conversion of cytosines to uracil indicating that no methylated cytosines were present in the *Fmr1* promoter region of the starting material (data not shown).

Discussion | 3.4

We have managed to obtain mice carrying full mutation length $(CGG)_n$ in the *Fmr1* locus through selective breeding of a line of mice that were created by knocking in 98 CGGs. In this study, we have characterised these animals for repeat instability during transmission and for expression of the *Fmr1* mRNA and protein. We find that mice carrying large $(CGG)_n$ alleles express increased amounts of mRNA, but significantly reduced levels of Fmrp. These findings are consistent with the observation that the large CGG alleles, which would be considered full mutations in humans, remain unmethylated in male mice.

$(CGG)_n$ instability

In humans, instability of a PM to a FM occurs exclusively upon maternal transmission [18]. Also general instability seems to be greater when the repeat allele is of maternal origin. No effect on instability was observed for gender of offspring in humans [19]. In our mouse model, we find a significant excess of repeat expansions over both contractions and stable transmissions, which is consistent with observations of human premutations. Statistical analysis revealed no significant differences in magnitude or direction of instability for the factors that defined transmissions groups, namely gender of offspring or parental allele of origin.

Different mouse models with random (autosomal) insertion of unstable human PM alleles (maximum uninterrupted CGG stretch of 97 repeats) showed a very high incidence of small instabilities and occasional large deletions, but no large expansions [20,21]. Random integration on an autosome, rather than on the X chromosome and the absence of necessary *cis*-acting factors might explain the different findings. Peier and Nelson described the generation of transgenic mice carrying PM-sized $(CGG)_n$ on a YAC, which enables them to study instability of these sequences within their chromosomal context. When looking at different constructs with varying (uninterrupted) $(CGG)_n$ lengths, they observed length-dependent instability. More expansions, however of smaller magnitude, were found than contractions. Instability of the $(CGG)_n$ was seen in about 17% of transmissions [22].

In humans, AGG interruptions in the $(CGG)_n$ have a stabilising role. Loss of AGG interruptions typically occurs often before a big jump towards the full mutation range, thus reflecting greater instability [23]. The expanded $(CGG)_n$ allele present in the knock-in mouse does not contain AGG interruptions [11]. Thus, it could be expected that greater instability already occurred right after the generation of the mouse model. Our initial report on the instability of an expanded $(CGG)_n$ in the *Fmr1* promoter described a much lower frequency of instability than currently seen [11]. However, in earlier studies a different method was applied to determine $(CGG)_n$ length, so no accurate comparison can be made with the methodology employed in this study. However, no obvious change in magnitude of repeat instability is observed over time. It seems that the risk of becoming unstable upon transmission becomes greater as the $(CGG)_n$ gets longer, rather than the magnitude of the instability.

Many questions remain unanswered as to how, why and when trinucleotide repeat instability occurs. However, some mechanisms have been proposed and some evidence has been given for the underlying mechanism of repeat instability. The observation in human fragile X syndrome that expansions to a full mutation (FM) only occur upon maternal transmission of a premutation allele,

means that repeat expansion must occur during meiosis in the oocyte. Alternatively, it could be due to mitotic events in the embryonic development of the germ line [24,25]. The mosaic pattern, as often seen in Fragile X patients, must result from mitotic contraction or expansion events in the developing embryo, leading to the presence of alleles with repeats in both the FM and PM range [11]. In male Fragile X patients, only PM alleles are present in sperm and testes [26,27], despite the presence of FM in peripheral lymphocytes and other tissues [28]. In a 13-week old FM foetus, no FMRP expression could be demonstrated in primordial germ cells (PGCs), while in a 17-week old FM foetus some primordial germ cells expressed FMRP. It was then concluded by the authors that a PM allele must have been present in the PGCs to support protein production. This suggests that contraction from FM to PM may occur during the maturation of the PGCs, after the initial replication event [25]. In both mouse and human zygotes, the paternal chromatin undergoes active demethylation soon after fertilisation [29]. This process, confined to the paternal pronucleus, occurs prior to DNA replication [30,31]. In mouse, the demethylation process continues up to the morula stage [29]. After completion of the first cell cycle passive demethylation occurs, due to the absence of a maintenance methylase [32]. Soon after implantation, DNA methylation is restored and maintained thereafter [33]. CpG methylation appears to stabilise the $(CGG)_n$ instability [34]. Hence, paternally derived alleles can be prone to deletions upon demethylation. Cells in the testis that have undergone contraction to PM lengths, thus producing FMRP, might be selectively maintained, as FMRP is thought to play a role in gonadal development [35].

Despite the abundance of repetitive DNA in the genome, no general microsatellite instability is found in fragile X patients, thus repeat instability as seen in the *FMR1* gene is limited to the disease locus only. This implies that expansions do not result from aberrant *trans*-acting factors involved in DNA replication, repair and recombination [36].

Repeat instability is dependent on both the repeat length and the purity of the $(CGG)_n$-tract, i.e. the number and locations of AGG interruptions, which in turn determine the likelihood and stability of alternate DNA structures such as hairpins [37]. Also, the location of transcription start sites has been shown to be affected. It was demonstrated that a shift to more upstream transcription initiation sites takes place as the $(CGG)_n$ expands to the premutation range. This was interpreted as that the $(CGG)_n$ might act as a downstream enhancer or modulator of transcription. This phenomenon was observed both in neuronal and non-neuronal cell lines [38]. The expanded $(CGG)_n$ mouse model as described here might provide insights about repeat instability *in vivo*, especially with the expanded range of repeat lengths now available.

Transcription and translation

Q-PCR analysis on RNA isolated from mouse brains revealed that *Fmr1* mRNA levels are two-fold higher in 100-week old wt animals, as opposed to 25-week old wt mice, illustrating that *Fmr1* mRNA is more abundant in old wt mice. On average a two-fold increase of *Fmr1* mRNA levels has been reported at different ages (1-72 weeks) in expanded $(CGG)_n$ mice, when compared with age-matched wt mice [15]. Here we report two-fold elevated *Fmr1* mRNA levels for mice with 110-200 CGGs, in comparison with age-matched wt mice, which is in line with earlier findings in the expanded $(CGG)_n$ mouse [15]. So, based on the current data, in contrast to observations in humans [5,8,9], in mice *Fmr1* mRNA levels do not seem to elevate further with added $(CGG)_n$ length. Interestingly, one exception to this observation was seen

in the mouse with over 230 $(CGG)_n$, which showed a five-fold increase. More mice with repeats of this length should be investigated in the future, in order to be able to assess to what extent mice resemble humans with regard to *Fmr1* mRNA levels. Also, it should be taken into account that in mice brain mRNA was measured, whereas findings in humans represent blood mRNA. Tassone and co-workers have shown that *Fmr1* mRNA levels, relative to a reference gene, are higher in brain than in blood in humans. However, the ratio of *Fmr1* mRNA levels in PM carriers is higher in blood (3.8x) than in brain (1.5x) [39]. Thus our observations of a two-fold elevation of Fmr1 mRNA levels in $(CGG)_n$ mice seems to mimic the human situation.

The underlying molecular mechanism of the elevated *FMR1* transcript levels has not been defined yet. Tassone and colleagues were the first to report elevated *FMR1* mRNA levels (at least five-fold) in leukocytes of carriers of the high PM range (100-200 CGGs), in combination with a reduced percentage of FMRP-immunoreactive cells [4]. They proposed a model wherein an increase of transcription compensates for a diminished translational efficiency that might occur due to an expanded $(CGG)_n$, in an attempt to overcome an arising protein deficit. A (linear) correlation between *FMR1* mRNA levels and repeat size was recently confirmed in a large cohort of both males and females, among whom were carriers of common, intermediate and PM alleles [8]. Similar correlations between *FMR1* transcript levels and repeat length were observed in human lymphoblastoid cell lines of PM carriers. Also, an inverse correlation was observed in these cell lines between FMRP levels and repeat length [5,9]. Primerano and co-workers found an up to five-fold increase of *Fmr1* mRNA in a cell line of a carrier with 195 repeats [9], while in the study by Kenneson and colleagues transcription levels did not exceed a three-fold increase when repeat length approached 200 units [5]. Both authors explained their observations by the occurrence of a translational defect that arises as the mRNA carries a longer CGG tract. Primerano *et al* revealed a dramatic change in translational efficiency of *Fmr1* mRNA specifically; they demonstrated a reduced percentage of *Fmr1* mRNA associated with polysomes in cell lines expressing a longer $(CGG)_n$ tract. However, they showed that a $(CGG)_n$ does not impair overall translational activity [9].

When comparing our own observations in mice with what has been described for human PM carriers, it appears that the positive linear relation between $(CGG)_n$ length and *Fmr1* transcript levels is absent. Furthermore, in humans the increase in *FMR1* mRNA levels as compared to controls was greater than in mice, although in the study performed by Kenneson and colleagues this increase was less pronounced [5]. However, the mouse carrying the largest CGG expansion detected in our lab thus far, did exhibit a five-fold increase in *Fmr1* mRNA levels, when compared to age-matched wild type control mice. It must be stressed that this was only observed in a single mouse. This mouse also showed a striking difference in protein expression level in brain, as visualised with immunohistochemistry. Brain tissue of a second mouse with approximately 230 CGGs showed the same decreased Fmrp expression, despite the two-fold increase of *Fmr1* mRNA. Throughout the brain a remarkable decrease in Fmrp expression could be detected, which was confirmed with Western blot. Interestingly, the CA2 and CA3 region of the hippocampus showed the strongest Fmrp expression. This is a region with the highest Fmrp expression in wt mice.

It is tempting to regard this particular expanded $(CGG)_n$ mouse, with the longest repeat tract as yet and the highest *Fmr1* mRNA levels, as a parallel to the human situation where at the end of the PM

range mRNA levels increase significantly and the number of FMRP-immunoreactive lymphocytes drops slightly, reflecting a decrease in translation [3,4]. However, it is too early to draw conclusions, since, due to limited availability of mice with repeat tracts this long; thus far this has only been a single observation. Hence, in the near future more mice with repeat lengths over 230 CGGs should be investigated in order to be able to distinguish a parallel to the human situation from an outlier.

To explain the impaired translation, many authors refer to a study performed by Feng *et al.* in 1995, in which they showed that the 40S ribosomal subunit is hampered in scanning for initiation codons when the *FMR1* mRNA contains over 200 CGGs [40]. However, individuals carrying an unmethylated full mutation have been reported, who showed normal FMRP expression in their EBV-transformed lymphoblasts and show normal cognitive functioning [41]. The observation that mice with repeat lengths of over 200 trinucleotide units, which in humans would constitute a full mutation, still express Fmrp confirms that in murine cells ribosomes are still capable of translating mRNAs carrying $(CGG)_n$ of this size.

When considering that a FM in humans is defined as a $(CGG)_n$ length greater than 200 CGG triplets, and that these alleles are typically hypermethylated [2,42], it can be concluded that a different situation occurs in mice carrying *Fmr1* $(CGG)_n$ of this length. It is as yet unknown whether a mechanism similar to that seen in humans might happen at a greater repeat length in mice, or whether a transition to a silenced *Fmr1* gene might never take place in mice. It is possible that certain methyltransferases that are responsible for CpG methylation of the *FMR1* promoter and the $(CGG)_n$ in humans are not present in mice. In embryonic development, differences exist in (de)methylation mechanisms between man and mouse. Active demethylation occurs up to a later stage in mouse, as compared with in humans [29]. After the first cell cycle, passive demethylation occurs, as a result of the absence of a maintenance methylase [32]. It could be that similar mechanisms play a role in mice with respect to the *Fmr1* gene, preventing this region from becoming methylated once the CGG tract exceeds 200 CGGs.

This mouse model provides an excellent tool to investigate whether methylation and silencing of the *Fmr1* gene takes place in mice. In case a mechanism similar to that in humans does occur, the events that trigger methylation, the timing and sequence of methylation and gene silencing events can be studied in detail with special emphasis to the gonads. Furthermore, it will be interesting to perform behavioural tests in mice with lowered protein expression and possibly in mice with silenced genes to investigate learning and memory processes.

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References |

1. Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS *et al.* 1991. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67:1047-1058.
2. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP *et al.* 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905-914.
3. Tassone F, Hagerman RJ, Chamberlain WD, Hagerman PJ. 2000. Transcription of the FMR1 gene in individuals with fragile X syndrome. Am J Med Genet 97:195-203.
4. Tassone F, Hagerman RJ, Taylor AK, Gane LW, Godfrey TE *et al.* 2000. Elevated levels of FMR1 mRNA in carrier males: A new mechanism of involvement in the Fragile-X syndrome. Am J Hum Genet 66:6-15.
5. Kenneson A, Zhang F, Hagedorn CH, Warren ST. 2001. Reduced FMRP and increased FMR1 transcription is proportionally associated with CGG repeat number in intermediate-length and premutation carriers. Hum Mol Genet 10:1449-1454.
6. Sherman SL. 2000. Premature Ovarian Failure among Fragile X Premutation Carriers: Parent-of-Origin Effect? Am J Hum Genet 67:11-13.
7. Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R *et al.* 2001. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 57:127-30.
8. Allen EG, He W, Yadav-Shah M, Sherman SL. 2004. A study of the distributional characteristics of FMR1 transcript levels in 238 individuals. Hum Genet 114:439-447.
9. Primerano B, Tassone F, Hagerman RJ, Hagerman P, Amaldi F *et al.* 2002. Reduced FMR1 mRNA translation efficiency in Fragile X patients with premutations. RNA 8:1-7.
10. Grigsby J, Brega AG, Jacquemont S, Loesch DZ, Leehey MA *et al.* 2006. Impairment in the cognitive functioning of men with fragile X-associated tremor/ataxia syndrome (FXTAS). J Neurol Sci. 248:227-33
11. Bontekoe CJ, Bakker CE, Nieuwenhuizen IM, van Der Linde H, Lans H *et al.* 2001. Instability of a (CGG)(98) repeat in the Fmr1 promoter. Hum Mol Genet 10:1693-9.
12. Van Dam D, Errijgers V, Kooy RF, Willemsen R, Mientjes E *et al.* 2005. Cognitive decline, neuromotor and behavioural disturbances in a mouse model for Fragile-X-associated tremor/ataxia syndrome (FXTAS). Behavioural Brain Research 162:233-239.
13. Gabel LA, Won S, Kawai H, McKinney M, Tartakoff AM *et al.* 2004. Visual Experience Regulates Transient Expression and Dendritic Localization of Fragile X Mental Retardation Protein. J Neurosci 24:10579-10583.
14. Bakker CE, de Diego Otero Y, Bontekoe C, Raghoe P, Luteijn T *et al.* 2000. Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse. Exp Cell Res 258:162-70.
15. Willemsen R, Hoogeveen-Westerveld M, Reis S, Holstege J, Severijnen L. *et al.*. 2003. The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. Hum Mol Genet 12:949-59.
16. Pietrobono R, Pomponi MG, Tabolacci E, Oostra B, Chiurazzi P *et al.* 2002. Quantitative analysis of DNA demethylation and transcriptional reactivation of the FMR1 gene in fragile X cells treated with 5-azadeoxycytidine. Nucleic Acids Res 30:3278-85.
17. Stoyanova V, Oostra BA. 2004. The CGG Repeat and the FMR1 Gene. Methods Mol Biol 277:173-84.
18. Rousseau F, Heitz D, Biancalana V, Blumenfeld S, Kretz C *et al.* 1991. Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. N Engl J Med 325:1673-1681.
19. Nolin SL, Brown WT, Glicksman A, Houck Jr GE, Gargano AD *et al.* 2003. Expansion of the Fragile X CGG Repeat in Females with Premutation or Intermediate Alleles. Am J Hum Genet 72:454-464.
20. Lavedan C, Grabczyk E, Usdin K, Nussbaum RL. 1998. Long uninterrupted CGG repeats within the first exon of the human FMR1 gene are not intrinsically unstable in transgenic mice. Genomics 50:229-240.
21. Fleming K, Riser DK, Kumari D, Usdin K. 2003. Instability of the fragile X syndrome repeat in mice: the effect of age, diet and mutations in genes that affect DNA replication, recombination and repair proficiency. Cytogenet Genome Res 100:140-6.
22. Peier A, Nelson D. 2002. Instability of a premutation-sized CGG repeat in FMR1 YAC transgenic mice. Genomics 80:423-432.
23. Poon PM, Chen QL, Zhong N, Lam ST, Lai KY *et al.* 2006. AGG interspersion analysis of the FMR1 CGG repeats in mental retardation of unspecific cause. Clin Biochem 39:244-248.
24. Moutou C, Vincent MC, Biancalana V, Mandel JL. 1997. Transition from premutation to full mutation in fragile X syndrome is likely to be prezygotic. Hum Mol Genet 6:971-979.
25. Malter HE, Iber JC, Willemsen R, De Graaff E, Tarleton JC *et al.* 1997. Characterization of the full fragile X syndrome mutation in fetal gametes. Nature Genet 15:165-169.

26. Tassone F, Hagerman RJ, Gane LW, Taylor AK. 1999. Strong similarities of the FMR1 mutation in multiple tissues: postmortem studies of a male with a full mutation and a male carrier of a premutation. Am J Med Genet 84:240-4.
27. Reyniers E, Martin JJ, Cras P, Van Marck E, Handig I *et al.* 1999. Postmortem examination of two fragile X brothers with an FMR1 full mutation. Am J Med Genet 84:245-9.
28. Reyniers E, Vits L, De Boulle K, Van Roy B, Van Velzen D *et al.* 1993. The full mutation in the FMR-1 gene of male fragile X patients is absent in their sperm. Nature Genet 4:143-146.
29. Fulka H, Mrazek M, Tepla O, Fulka J, Jr. 2004. DNA methylation pattern in human zygotes and developing embryos. Reproduction 128:703-8.
30. Mayer W, Niveleau A, Walter J, Fundele R, Haaf T. 2000. Demethylation of the zygotic paternal genome. Nature 403:501-2.
31. Oswald J, Engemann S, Lane N, Mayer W, Olek A *et al.* 2000. Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10:475-8.
32. Monk M, Adams RL, Rinaldi A. 1991. Decrease in DNA methylase activity during preimplantation development in the mouse. Development 112:189-92.
33. Monk M, Boubelik M, Lehnert S. 1987. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development 99:371-82.
34. Nichol Edamura K, Pearson CE. 2005. DNA Methylation and Replication: Implications for the "Deletion Hotspot" Region of FMR1. Hum Genet 118:301-304.
35. Tamanini F, Willemsen R, van Unen L, Bontekoe C, Galjaard H *et al.* 1997. Differential expression of FMR1, FXR1 and FXR2 proteins in human brain and testis. Hum Mol Genet 6:1315-1322.
36. Pearson CE, Edamura KN, Cleary JD. 2005. Repeat instability: mechanisms of dynamic mutations. Nat Rev Genet 6:729-42.
37. Pearson CE, Eichler EE, Lorenzetti D, Kramer SF, Zoghbi HY *et al.* 1998. Interruptions in the triplet repeats of SCA1 and FRAXA reduce the propensity and complexity of slipped strand DNA (S-DNA) formation. Biochemistry 37:2701-8.
38. Beilina A, Tassone F, Schwartz PH, Sahota P, Hagerman PJ. 2004. Redistribution of transcription start sites within the FMR1 promoter region with expansion of the downstream CGG-repeat element. Hum Mol Genet 13:543-549.
39. Tassone F, Hagerman RJ, Garcia-Arocena D, Khandjian EW, Greco CM *et al.* 2004. Intranuclear inclusions in neural cells with premutation alleles in fragile X associated tremor/ataxia syndrome. J Med Genet 41:E43.
40. Feng Y, Zhang FP, Lokey LK, Chastain JL, Lakkis L *et al.* 1995. Translational suppression by trinucleotide repeat expansion at FMR1. Science 268:731-734.
41. Smeets H, Smits A, Verheij CE, Theelen J, Willemsen R *et al.* 1995. Normal phenotype in two brothers with a full FMR1 mutation. Hum Mol Genet 4:2103-2108.
42. Oberlé I, Rousseau F, Heitz D, Kretz C, Devys D *et al.* 1991. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. Science 252:1097-1102.

CHAPTER 4

Lamin A/C dysregulation and cellular stress response in fragile X-associated tremor/ataxia syndrome

Submitted in extended version to Brain

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Abstract |

Fragile X-associated tremor/ataxia syndrome (FXTAS) is an adult-onset neurodegenerative disorder that is limited almost exclusively to adult carriers of premutation alleles (55 to 200 CGGs) of the *FMR1* gene. We have discovered that the neurofilament protein isoforms, lamin A/C, are dysregulated in CNS tissue from nine patients with FXTAS, generally with dramatic reductions in lamin A/C solubility, compared to CNS tissue from three controls. Moreover, there also appears to be a cellular stress response accompanying expression of the expanded $(CGG)_n$ *FMR1* mRNA in neural tissues of six *post mortem* cases of FXTAS, as evidenced by altered expression of the heat shock proteins (Hsp), αB-crystallin, Hsp70, and Hsp27. We have also observed remarkably similar features of cellular pathology in cultured skin fibroblasts from four patients with FXTAS, including altered nuclear architecture of lamin A/C, abnormal nuclear morphology, and an elevated Hsp response. Thus, dysregulation in skin fibroblasts may represent an important peripheral manifestation of the pathogenesis of FXTAS. Finally, in mice harboring large premutation expansions (~200 CGGs), we observe a corresponding lamin A/C dysregulation in adult as well as embryonic fibroblasts; this last observation raises the possibility that, in humans, at least some of the neurodevelopmental abnormalities observed in a limited number of children with premutation alleles may be due to a similar pathogenic mechanism involving RNA toxicity.

Introduction | 4.1

Fragile X-associated tremor/ataxia syndrome (FXTAS) is an adult-onset neurodegenerative disorder that is largely restricted to older adult carriers of premutation alleles (55 to 200 CGGs) of the fragile X mental retardation 1 (*FMR1*) gene (OMIM *309550)(recent reviews on FXTAS [2,3-5]). The principal features of FXTAS include action tremor and gait ataxia; with associated, more variable features that include cognitive decline with disinhibition and executive function deficits, mild Parkinsonism, peripheral neuropathy and autonomic dysfunction [1,2,6-13]. Radiological features of FXTAS include loss of brain volume involving the cerebrum, brain stem and cerebellum, and white matter disease in the periventricular and subcortical regions, with bilateral high signal lesions in the middle cerebellar peduncles on T2-weighted and FLAIR MRI images (MCP sign) observed in approximately 60% of males and 13% of females with FXTAS [7,9,14-16].

Neuropathological studies of *post mortem* FXTAS brains demonstrate significant white matter disease with spongiosis in both the cerebrum and cerebellum and subcortical astroglial activation, Purkinje cell loss in the cerebellum, and the presence of eosinophilic, intranuclear inclusions in neurons and astrocytes throughout the cortex, subcortical regions, and brainstem (excluding cerebellar Purkinje cells) [17,18]. Studies of the composition of isolated inclusions have revealed the presence of at least thirty proteins, including ubiquitin, lamin A/C isoforms, and the heat shock proteins (Hsps) αB-crystallin, Hsp27, and Hsp70 [19]. Importantly, the inclusions contain the *FMR1* mRNA itself [20], in accord with the proposed RNA-toxicity model for FXTAS [8,18,21,22], wherein the expanded $(CGG)_n$ RNA itself triggers the pathogenic process leading to FXTAS.

We have previously reported the presence of lamin A/C protein isoforms (*LMNA* gene; OMIM *150330) within the intranuclear inclusions of neurons and astrocytes from patients with FXTAS, as well as disruption of the nuclear lamin A/C architecture in both neural cells from patients with FXTAS and in neural cell culture [19,23]. Given that specific mutations in the *LMNA* gene cause a form of peripheral neuropathy (Charcot-Marie-Tooth type 2B1; OMIM *605588), and that peripheral neuropathy is a prominent clinical feature of FXTAS [24-26], we have proposed that FXTAS may represent, at least in part, a functional laminopathy [23]. However, to date, the fundamental limitation with the study of FXTAS pathogenesis has been that research in patients with FXTAS was limited to studies of either CNS neural cells (neurons and astrocytes) from *post mortem* brain tissue, or to studies of transfected neural cells in culture.

We now report that two of the central features of CNS cellular dysfunction in FXTAS, namely, disorganisation of the lamin A/C nuclear architecture and a cellular stress response, are recapitulated in skin fibroblasts from patients with FXTAS. Therefore, skin fibroblasts may provide a powerful tool to study the CNS cellular dysregulation in FXTAS.

Materials and Methods | 4.2

Subjects

Post mortem tissue samples were obtained in accordance with University of California, Davis, IRB approved protocols. Brain autopsies were performed in a standard fashion as described [17]. All subjects contributing skin biopsies were participants in a multi-centre study to characterise neurological findings in premutation carriers, after signing

informed consent. Institutional Review Boards at RUSH University Medical Centre and the University of California, Davis, School of Medicine approved the study. Characteristics of individuals contributing autopsy or biopsy materials are listed in Table 1a.

Post mortem cases were referred to our centre because of FXTAS symptoms including tremor and/or ataxia. The clinical and neuropathological features of FXTAS Cases 1, 5 - 9, and 11 have been described previously [17,18], and correspond in case number to those presented in Greco *et al*. [17]. Clinical features have also been described for Case 10 [8,27] and Case 12 (Greco *et al*., unpublished). The following cases were not previously reported.

Case 2 was characterised by family members as a brilliant professional who had life-long social anxiety, with alcoholism and obsessional thinking. At age 60, he had a thalamic stroke, followed by neurological deterioration with increasing difficulty swallowing. He had also experienced severe neuropathic pain in both legs for a number of years. Ataxia began at 65 yr; intension tremor was evident but sporadic. He developed cognitive decline and dementia over several years prior to his death. At autopsy, his brain (left hemisphere) showed multiple, scattered cystic infarcts and involving cerebral white matter, basal ganglia, and pons. In addition to vascular hyalinisation, confirmation of the remote ischemic nature of the above-described cystic lesions, multiple microinfarcts were identified. Rare intranuclear inclusions were identified only in astrocytes of the hippocampal endplate.

Case 3 had a long history of ataxia, frequent falling, and deterioration in his handwriting. His MRI demonstrated moderate volume loss and severe white matter disease in basal ganglia, anterior capsule, subcortical regions; and a prominent MCP sign, which confirmed his clinical diagnosis of FXTAS before his death at age 76. *Post mortem* examination of his brain (right cerebrum) showed moderate frontoparietal atrophy. Histopathological evaluation showed pallor and spongiosis of cerebral white matter. Eosinophilic intranuclear inclusions were identified in neurons and astrocytes of the hippocampal endplate.

Case 4 had onset of rest tremor at age 70 and shuffling gait 5 years later, when he was diagnosed as having Parkinson disease. Between ages 79 and 80 he developed increasingly severe dysarthria and dysphagia, requiring feeding tube placement. He was ambulatory, with very occasional falls until age 80 when he deteriorated rapidly, required a walker and, two months after an episode of sepsis, became bedridden and died. There was no reported evidence of cognitive impairment. MRI at age 79 revealed moderate brain atrophy and subcortical white matter disease with punctate lesions in the pons, typical of neuroimaging findings in FXTAS, although he did not have the MCP sign. At autopsy, his brain showed mild, generalised atrophy, and ventriculomegaly. Pigmentation of the substantia nigra was diminished. Microscopic evaluation of sections of frontal cortex stained with hematoxylin and eosin showed intranuclear inclusions in 1% of astrocytes, although no inclusions were observed in cortical neurons. Microscopic studies did confirm the presence of Lewy bodies in the substantia nigra, basal forebrain, and parahippocampal cortex. Amyloid cores, indicative of Alzheimer changes, were identified in the temporal cortex.

Case (PSP) had onset of falls and occasional choking at age 64. At autopsy, the brain (left hemisphere) showed no gross abnormalities. Microscopically, the substantia nigra and locus coeruleus were depleted of dopaminergic neurons and showed reactive astrocytes. Neuronal loss, reactive astrocytosis, and grumose degeneration were seen in the dentate nucleus. Immunostaining with anti-tau antibody identified positive-staining cytoplasmic aggregates in neurons and primarily astrocytes of the basal ganglia, substantia nigra, pons, and dentate nucleus. Flame-shaped tangles were also present. Intranuclear inclusions were not seen. This subject was not affected with FXTAS. The final neuropathological diagnosis was progressive supranuclear palsy (PSP).

	Code	Gender	Age of death	CGG repeats	FXTAS clinical stage[a]	CNS neural inclusions	Reference
Control[b]							
Control 1	946	M	69	30	0	No	
Control 2	1206	M	57	28	0	No	
Control 3	1578	M	53	21	0	No	
FXTAS							
Case 1	1	M	70	113	6	Yes	Case 1 [63,64]
Case 2	1006-05	M	72	62	6	Rare	
Case 3	1007-05	M	75	78	6	Yes	
Case 4	1012-05	M	80	67	6	Rare	
Case 5	142-02	M	66	105	5	Yes	Case [63,64]
Case 6	13-03	M	77	77	6	Yes	Case 6 [63,64]
Case 7	125-02	M	76	88	6	Yes	Case 7 [63,64]
Case 8	58-02	M	75	92	5	Yes	Case 8 [63,64]
Case 9	334-03	M	81	88	5	Yes	Case 9 [63,64]
Case 10	1015-05	M	68	98	5	Yes	Case 10 [63,64]
Case 11	1002-04	M	87	65	4	Yes	Case 11 Case 9 [63,64]
Case 12[c]	468-05	F	52	30, 75	6	NA	Greco *et al* 2007 unpublished results
Other Neurological Disorders							
PSP[d]	30-03	M	70	42	0	No	

Table 4.1a | Characteristics of individuals contributing autopsy material.

[a] FXTAS clinical stages: stages 0/1, uninvolved/equivocal involvement; stage 2, definite tremor or ataxia not interfering with ADLs; stage 3, tremor or ataxia interfering with ADLs; stage 4, use of cane or walker; stage 5, use of a wheelchair; stage 6, bedridden [1], [b] From Maryland Brain Bank, [c] FXTAS concurrent with multiple sclerosis. Tissue analysed was skin biopsy, [d] PSP, Progressive Supranuclear Palsy

Cultured human fibroblasts

Full thickness skin biopsies were performed under local anaesthesia with lidocaine. The biopsy was placed in culture media and later diced under sterile conditions on a culture plate and then plated in T25 flasks in AmnioMAX™-C100 Basal Medium (Gibco, Grand Island, NY) containing 15% AmnioMAX™-C100 Supplement (Gibco). After cells grew out, longer-term cultures were maintained in RPMI-1640 supplemented with 10% foetal bovine serum and 1X penicillin-streptomycin (pen-strep) media. Synchronisation of primary fibroblast cultures was achieved by growing the cell cultures in RPMI-1640 media without foetal bovine serum, at 37° C, 5% CO_2, for four days.

FXTAS Rating Scale

Videotapes from the structured neurological examination, designed to capture the major motor features of FXTAS: tremor, cerebellar dysfunction, and parkinsonism, were scored by a movement disorder neurologist, blinded to the subject's premutation status, utilising the FXTAS Rating Scale, Version 1.0 [28].

Code	Age	CGG repeats (Cultured fibroblasts)	CGG repeats (Blood samples)	FXTAS clinical stage [a] (0-6)	FXTAS Rating Scale [b] (0-226)
Control					
C1 (1027-07)	57	30	30	0	0
C2 (C170)	75	21	20	0	0
C3 (1028-07)	87	22	NA	0	0
P1 (C082)	70	81	79	0	6
P2 (C124)	75	62	60	0	7
P3 (C146)	64	89	80	2	30
P4 (C154)	63	95	91	3	49
P5 (1025-07)	60	27, 106	98	3	72
P6 (C061)	80	98, 113	107	5	136

Table 4.1b | Cultured fibroblasts samples derived from male subjects
[a] FXTAS clinical stages: stages 0/1, uninvolved/equivocal involvement; stage 2, definite tremor or ataxia not interfering with ADLs; stage 3, tremor or ataxia interfering with ADLs; stage 4, use of cane or walker; stage 5, use of a wheelchair; stage 6, bedridden [1]. [b] FXTAS Rating Scale for major motor features, in subjects who underwent videotaping sessions. The ranges for sub domains scored are: Tremor (0-53), ataxia (0-73), and Parkinsonism (0-100) [2]. Fibroblasts were derived from other subjects than the cases summarised in table 4.1a.

FXTAS Clinical Staging Scale

Subjects included in this study were also assigned a rating on a 6-point FXTAS clinical staging scale (Table 1) based on the description of the degree of movement and gait problems, as previously described [1,14].

Molecular Data

***Determination of* (CGG)$_n$ *length* – Genomic DNA** from peripheral blood lymphocytes or fibroblasts was amplified using an enhanced PCR technique as described [29], followed by sizing on polyacrylamide gels [30].

***Quantification of mRNA levels* -** All quantitative analysis of *FMR1* mRNA levels utilised quantitative RT-PCR, using the 7900 Sequence Detector (Applied Biosystems, Foster City, CA), as described [31]. Lamin A/C, αB-crystallin, Hsp27, and Hsp70 mRNA levels were determined by quantitative RT-PCR using gene-specific probe/primer sets synthesised by (Applied Biosystems, Assays On Demand, Foster City, CA). Quantification of mRNA levels in human fibroblasts, and on mouse embryonic fibroblasts (MEF) and adult mouse fibroblasts, was performed on synchronised cell cultures.

Generation of stable, expanded (CGG)$_n$ mouse embryonic fibroblast (MEF) cell lines

The knock-in mice harbouring large expansions (~100-250 CGGs) of the (CGG)$_n$ element in the *Fmr1* gene have been described [32]. A timed mating was set up between a male mouse (~220 CGGs) and a female mouse homozygous for the expanded (CGG)$_n$ (~180 and ~210 CGGs). The pregnant female was sacrificed at day 14-post coitum. Six embryos were processed to make six mouse embryonic fibroblast (MEF) lines. The embryonic sacs and placenta were removed from each embryo. The embryos were placed in PBS. After removal of PBS, the remainder of the embryo was minced. Five ml of trypsin/EDTA (TE) was added per embryo and incubated for 10 minutes at 37˚C while

shaking. The cell-TE suspension was removed and added to DMEM (BioWhittaker) with 10% FCS and 1% pen/strep to neutralise the trypsin. After incubation for 30 minutes at 37˚C with shaking, the cells were filtered and spun down, resuspended in DMEM with 10% FCS and 1% pen-strep, and plated onto a culture dish for each embryo. Cells were split upon reaching confluency.

Mouse adult skin fibroblast lines were generated by cutting a piece of shaved skin of a mouse harbouring a $(CGG)_n$ of about 200 CGGs, and a wild type (wt) control, which were sacrificed at 20 weeks of age. The pieces of skin were minced and left to proliferate in DMEM with 10% FCS and 1% pen-strep. Cells were split upon reaching confluency and cultured until they became established fibroblast lines. MEFs and adult fibroblasts from wt and expanded CGG mice were grown on cover slips and further evaluated for the cellular distribution of lamin A/C and Hsps.

Immunofluorescence microscopy

Cultured cells **-** Primary cultures of dermal human fibroblasts and mouse embryonic and adult skin fibroblasts (10000 cells/ cover slip) were grown on individual glass cover slips in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (JR Scientific, Woodland, CA) and 1X pen-strep (Gibco), and incubated for 48 hours at 37°C and 5.0% CO_2. When cells reached 70% confluency, they were fixed, followed by blocking (Arocena *et al*., 2005). Blocked cover slips were incubated overnight (4°C) with rabbit anti-lamin C (GeneTex Inc., San Antonio, TX; 1:500 dilution) and mouse anti-αB-crystallin (Stressgen, Ann Arbor MI; 1:500 dilution) antibodies. The cellular distributions of Hsp27 and Hsp70, relative to lamin, were studied by incubating fibroblasts with a combination of rabbit anti-lamin C (GeneTex Inc.; 1:500) and mouse anti-Hsp27 (Stressgen; 1:500) antibodies, or mouse anti lamin A/C (BD Transduction Laboratories, CA; 1: 500) and rabbit anti-Hsp70 (Stressgen; 1:1000) antibodies, respectively. Cover slips were washed in PBS-T, and then incubated with secondary antibodies: Alexa 488 goat anti-rabbit (Molecular Probes, Carlsbad, CA; 1:500), and Alexa 555 goat anti-mouse (Molecular Probes; 1:1000) for Hsp27; and Alexa 488 goat anti-mouse (Molecular Probes; 1:500) and Alexa 555 goat anti-rabbit (Molecular Probes; 1:1000) for Hsp70. All samples were counterstained with DAPI (Sigma-Aldrich, St. Louis MO) to define nuclear shape and volume. Slides were examined by fluorescence microscopy (Provis Olympus, Center Valley, PA) to characterise lamin organisation (± uniform ring-like structure) and for presence of Hsp aggregates [23], scoring an average of ~740 cells per subject. Three independent staining experiments were performed per cell line.

Whole tissue – Tissues from *post mortem* brain or skin biopsies were formalin-fixed, and processed for paraffin embedding in standard fashion. Antigen retrieval was accomplished by heating of slides for 30 minutes at 95°C in 0.05 M Tris HCl, pH 9.5 with 0.05% polyoxyethylene (20) sorbitan monolaurate. Slides were allowed to cool to room temperature and washed with PBS-T Slides were incubated in blocking solution (5% goat serum in PBS-T); staining proceeded as outlined above for cultured cells.

One-dimensional Western blot analysis

Frozen frontal cortex was powdered in the presence of liquid N_2, and homogenised (Dounce) in lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 0.1% SDS, 0.05% deoxycholate, 1% triton X-100, pH 7.4). Homogenates were spun at 16,000 *g* at 4°C. Protein concentrations of the supernatants and soluble fractions were quantified using a protein assay kit (#23225, BCA Protein Assay Kit, Pierce Biotechnology, Rockford, IL). Insoluble fractions (pellets) were resuspended in Laemmli sample buffer (62.5 mM Tris-HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue, pH 6.8) and heated at 95°C for 5 minutes. Soluble fractions were diluted in Laemmli sample buffer and heated for 5 minutes at 95°C. Following electrophoresis on 10.5-14% linear gradient Criterion Tris-HCl gels (BioRad, Hercules, CA), proteins were transferred to nitrocellulose membranes. The membranes were subsequently blocked with BLOTTO (5% non-fat dry milk in 100 mM Tris-HCl, 0.9% NaCl, 0.1% polyoxyethylene (20) sorbitan monolaurate, pH 7.5) followed by overnight incubation with primary antibodies: mouse monoclonal anti-lamin A (MAB3540, Chemicon International, Temecula, CA), rabbit polyclonal anti-lamin C (GTX28981, GeneTex, Inc.), rabbit polyclonal anti-Hsp70

(SPA-812, Stressgen), mouse monoclonal anti-Hsp27 (SPA-800, Stressgen), mouse monoclonal anti-αB-crystallin (SPA-222, Stressgen), or mouse monoclonal anti- glyceradehyde 3-phosphate dehydrogenase (GAPDH) (GTX29484, GeneTex, Inc.), diluted in BLOTTO. Following washes with T-TBS (100mM Tris, 150mM NaCl, 0.1% polyoxyethylene (20) sorbitan monolaurate), blots were incubated with horseradish peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Detection of antibodies was accomplished with SuperSignal West Dura Extended Duration Substrate (#34075, Pierce Biotechnology). Autoradiograms were quantified by densitometry using ImageJ [33].

Laser scanning cytometry (LSC)

A set of immunofluorescent stained slides, which included fibroblasts (10000 cells per coverslip) derived from five premutation carriers, with and without FXTAS symptoms, and three age-matched controls (Table 4.1b), were subjected to morphometric analysis of the fibroblast nuclei using an LSC-1 microscope and Wincyte software (Compucyte Corp., Cambridge, MA), as described [23]. The values for the area of the nuclei were acquired based on the contour of DAPI fluorescence. At least 1000 nuclei were acquired and analysed per subject.

Statistics

The analysis of mRNA levels determined by quantitative RT-PCR was as described in Tassone *et al*. [31]. Independent analyses were performed for relative mRNA levels of *FMR1*, Hsp27, Hsp70, lamin A/C and αB-crystallin. No correction for multiple outcomes was applied across independent experiments. A Student's t-test was used to analyze the pair-wise differences between phenotypic groups.

For the analysis of the numbers of normal lamin C rings in cultured human fibroblasts, an average of ~740 cells were counted per subject and scored for the presence of a uniform ring-like structure (a complete/closed ring of lamin staining the inner nuclear membrane). The percentages of cells with complete lamin rings were grouped according to clinical phenotype: FXTAS, stages 3 or greater; asymptomatic carrier; and control. The average percent of rings in three independent staining experiments of premutation carriers and controls were graphed and the standard deviations indicated by error bars. Pair-wise inter-group comparisons used the Student's t-test. For LSC analysis, we scored at least 1,000 nuclei per subject and performed group-wise comparisons using a chi-squared statistical analysis.

Results | 4.3

Post mortem frontal cortex from patients with FXTAS demonstrates both an absolute loss of lamin A/C protein and a repartitioning of the protein into a low-solubility fraction upon extraction

The nuclear intermediate filament protein, lamin A/C was originally identified as a possible participant in the pathogenesis of FXTAS by virtue of its presence in the intranuclear inclusions found in the *post mortem* brain tissue of patients with FXTAS [17-19]. It was subsequently demonstrated that similar inclusions form in cultured neural cells upon expression of reporter mRNAs with expanded $(CGG)_n$ in the 5'UTR region of the message [23]. Although inclusions formed in only a small percentage of the cultured cells, there was a more pervasive and striking collapse of the normal ring-like nuclear architecture of lamin A/C, suggesting that lamin disorganisation and aggregation may participate in the pathogenesis of FXTAS.

To determine whether a relationship exists between the FXTAS phenotype and altered structural organisation of lamin A/C, we examined the properties of these lamin isoforms in post mortem brain tissue from ten males who had died with FXTAS. Included in this initial study were three male controls of comparable ages and a subject who died with an unrelated neurodegenerative disorder,

PSP (Table 4.1a). There was a striking difference between FXTAS cases and controls with respect to absolute lamin levels and the ratio of soluble/insoluble lamin isoforms in the frontal cortex. Western blot analysis of the soluble fraction of protein extracts of *post mortem* frontal cortex tissue from cases with FXTAS and controls (Figure 4.1A) reveals dramatic decreases in the levels of both lamin A and lamin C protein isoforms in nine FXTAS cases. The striking exception is a single case of severe FXTAS (Case 10; lane 13) where there does not appear to be detectable repartitioning of lamin A/C. This case will be considered further below. Interestingly, the PSP case (lane 9), which does not possess the intranuclear inclusions, also demonstrates loss of soluble lamin, suggestive of parallel disease processes in these two neurodegenerative disorders. A subset of these brain tissue samples, 3 FXTAS, 1 PSP, and 2 controls, were analysed further, revealing that at least some of the reduction of lamin A/C observed in patients with FXTAS (and the single PSP case) on the initial Western blots was due to repartitioning of lamin proteins from the soluble extract to a much less soluble form, one that requires boiling the protein extracts in SDS buffer for solubilisation (Figure 4.1B). This redistribution of lamin is consistent with the observed aggregation and change in nuclear lamin architecture seen in cultured cells containing the expanded $(CGG)_n$ [23].

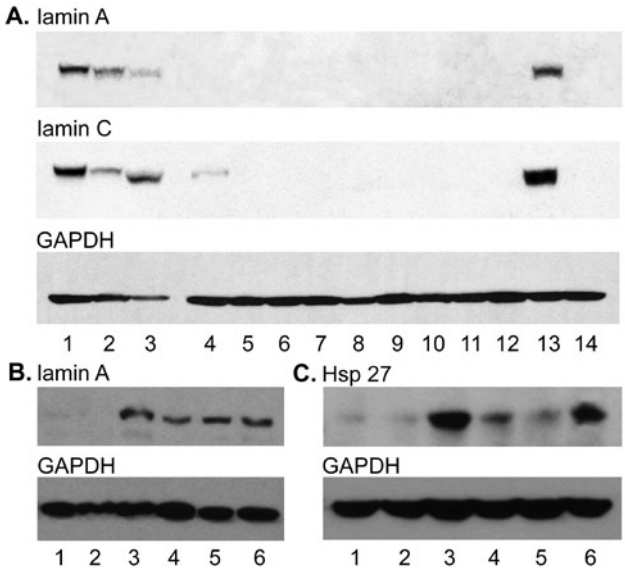

Figure 4.1 | *Post mortem* brain (cerebral cortex) tissue samples from FXTAS patients show reduced levels of soluble lamin A and lamin C, and increased levels of Hsp 27. (**A**) 100 μg of soluble protein from frontal cortex was loaded into each lane. Lamin A and lamin C are detectable in all controls (lanes 1-3). Lamin A is detected in only one case (lane 13, Case 10). Two of the FXTAS cases (lanes 4 and 13, Cases 9 and 10) contain detectable lamin C. Neither lamin A nor lamin C is detectable in the soluble fraction obtained from a PSP patient (lane 9). (**B**) Western blot of the insoluble fractions of the *post mortem* tissue reveals an increase in lamin A in FXTAS (lanes 3, 4,and 6) and PSP (lane 5) patients versus controls (lanes 1 and 2). (**C**) 40 μg of soluble protein from frontal cortex was loaded into each lane. FXTAS cases (lanes 3-6) show increased levels of Hsp27 compared to controls (lanes 1 and 2); relative Hsp27 levels (GAPDH loading standard) are 0.9, 1.1, 7.0, 4.1, 2.6 and 6.6, for lanes 1 – 6, respectively, relative to the average expression of the controls. *Post mortem* intervals for FXTAS/PSP cases were shorter (approximately 12 hours or less) than control cases (all greater than 12 hours).

To determine whether the reductions in lamin protein were a consequence of reduced transcription of the *LMNA* gene, *LMNA* mRNA levels from *post mortem* frontal cortex of six FXTAS cases (including Case 10) and two controls were quantified by real time (TaqMan) RT-PCR. There was no reduction in *LMNA* mRNA level for any of the FXTAS cases tested, ruling out reduced mRNA levels as an explanation for the decreased levels of soluble lamin protein. In fact, excluding Case 10, we observed an average 1.7-fold increase (range, 1.2 - 2.5; t-test, p=0.041) in *LMNA* mRNA levels among the cases with FXTAS; inclusion of Case 10, which displayed no increase in *LMNA* mRNA reduced the average increase to 1.6-fold (range, 0.8 - 2.5; p=0.078).

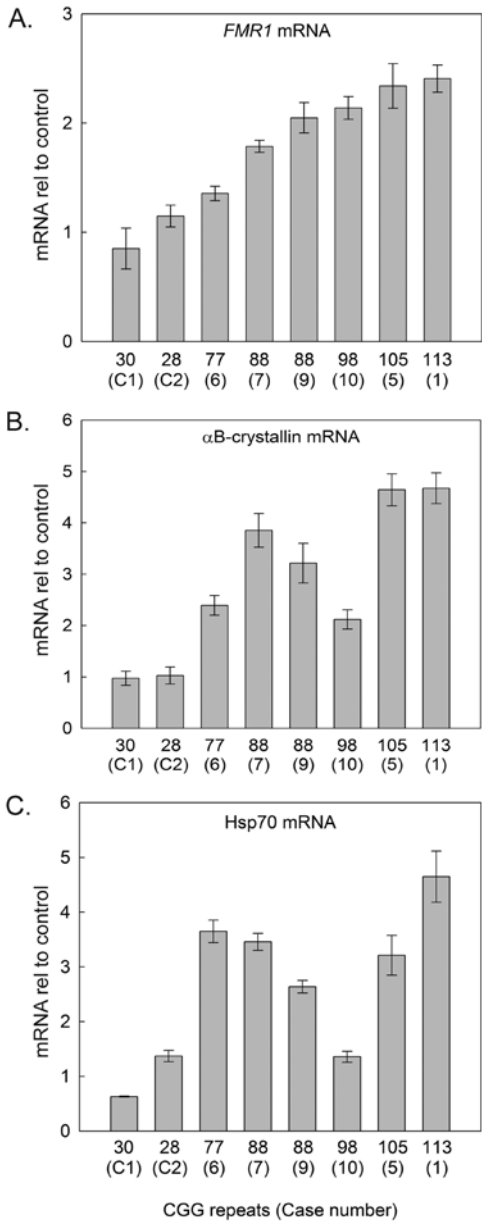

Figure 4.2 | Brain tissue from patients affected with FXTAS express higher levels of *FMR1* mRNA and stress response genes compared to the controls in the same age range. (**A**) *FMR1;* (**B**) αB-crystallin; (**C**) Hsp70. Cases are displayed according to increasing number of CGG repeats (case number).

To confirm that the FXTAS samples being tested were expressing higher than normal levels of *FMR1* mRNA, as expected for neural cells expressing from premutation alleles, the same tissue samples from 6 patients with FXTAS and 2 control subjects were analysed for levels of *FMR1* mRNA. There was an average increase of 2.0-fold compared to controls (range, 1.4 - 2.4; p=0.012), including Case 10, which itself was elevated by 2.1-fold. Thus, the absence of a lamin repartitioning in Case 10 was not due to lowered *FMR1* mRNA expression (Figure 4.2A). These results are consistent with previous findings of increased *FMR1* mRNA expression in FXTAS brain tissue [20].

FXTAS also involves a cellular stress response with induction of heat-shock proteins αB-crystallin, Hsp 27, and Hsp 70

Like lamin A/C, αB-crystallin is also found within the inclusions formed in cultured neural cells upon expression of transfected expanded $(CGG)_n$ reporter RNA [23]. To determine whether αB-crystallin is induced in FXTAS brain tissue, relative levels of αB-crystallin mRNA were determined in frontal cerebral cortex samples from six FXTAS and two control cases. The samples from FXTAS brain tissue express higher levels of αB-crystallin mRNA compared to the controls: an average 3.5-fold increase (range, 2.1-4.7-fold; p = 0.0026), including Case 10 (Figure 4.2B).

The same set of samples was also analysed to quantify the expression levels of Hsp70 and Hsp27 mRNAs. Our results indicate that mRNA levels of Hsp70 are significantly elevated in FXTAS frontal cortex, compared to age-matched controls; an average of 3.2-fold increase (range, 1.4 – 4.7-fold; p=0.018), again including Case 10 (Figure 4.2C). There was no significant increase in Hsp27 mRNA level for the current samples, due in part to greater variation in Hsp27 mRNA levels within the control group (data not shown). However, examination of Hsp27 protein levels in frontal cortex revealed increases in several of the FXTAS cases compared to controls (Figure 4.1C). Taken together, the current data provide evidence for a stress (heat-shock) response to the expanded $(CGG)_n$ *FMR1* mRNA, as reflected by either/both increased Hsp mRNA or protein levels.

Dysregulation of lamin A/C in human skin fibroblasts mirrors the abnormalities found in neural cells

One of the fundamental limitations inherent in characterisation of the FXTAS cellular phenotype is the inaccessibility of CNS neural tissue. However, based on the knowledge that a number of laminopathies also display altered lamin organisation in dermal fibroblasts, with formation of lamin-containing nuclear aggregates [34-39], we explored the possibility that lamin A/C architecture is similarly altered in the skin fibroblasts of premutation carriers with FXTAS. Our initial approach was to examine dermal fibroblasts *in situ* from a 52 year old female carrier with alleles of 30 and 75 CGGs (Greco *et al*., unpublished results) who had died with FXTAS and multiple sclerosis, and from a male age-matched control (52 yr) (Figure 4.3). In the control skin fibroblasts, lamin A/C staining was uniform, with a heavier ring-like pattern at the nuclear periphery. By contrast, in the FXTAS case, the normal ring-like distribution of lamin A/C was frequently disrupted or distorted. Interestingly, some nuclei in the patient with FXTAS appear to have essentially normal morphology, presumably representing those cells where the normal allele ($(CGG)_{30}$) is active. The immunofluorescent patterns, using antibodies directed to either lamin A/C, lamin A or lamin C, gave the same results. These observations strongly indicate that expression of the expanded $(CGG)_n$ *FMR1* mRNA leads to cellular dysregulation in human dermal fibroblasts.

To extend these results obtained with *post mortem* skin cells to living cells, fibroblasts were cultured from four affected male carriers (FXTAS ratings of 30, 49, 72, and 136; clinical stages of 2, 3, 3, and 5, respectively), two premutation males with no clinical signs of FXTAS, and three male controls with normal alleles (Table 4.1b). The $(CGG)_n$ numbers present in the cultured fibroblasts are approximately the same as the alleles observed in blood samples from the same subjects; with the exception of subject P5, who displays allele-size mosaicism (27 and 106 CGGs) in cultured fibroblasts. The levels of *FMR1* mRNA were quantified for each fibroblast culture, demonstrating that *FMR1* expression is upregulated by an average of 1.7-fold (p=0.025) in fibroblasts derived from the premutation carriers

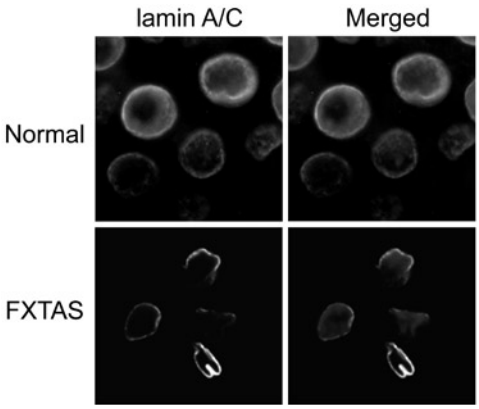

Figure 4.3 | *Post mortem* skin biopsy in a case (468-05) with **FXTAS displays frequently distorted nuclear ring-like distribution of lamin A/C** (lower panel) compared to an age-matched control (upper panel). Green, lamin A/C; blue, DAPI staining of nuclei. See page 163 onwards for colour version of this figure.

(n= 6) with and without FXTAS, relative to normal controls, in agreement with previous observations in both neural and non-neural cells [20,31].

Immunocytochemical staining with lamin C antibodies revealed a striking disruption of the normal nuclear shape and the normal ring-like pattern in fibroblasts from three cases with moderate to severe FXTAS (P3-P6 with clinical stage 3 or greater) (Figure 4.4). For these three cases, we observed the ring-like lamin architecture in an average of only 26 (± 7)% of 2,667 cells scored, relative to an average of 74 (± 13)% of 2,008 cells from controls (t-test; p=0.0095). This result mirrors our earlier observation of lamin disruption in cultured neural cells transfected with expanded $(CGG)_n$ [23]. Remarkably, we see an average decrease in lamin rings for the two asymptomatic carriers (P1 and P2) and the mildly affected case (P3; stage 2) relative to the normal controls (number of lamin ring nuclei/total nuclei: 1466/2008, controls; 825/1975, non- and mildly-affected premutation carriers; Fisher's exact test; p <0.0001); a comparison of the means leads to the same conclusion (student's t-test, p = 0.011). These preliminary results suggest that skin fibroblasts may report early states of the cellular dysfunction that eventually leads to FXTAS. Given the small number of asymptomatic carriers examined thus far, it would be premature to draw conclusions regarding incipient pathology or the likelihood of progression; however, our observations with asymptomatic carriers provide motivation for additional molecular-clinical and longitudinal studies.

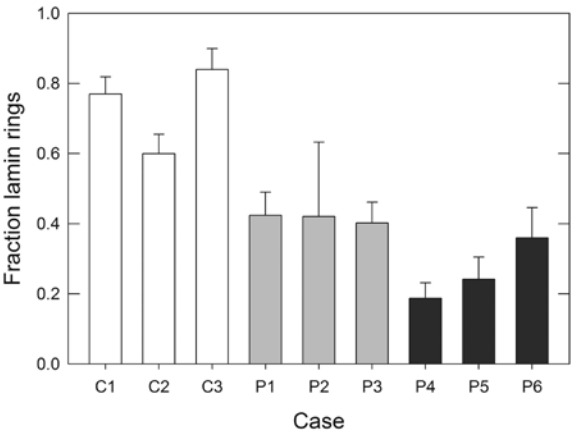

Figure 4.4 | Reduction of normal lamin rings in cultured fibroblasts derived from FXTAS patients (Table 1b). Open bars, controls; light grey bars, premutation carriers with no symptoms of FXTAS (P1, P2) or mild features (clinical stage 2; P3); dark grey bars, individuals with moderate to severe FXTAS (P4-P6).

A second morphological abnormality previously associated with expression of the expanded $(CGG)_n$ in cultured neural cells is increased nuclear volume [23]. We therefore examined the cultured fibroblasts from cases with FXTAS for corresponding changes. For the three cases with FXTAS with moderate to severe FXTAS (stage 3-5), we find an average increase in area of 1.4 (± 0.2) fold (p=0.005). A comparison of fibroblasts derived from a clinical stage 5 FXTAS case (P6; 80 yr) and a normal control (C3; 87 yr) is presented in figure 4.5.

The cellular phenotype of dermal fibroblasts also parallels CNS neural cell dysregulation in FXTAS with respect to a stress response involving over-expression of Hsp27 mRNA

The induction of a cellular stress response in the CNS of FXTAS cases raises the possibility of a corresponding stress response in dermal fibroblasts, since these peripheral cells also over-express the expanded $(CGG)_n$ *FMR1* mRNA. In all fibroblast cultures from patients with FXTAS (n=4; FXTAS clinical stage ≥ 2), the level of Hsp27 mRNA is also elevated by an average 2.7-fold (range 1.7 – 4.1-fold; p=0.037). These results demonstrate that the response to expression of the expanded $(CGG)_n$ in

fibroblasts involves both morphologic changes that parallel those in neural cells, and corresponding biochemical changes involving a stress response.

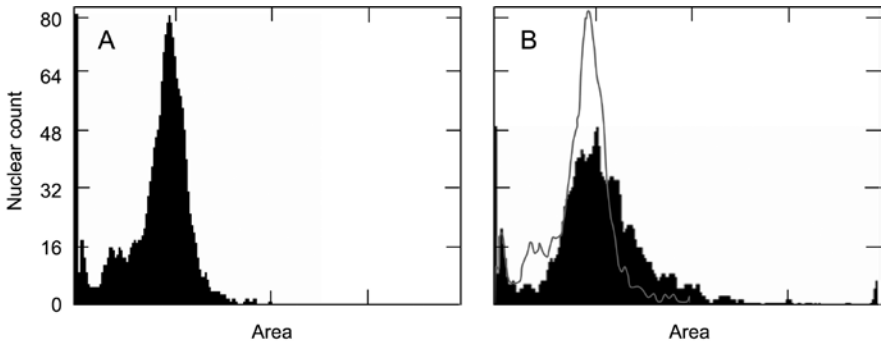

Figure 4.5 | Increased nuclear area in human fibroblasts from a patient with FXTAS. Morphometric values of individual human skin fibroblasts contoured on the basis of DAPI fluorescence. (**A**) Distribution of the nuclear areas of 2013 fibroblasts derived from an 87-year old normal control (C3). (**B**) The nuclear areas of 2009 fibroblasts derived from an 80-year old patient with severe FXTAS (P6) exhibit a broader distribution of nuclear areas, with an average 1.3-fold increase, compared to the age-matched control (overlay tracing of the distribution in part A). See page 163 onwards for colour version of this figure.

Mouse embryonic fibroblasts harbouring large premutation expansions (~200 CGGs) also display an abnormal cellular phenotype involving nuclear lamin A/C dysregulation

Studies of the cellular phenotypes induced by the expanded $(CGG)_n$ *FMR1* mRNA have thus far been largely restricted to tissue samples obtained from adult carriers and controls. However, cellular phenotypes can be induced in a much shorter time-frame in neural cell culture [23], raising the possibility of an early-onset cellular phenotype. As an initial approach to address this possibility, we utilised mouse embryonic fibroblasts (MEFs) derived from knock-in mice with expanded (~200 CGGs), unmethylated *Fmr1* alleles [32]. These animals show elevated *Fmr1* mRNA levels and mildly reduced FMRP levels characteristic of large premutation alleles in humans [31,32].

We quantified the levels of *Fmr1* mRNA in cultured mouse fibroblasts, derived from embryos and adult mice with expanded $(CGG)_n$, relative to wt control fibroblasts. MEFs derived from expanded $(CGG)_n$ mice express a four-fold elevation ($p< 0.001$) in *Fmr1* mRNA levels, compared to MEFs derived from wt controls. Moreover, premutation MEFs express 4.6-fold ($p< 0.01$) more *Fmr1* mRNA than premutation adult mouse fibroblasts, indicating that *Fmr1* expression is very high in fibroblasts during early development and decreases in adulthood. Interestingly, we observed no significant difference in *Fmr1* mRNA levels in adult cultured mouse fibroblasts between expanded-repeat and wt mice.

Remarkably, the MEFs from premutation mice already display evidence of lamin dysregulation, with a 4.7 fold decrease in the number of complete lamin rings ($p < 0.001$, Fisher's exact test) relative to cells derived from wildtype MEFs. Fibroblasts derived from adult expanded repeat mice also display a significant, albeit less pronounced lamin C dysregulation than their MEF counterparts (50% vs 18% of complete lamin rings; $p < 0.001$, Fisher's exact test)**.** This result suggests that the fundamental processes

leading to lamin dysregulation may be at least in part reversible, since the adult mice developing from such embryos do not display the degree of abnormality exhibited by the MEFs.

In addition, we have observed a greater degree of perinuclear αB-crystallin aggregation in the MEFs (Figure 4.6) relative to adults with the same range of expanded repeats. Indeed, although the number of MEFs from expanded repeat mice displaying *both* lamin C and αB-crystallin aggregates is a small percentage of the total cells examined, the reduced number found in adult cells is highly significant (47/380, MEFs; 4/398 adult mice; Fisher exact test, $p < 0.0001$).

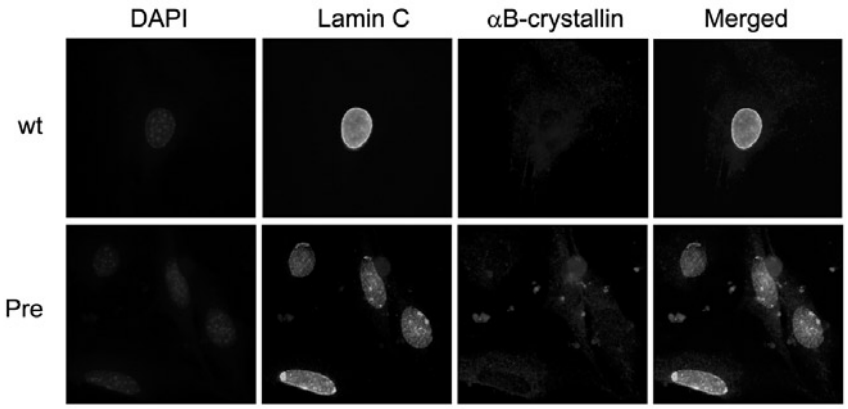

Figure 4.6 | Immunocytochemical localisation of lamin C and αB-crystallin in mouse embryonic fibroblasts. Lower panel: mouse fibroblasts harbouring the expanded-CGG-repeat display irregular nuclear morphology (nuclei counterstained with DAPI, blue), marked lamin C disruption and aggregation with loss of the normal ring-like architecture (green), and an increased number of perinuclear aggregates containing αB-crystallin (red). See page 163 onwards for colour version of this figure.

Discussion | 4.4

To determine whether there is a broader lamin A/C abnormality than simply its presence in intranuclear inclusions [19], which are present in a minority of neural cells, we determined the expression pattern of this protein in frontal cortex from patients with FXTAS and normal controls. Patients with FXTAS clearly express increased levels of *LMNA* mRNA; however, levels of soluble lamin A/C were dramatically reduced, consistent with its aggregation in FXTAS cells. Thus, altered expression of lamin A/C is clearly a component of the CNS cellular response to expression of the expanded-repeat *FMR1* mRNA.

We also demonstrate that the clinical presentation of FXTAS is associated with a cellular stress response that involves at least three Hsps, αB-crystallin, Hsp27 and Hsp70. In frontal cortex from patients with FXTAS and age-matched controls, the transcription levels of αB-crystallin and Hsp70 are significantly elevated (Figure 4.2) though the overall levels of protein seem to be unchanged, perhaps due to altered post-translational regulatory events. By contrast, Hsp27 protein levels are clearly elevated in the frontal cortex from patients with FXTAS (Figure 4.1C). It has long been known that the Hsps function as chaperones in mitigating protein misfolding and aggregation [40-42]. More recently, Hsps have been shown to interact with elements of the cytoskeleton that are adversely affected by several

types of cellular stress ([43-46], reviewed in [47,48]). Our observations suggest that neurons and astrocytes in patients with FXTAS are responding to a form of stress that is triggered by elevated levels of *FMR1* mRNA, and which also leads to altered cytoskeletal structure.

Whereas Case 1 possesses the highest levels of Hsp and lamin A/C mRNAs, and the highest numbers of inclusions in both neurons and astrocytes [17], this patient displayed the mildest neuropathological findings of the five cases [17]. By contrast, Case 6, which has the lowest levels Hsp mRNAs and lamin A/C, displays the most extensive brain abnormalities, involving severe white matter spongiosis with axonal and myelin loss, and extensive cerebellar Purkinje cell dropout. Although there appears to be a similar trend in Cases 5, 7, 9, and 10, the significance of this trend has not been quantified. Such a trend, if established in a larger series, would be in accord with the recently described neuroprotective action of αB-crystallin, Hsp27, and Hsp70 [49-51].

Overexpression of Hsp27 and Hsp70 has been shown to prevent neuronal death in some studies in animal models of neurodegenerative diseases reviewed by [22,49]. However, therapies targeting FXTAS progression (and reversal) will likely need to do more than simply boosting the heat shock response, as recently exemplified in a mouse model for multiple sclerosis [52]. Efforts should be made to target earlier events in FXTAS pathogenesis, including the reduction of excess *FMR1* mRNA in susceptible cells. In view of the significant fraction of FXTAS cases that have peripheral neuropathy [24-26], it is interesting to note that one form of Charcot-Marie-Tooth disease (CMT2F) is due to a missense mutation in the gene encoding Hsp27; the mutant form of the gene appeared to disrupt neurofilament (intermediate filament) assembly [53].

Willemsen *et al*. [54] have demonstrated that mice with expanded repeats (~100 CGGs) developed neuronal intranuclear inclusions in broad distribution throughout the brain. In the current work, we demonstrate that mouse embryonic fibroblasts (MEFs) with expanded $(CGG)_n$ already display dramatic evidence of early lamin dysregulation, although there is no evidence of any upregulation of αB-crystallin mRNA levels.

By analogy to the mouse model demonstrating altered lamin A/C architecture, we propose that, in humans, the impaired cytoskeletal network induced by overexpression of the expanded $(CGG)_n$ *FMR1* RNA will likely have an adverse impact on neural development and differentiation, during which extensive neurofilament rearrangement takes place [43]. Cytoskeletal components have been shown to play a major role in the elaboration of axons and dendrites, and in the proper maintenance of neuronal connectivity and dynamics through adulthood [55]. Many of the clinically affected children who carry a premutation allele have autism spectrum disorders (ASD) [56]. An important neuropathological mechanism leading to ASD involves connectivity deficits, which have been studied by several authors [57,58]. Impaired functional connectivity has been demonstrated on fMRI studies of the amygdala response to fearful stimuli in young adult males with the premutation who do not have neurological symptoms [59]. Therefore, by analogy with our current observations with MEFs, the lamin A/C dysregulation in human embryonic and/or fetal cells may impact the subsequent development of connectivity in the brain of premutation carriers [56,60,61].

In addition to the proposed effects in early development, impaired organisation of the cytoplasmic cytoskeleton impedes axonal transport of organelles and nutrients, eventually leading to

neuronal death [62]. This phenomenon could explain the formation of pathological axonal torpedoes in FXTAS [17,18].

Finally, our observation of parallel cellular phenotypes in CNS and dermal fibroblasts (e.g. lamin disorganisation/aggregation, heat-shock response) indicates that the latter cell type provides an accessible (peripheral) cellular marker of the *FMR1* mRNA-induced cellular pathology that underlies FXTAS. In particular, the cellular status of dermal fibroblasts in premutation carriers may be linked to eventual clinical progression, and may also report efficacy of therapeutic intervention. These possibilities clearly require further investigation.

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References |

1. Bacalman S, Farzin F, Bourgeois JA, Cogswell J, Goodlin-Jones BL *et al.* 2006. Psychiatric Phenotype of the Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) in Males: Newly Described Fronto-Subcortical Dementia. J Clin Psychiatry. 67:87-94.
2. Berry-Kravis E, Abrams L, Coffey SM, Hall DA, Greco C *et al.* 2007. Fragile X-associated tremor/ataxia syndrome: Clinical features, genetics, and testing guidelines. Mov Disord.
3. Hagerman PJ, Hagerman RJ. 2007. Fragile X-associated tremor/ataxia syndrome--an older face of the fragile X gene. Nature clinical practice 3:107-112.
4. Jacquemont S, Hagerman RJ, Hagerman PJ, Leehey MA. 2007. Fragile-X syndrome and fragile X-associated tremor/ataxia syndrome: two faces of FMR1. Lancet neurology 6:45-55.
5. Willemsen R, Mientjes E, Oostra BA. 2005. FXTAS: a progressive neurologic syndrome associated with Fragile X premutation. Current neurology and neuroscience reports 5:405-410.
6. Berry-Kravis E, Lewin F, Wuu J, Leehey M, Hagerman R *et al.* 2003. Tremor and ataxia in fragile X premutation carriers: blinded videotape study. Ann Neurol 53:616-623.
7. Brunberg JA, Jacquemont S, Hagerman RJ, Berry-Kravis EM, Grigsby J *et al.* 2002. Fragile X premutation carriers: characteristic MR imaging findings of adult male patients with progressive cerebellar and cognitive dysfunction. AJNR Am J Neuroradiol 23:1757-1766.
8. Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R *et al.* 2001. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 57:127-130.
9. Jacquemont S, Farzin F, Hall D, Leehey M, Tassone F *et al.* 2004. Aging in individuals with the *FMR1* mutation. Am J Ment Retard 109:154-164.
10. Jacquemont S, Hagerman RJ, Leehey M, Grigsby J, Zhang L *et al.* 2003. Fragile X premutation tremor/ataxia syndrome: molecular, clinical, and neuroimaging correlates. Am J Hum Genet 72:869-878.
11. Jacquemont S, Hagerman RJ, Leehey MA, Hall DA, Levine RA *et al.* 2004. Penetrance of the fragile X-associated tremor/ataxia syndrome in a premutation carrier population. Jama 291:460-469.
12. Leehey MA, Hagerman RJ, Landau WM, Grigsby J, Tassone F *et al.* 2002. Tremor/ataxia syndrome in fragile X carrier males. Mov Disord 17:744-745.
13. Grigsby J, Brega AG, Leehey MA, Goodrich GK, Jacquemont S *et al.* 2007. Impairment of executive cognitive functioning in males with fragile X-associated tremor/ataxia syndrome. Mov Disord 22:645-650.
14. Adams JS, Adams PE, Nguyen D, Brunberg JA, Tassone F *et al.* 2007. Volumetric brain changes in females with fragile X-associated tremor/ataxia syndrome (FXTAS). Neurology 69:851-859.
15. Cohen S, Masyn K, Adams J, Hessl D, Rivera S *et al.* 2006. Molecular and imaging correlates of the fragile X-associated tremor/ataxia syndrome. Neurology. 67:1426-1431.
16. Loesch DZ, Litewka L, Brotchie P, Huggins RM, Tassone F *et al.* 2005. Magnetic resonance imaging study in older fragile X premutation male carriers. Ann Neurol 58:326-330.
17. Greco CM, Berman RF, Martin RM, Tassone F, Schwartz PH *et al.* 2006. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). Brain. 129:243-255. Epub 2005 Dec 2005.
18. Greco CM, Hagerman RJ, Tassone F, Chudley AE, Del Bigio MR *et al.* 2002. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. Brain 125:1760-1771.
19. Iwahashi CK, Yasui DH, An HJ, Greco CM, Tassone F *et al.* 2006. Protein composition of the intranuclear inclusions of FXTAS. Brain. 129:256-271. Epub 2005 Oct 2024.
20. Tassone F, Hagerman RJ, Garcia-Arocena D, Khandjian EW, Greco CM *et al.* 2004. Intranuclear inclusions in neural cells with premutation alleles in fragile X associated tremor/ataxia syndrome. J Med Genet 41:e43.
21. Hagerman PJ, Hagerman RJ. 2004. The fragile-X premutation: a maturing perspective. Am J Hum Genet 74:805-816. Epub 2004 Mar 2029.
22. Jin P, Zarnescu DC, Zhang F, Pearson CE, Lucchesi JC *et al.* 2003. RNA-mediated neurodegeneration caused by the fragile X premutation rCGG repeats in *Drosophila*. Neuron 39:739-747.
23. Arocena DG, Iwahashi CK, Won N, Beilina A, Ludwig AL *et al.* 2005. Induction of inclusion formation and disruption of lamin A/C structure by premutation CGG-repeat RNA in human cultured neural cells. Hum Mol Genet 14:3661-3671. Epub 2005 Oct 3620.
24. Berry-Kravis E, Goetz CG, Leehey MA, Hagerman RJ, Zhang L *et al.* 2007. Neuropathic features in fragile X premutation carriers. American journal of medical genetics 143:19-26.

25. Hagerman RJ, Coffey SM, Maselli R, Soontarapornchai K, Brunberg JA *et al.* 2007. Neuropathy as a presenting feature in fragile X-associated tremor/ataxia syndrome. American journal of medical genetics 143A:2256-2260.
26. Soontarapornchai K, Maselli R, Fenton-Farrell G, Tassone F, Hagerman PJ *et al.* 2007. Abnormal nerve conduction features in fragile X premutation carriers. Ann Neurol In Press.
27. Bourgeois JA, Farzin F, Brunberg JA, Tassone F, Hagerman P *et al.* 2006. Dementia with mood symptoms in a fragile X premutation carrier with the fragile X-associated tremor/ataxia syndrome: clinical intervention with donepezil and venlafaxine. J Neuropsychiatry Clin Neurosci. 18:171-177.
28. Leehey MA, Goetz CG, Berry-Kravis E, Zhang L, Hall DA *et al.* 2007. Development of the FXTAS Rating Scale for quantitative motor analysis. Mov Disord In Press.
29. Saluto A, Brussino A, Tassone F, Arduino C, Cagnoli C *et al.* 2005. An enhanced polymerase chain reaction assay to detect pre- and full mutation alleles of the fragile x mental retardation 1 gene. J Mol Diagn 7:605-612.
30. Tassone F, Pan R, Amiri K, Taylor A, Hagerman PJ. 2007. A rapid PCR-based screening method for identification of all expanded alleles of the fragile X (*FMR1*) gene in newborn and high-risk populations. J Mol Diagn:In Press.
31. Tassone F, Hagerman RJ, Taylor AK, Gane LW, Godfrey TE *et al.* 2000. Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. Am J Hum Genet 66:6-15.
32. Brouwer JR, Mientjes EJ, Bakker CE, Nieuwenhuizen IM, Severijnen LA *et al.* 2007. Elevated Fmr1 mRNA levels and reduced protein expression in a mouse model with an unmethylated Fragile X full mutation. Experimental cell research 313:244-253.
33. Rasband WS. ImageJ. Bethesda, MD: U.S. National Institutes of Health; 1997-2007.
34. Favreau C, Dubosclard E, Ostlund C, Vigouroux C, Capeau J *et al.* 2003. Expression of lamin A mutated in the carboxyl-terminal tail generates an aberrant nuclear phenotype similar to that observed in cells from patients with Dunnigan-type partial lipodystrophy and Emery-Dreifuss muscular dystrophy. Experimental cell research 282:14-23.
35. Markiewicz E, Venables R, Mauricio Alvarez R, Quinlan R, Dorobek M *et al.* 2002. Increased solubility of lamins and redistribution of lamin C in X-linked Emery-Dreifuss muscular dystrophy fibroblasts. Journal of structural biology 140:241-253.
36. Paradisi M, McClintock D, Boguslavsky RL, Pedicelli C, Worman HJ *et al.* 2005. Dermal fibroblasts in Hutchinson-Gilford progeria syndrome with the lamin A G608G mutation have dysmorphic nuclei and are hypersensitive to heat stress. BMC cell biology 6:27.
37. Sullivan T, Escalante-Alcalde D, Bhatt H, Anver M, Bhat N *et al.* 1999. Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. The Journal of cell biology 147:913-920.
38. Vigouroux C, Auclair M, Dubosclard E, Pouchelet M, Capeau J *et al.* 2001. Nuclear envelope disorganization in fibroblasts from lipodystrophic patients with heterozygous R482Q/W mutations in the lamin A/C gene. Journal of cell science 114:4459-4468.
39. Worman HJ, Courvalin JC. 2005. Nuclear envelope, nuclear lamina, and inherited disease. International review of cytology 246:231-279.
40. Ellis RJ. 1990. The molecular chaperone concept. Seminars in cell biology 1:1-9.
41. Gething MJ, Sambrook J. 1992. Protein folding in the cell. Nature 355:33-45.
42. Lee JS, Lee JJ, Seo JS. 2005. HSP70 deficiency results in activation of c-Jun N-terminal Kinase, extracellular signal-regulated kinase, and caspase-3 in hyperosmolarity-induced apoptosis. The Journal of biological chemistry 280:6634-6641.
43. Djabali K, de Nechaud B, Landon F, Portier MM. 1997. AlphaB-crystallin interacts with intermediate filaments in response to stress. Journal of cell science 110 (Pt 21):2759-2769.
44. Kato K, Ito H, Inaguma Y, Okamoto K, Saga S. 1996. Synthesis and accumulation of alphaB crystallin in C6 glioma cells is induced by agents that promote the disassembly of microtubules. The Journal of biological chemistry 271:26989-26994.
45. van Bergen en Henegouwen PM, Linnemans AM. 1987. Heat shock gene expression and cytoskeletal alterations in mouse neuroblastoma cells. Experimental cell research 171:367-375.
46. Wiesmann KE, Coop A, Goode D, Hepburne-Scott HW, Crabbe MJ. 1998. Effect of mutations of murine lens alphaB crystallin on transfected neural cell viability and cellular translocation in response to stress. FEBS letters 438:25-31.
47. Head MW, Goldman JE. 2000. Small heat shock proteins, the cytoskeleton, and inclusion body formation. Neuropathol Appl Neurobiol. 26:304-312.
48. Launay N, Goudeau B, Kato K, Vicart P, Lilienbaum A. 2006. Cell signaling pathways to alphaB-crystallin following stresses of the cytoskeleton. Exp Cell Res. 312:3570-3584. Epub 2006 Aug 3579.
49. Benn SC, Woolf CJ. 2004. Adult neuron survival strategies--slamming on the brakes. Nat Rev Neurosci 5:686-700.
50. Luo GR, Chen S, Le WD. 2007. Are heat shock proteins therapeutic target for Parkinson's disease? International journal of biological sciences 3:20-26.
51. Muchowski PJ, Wacker JL. 2005. Modulation of neurodegeneration by molecular chaperones. Nat Rev Neurosci 6:11-22.

52. Ousman SS, Tomooka BH, van Noort JM, Wawrousek EF, O'Conner K *et al.* 2007. Protective and therapeutic role for alphaB-crystallin in autoimmune demyelination. Nature 448:474-479.
53. Evgrafov OV, Mersiyanova I, Irobi J, Van Den Bosch L, Dierick I *et al.* 2004. Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. Nature genetics 36:602-606.
54. Willemsen R, Hoogeveen-Westerveld M, Reis S, Holstege J, Severijnen LA *et al.* 2003. The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. Hum Mol Genet. 12:949-959.
55. Bettencourt da Cruz A, Schwarzel M, Schulze S, Niyyati M, Heisenberg M *et al.* 2005. Disruption of the MAP1B-related protein FUTSCH leads to changes in the neuronal cytoskeleton, axonal transport defects, and progressive neurodegeneration in Drosophila. Molecular biology of the cell 16:2433-2442.
56. Farzin F, Perry H, Hessl D, Loesch D, Cohen J *et al.* 2006. Autism spectrum disorders and attention-deficit/hyperactivity disorder in boys with the fragile X premutation. J Dev Behav Pediatr. 27:S137-144.
57. Herbert MR, Ziegler DA, Deutsch CK, O'Brien LM, Kennedy DN *et al.* 2005. Brain asymmetries in autism and developmental language disorder: a nested whole-brain analysis. Brain 128:213-226.
58. Just MA, Cherkassky VL, Keller TA, Minshew NJ. 2004. Cortical activation and synchronization during sentence comprehension in high-functioning autism: evidence of underconnectivity. Brain 127:1811-1821.
59. Hessl D, Rivera S, Koldewyn K, Cordeiro L, Adams J *et al.* 2006. Amygdala dysfunction in men with the fragile X premutation. Brain 12:12.
60. Aziz M, Stathopulu E, Callias M, Taylor C, Turk J *et al.* 2003. Clinical features of boys with fragile X premutations and intermediate alleles. Am J Med Genet B Neuropsychiatr Genet 121:119-127.
61. Goodlin-Jones BL, Tassone F, Gane LW, Hagerman RJ. 2004. Autistic spectrum disorder and the fragile X premutation. J Dev Behav Pediatr 25:392-398.
62. Williamson TL, Cleveland DW. 1999. Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. Nature neuroscience 2:50-56.

CHAPTER 5

Altered hypothalamo-pituitary-adrenal axis regulation in the expanded CGG-repeat mouse model for fragile X-associated tremor/ataxia syndrome

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Abstract |

The human *FMR1* gene contains an unstable CGG-repeat ($(CGG)_n$) in its 5' untranslated region. The repeat length in the normal population is polymorphic (5-54 CGGs). Individuals carrying lengths beyond 200 CGGs (i.e. the full mutation) show hypermethylation and as a consequence gene silencing of the *FMR1* gene. The absence of the gene product FMRP causes the fragile X syndrome, the most common inherited form of mental retardation. Elderly carriers of the premutation (PM), which is defined as a repeat length between 55-200 CGGs, can develop a progressive neurodegenerative syndrome: fragile X-associated tremor/ataxia syndrome (FXTAS). The high *FMR1* mRNA levels observed in cells from PM carriers have led to the hypothesis that FXTAS is caused by a pathogenic RNA gain-of-function mechanism. Apart from tremor/ataxia, specific psychiatric symptoms have been described in PM carriers with or without FXTAS. Since these symptoms could arise from elevated stress hormone levels, we investigated hypothalamo-pituitary-adrenal (HPA) axis regulation using a knock-in mouse model with an expanded $(CGG)_n$ in the PM range (>98 repeats) in the *Fmr1* gene, which shows repeat instability, and displays biochemical, phenotypic and neuropathological characteristics of FXTAS. We show elevated levels of corticosterone in serum and ubiquitin-positive inclusions in both the pituitary and adrenal gland of 100-week old animals. In addition, we demonstrate ubiquitin-positive inclusions in the amygdala from aged expanded $(CGG)_n$ mice. We hypothesise that altered regulation of the HPA axis and the amygdala and higher stress hormone levels in the mouse model for FXTAS may explain associated psychological symptoms in humans.

Introduction | 5.1

The fragile X mental retardation gene 1 (*FMR1*), located on the X chromosome, harbours a $(CGG)_n$ in its 5' untranslated region (5'UTR). This repeat may become unstable upon transmission to the next generation. Different clinical outcomes occur depending on the length of this trinucleotide repeat. Normal individuals carry a repeat of up to 44 CGG units, which remains stable on transmission [1]. Alleles with between 45 and 54 repeats are considered intermediate size alleles, which are associated with some degree of instability. Individuals with over 200 CGGs have the full mutation, which usually leads to methylation of both the promoter region and the $(CGG)_n$, and consequent transcriptional silencing of the gene. The absence of the gene product fragile X mental retardation protein (FMRP) is the cause of the mental retardation seen in fragile X patients [2]. Individuals with the premutation (PM) have between 55 and 200 CGGs. Female carriers of the PM are at increased risk of developing premature ovarian failure (POF) [3]. PM carriers also are at risk for fragile X-associated tremor/ataxia syndrome (FXTAS), which has been observed in elderly men over age 50, and less often in female PM carriers [4-6].

FXTAS is a progressive neurodegenerative disorder, believed to be the result of a pathogenic RNA gain-of-function mechanism, as PM carriers produce 2-8 fold elevated levels of *FMR1* mRNA in their lymphocytes. While transcription is increased, translation is hampered, resulting in slightly lower FMRP levels in individuals with high $(CGG)_n$ alleles within the PM range [7-10]. Patients with FXTAS usually present with tremor and ataxia, but may develop other neurological symptoms such as Parkinsonism, autonomic dysfunction and peripheral neuropathy and may suffer from cognitive decline including formal dementia. *Post mortem* studies of brains from patients with FXTAS reveal intranuclear inclusions in neurons and astrocytes in multiple brain areas [11]. These inclusions contain several proteins, including ubiquitin, heat shock proteins including αB-crystallin, the RNA binding proteins hnRNP-A2 (heterogeneous nuclear ribonucleoprotein A2) and MBNL1 (muscle blind-like protein 1) and a number of neurofilaments, among which are lamin A/C [12] and *FMR1* mRNA [13]. Very recently, Pur α has been identified as a component of the ubiquitin-positive inclusions in FXTAS brain [14]. Proteins that are sequestered into the inclusions may be prevented from exerting their normal function, thus resulting in cellular dysfunction, ultimately leading to neurodegeneration [14-16].

Recent studies have documented that the abnormal elevation of *FMR1* mRNA is associated with increased psychological symptoms, such as anxiety, depression, and irritability in adult PM carriers, with or without symptoms of FXTAS, especially males [17-20]. These psychological symptoms could arise from elevated stress hormone levels, thus aberrant regulation of the hypothalamo-pituitary-adrenal gland (HPA) axis. More evidence suggestive of altered regulation of the HPA axis by the PM comes from the observation that ubiquitin-positive intranuclear inclusions are also present in the anterior and posterior lobes of the pituitary gland of patients with FXTAS [21,22]. A link has been suggested between pituitary inclusions and dysregulated neuroendocrine function in patients with FXTAS. Increased Follicle Stimulating Hormone (FSH) [22-24] and decreased inhibin A and B levels in female PM carriers were reported even in those who are cycling normally, suggestive of early ovarian aging and ovarian compromise [25]. Elevated levels of FSH have been found to reflect decreasing ovarian reserve [26], which can be correlated to the risk of developing POF seen in female PM carriers. Interestingly, intranuclear inclusions have been reported in the testicles of two men with FXTAS; inclusions were present in the

anterior and posterior pituitary gland of one of these for whom the pituitary gland was available [22]. Finally, a reduced amygdala response has been reported in PM male carriers which may explain the aetiology of psychological symptoms involving emotion and social cognition as well [27].

An expanded $(CGG)_n$ knock-in ($(CGG)_n$) mouse model has been generated [28,29], by substituting the endogenous mouse $(CGG)_8$ with a human $(CGG)_{98}$. The $(CGG)_n$ in the mouse model shows instability upon transmission to the next generation [30], similar to humans. Also, the $(CGG)_n$ mice show elevated *Fmr1* mRNA levels [30], as well as ubiquitin-positive neuronal inclusions throughout the brain [28,29]. Aberrant behaviour in mice was described by Van Dam and colleagues, including mild learning deficits and increased anxiety [31].

We explored HPA axis physiology in expanded $(CGG)_n$ mice for two reasons: 1) increased anxiety has been observed in our mouse model [31] and PM carriers, especially those with elevated *FMR1* mRNA experience more psychological distress [19], 2) ubiquitin-positive inclusions have been found in the pituitary gland of male patients with FXTAS [22]. Here we further characterise the expanded $(CGG)_n$ mouse model for FXTAS and more specifically we demonstrate the presence of inclusions in several endocrine organs of the $(CGG)_n$ mice and in the amygdala. The amygdala plays an important role in the regulation of the secretion of HPA axis-related hormones in the hypothalamus [32]. We also report elevated corticosterone levels in response to a mild stressor. Thus we present evidence that HPA axis physiology is disturbed in $(CGG)_n$ mice, which might explain molecular mechanisms underlying psychopathology in PM carriers and/or patients with FXTAS.

Materials and methods | 5.2

Mice

Both the expanded $(CGG)_n$ knock-in ($(CGG)_n$) mice and the wild type (wt, with an endogenous $(CGG)_8$-repeat) mice were housed under standard conditions. All experiments were carried out with permission of the local ethical committee. $(CGG)_n$ lengths were determined for the whole colony, but only male mice were used for experiments. All mice had a mixed C57/BL6 and FVB/n genetic background.

Genotyping of the mice

DNA was extracted from mouse tails as described previously [30]. Determination of the $(CGG)_n$ length was performed by means of PCR using the Expand High Fidelity Plus PCR System (Roche Diagnostics), with forward primer 5'- CGGAGGCGCCGCTGCCAGG-3' and 5'-TGCGGGCGCTCGAGGCCCAG-3' as reverse. PCR products were visualised on a 6% polyacrylamide gel. As these primers are specific to the knock-in allele, a separate PCR is performed for the wt allele. The wt allele was detected using TaKaRa LA Taq polymerase (using GC buffer II), according to manufacturer's instructions (Takara Bio Inc), with 5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3' as forward primer and 5'-AGCCCCGCACTTCCACCACCAGCTCCTCCA-3' as reverse primer (detailed description in [30]). The 100-week old mice used in this study had $(CGG)_n$ lengths between 100 to 160 CGGs, while 25-week old mice had 160-200 CGGs.

Western blotting

Half brains (sagittal) were homogenised in HEPES buffer. We loaded 100 µg of protein onto an 8% SDS-PAGE gel, which was then electroblotted onto a nitrocellulose membrane. The membrane was incubated overnight at 4°C with the monoclonal 2F5-1 antibody specific for Fmrp [33] and a monoclonal antibody against Gapdh (Chemicon), which served as a loading control. The next day the membrane was incubated with a horseradish peroxidase conjugated

rabbit-anti-mouse antibody (DAKO), allowing visualisation by chemilluminescence using an ECL kit (Amersham) (for details, see [30]). Quantification of protein bands was performed using TotalLab software (Nonlinear Dynamics Ltd). All three Fmrp isoforms were taken into account in the calculations. Multiple comparable series of increasing repeat lengths were performed, both for young and old mice. One series, representative of many repeated experiments, is shown.

Blood collection and hormone levels

In the morning, mice were transported from the animal facility to the laboratory in their home cage, which can be considered a mild stressor. After arrival in the laboratory they were left to acclimate for at least 30 minutes. The order of sacrifice was random with respect to genotype. After sacrifice by cervical dislocation, blood was collected immediately from the thoracic cavity by perforating the right cardiac atrium and collecting the resulting blood flow. Blood was kept at 4°C overnight and centrifuged the next day at 4°C for 15 minutes. The supernatant was stored at -20°C until analysis. Serum corticosterone levels were estimated using radio immunoassays or enzyme immunoassays for the determination of corticosterone in serum provided by Diagnostic Systems Laboratories (Webster, TX). Both assays use the same antiserum and yielded identical results for the internal quality control sera. Intra-assay and inter-assay variation coefficients were below 7 and 13%, respectively. Thus, all data were analyzed together.

Measured serum corticosterone levels did not follow a normal distribution (positively skewed: standard deviation > ½ * mean). Therefore measured corticosterone levels were subjected to logarithmic transformation. The lognormal plot showed a strongly improved distribution, hence the log-transformed data were used for further analysis. Back-transformation of the means of the values by calculating 10^{mean} gives the geometrical mean (GM). Analysis of variance (ANOVA) was performed on log-transformed corticosterone levels to test the difference between wt and $(CGG)_n$ mice, at 25 and 100 weeks of age. Since significance was revealed, posthoc pair wise comparisons were performed with Bonferroni correction for multiple comparisons. Geometrical mean differences (GMD) are presented with their respective corrected p-value. GMDs were calculated by subtracting back-transformed mean log-transformed corticosterone levels. The correlation between corticosterone levels and repeat length was also examined. The statistical software package SPSS (version 11) was used for all analyses. The level of statistical significance was set at $p<0.05$.

Immunohistochemistry

After mice were sacrificed brains were dissected, followed by the pituitary gland and adrenal gland and fixed overnight in 3% paraformaldehyde. Tissues were embedded in paraffin according to standard protocols. Sections (7 µM) were deparaffinised followed by antigen retrieval using microwave treatment in 0.01 M sodium citrate solution. Endogenous peroxidase activity blocking and immunoincubation were performed as described before [34], using a polyclonal rabbit antibody against ubiquitin (Dako, ZO458), a monoclonal antibody against FMRP (1C3)[34] and a polyclonal antibody against glucocorticoid receptor (GR: Affinity BioReagents PA1-511A). Ubiquitin-positive intranuclear inclusion counts in amygdala, pituitary and adrenal gland were determined by counting the number of inclusion-bearing cells in a field of 100 neurons counted. Per area of interest three fields of 100 neurons were counted. Mean counts and the range are given.

RNA isolation, RT and Q-PCR

RNA of pituitary glands of 100-week old animals was extracted using a RNeasy Mini Kit, according to manufacturer's instructions (Qiagen). RNA concentration and purity were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Reverse transcriptase was performed on 1 µg RNA using iScript cDNA Synthesis Kit (BioRad) according to manufacturer's instructions. Detailed description of RNA isolation and RT can be found in Brouwer *et al* (2007). Q-PCR was performed on 0.1 µl of RT product. Primers used for Q-PCR were as follows: *Fmr1* (transition exon 7/8: forward: 5'- TCTGCGCACCAAGTTGTCTC -3', reverse: 5'-CAGAGAAGGCACCAACTGCC-3'), *Gapdh* (forward: 5'-AAATCTTGAGGCAAGCTGCC-3', reverse: 5'-GGATAGGGCCTCTCTTGCTCA-3'), GR (nuclear receptor

subfamily 3, group C, member 1: *Nr3c1*: transition exon 4/5: forward: 5'-CAAAGGCGATACCAGGATTCA-3', reverse: 5'- GGGTCATTTGGTCATCCAGGT-3'), Corticotrophin releasing hormone receptor type 1 (*Crhr1:* transition exon 2/3: forward: 5'-TCTGACAATGGCTACCGGG-3', reverse: 5'-AATAATTCACACGGGCTGCC-3'), Arginine vasopressin receptor 1b (*Avpr1b*: transition exon 1/2: forward: 5'-GGACGAGAATGCCCCTAATGA-3', reverse: 5'-TCGAGATGGTGAAAGCCACAT-3') and Proopiomelanocortin (*Pomc:* transition exon 2/3: forward: 5'-AACCTGCTGGCTTGCATCC-3', reverse: 5'-TGACCCATGACGTACTTCCG-3'). Efficiencies of the different primer sets were checked and found to be comparable. Ct values of the reference gene were subtracted from the Ct value of the target gene for each sample, which gives the ΔCt. ΔCt of wt was then subtracted from ΔCt of $(CGG)_n$ mice, which is designated as the ΔΔCt. $2^{-\Delta\Delta Ct}$ then gives fold change.

Results | 5.3

All knock-in mice have a $(CGG)_n$ length in the PM range, and they all show Fmrp expression as illustrated by Western blot analysis (figure 5.1). Analysis of different $(CGG)_n$ animals shows that Fmrp levels decrease mildly with increasing repeat length and a transition to reduced levels seems to occur around 170 CGGs. Western blot was performed on multiple series of brain lysates of mice with increased repeat lengths. All experiments showed the same pattern, both when brains from old (72- or 100-weeks old) and young (25-weeks old) were used. Figure 5.1 shows a series representative of what was seen in all experiments. For the samples used in figure 5.1, quantification of the amount of Fmrp in each sample relative to wt brain revealed 100% for 112 and 129 CGGs and 80% and 70% for 174 and 185 CGGs respectively.

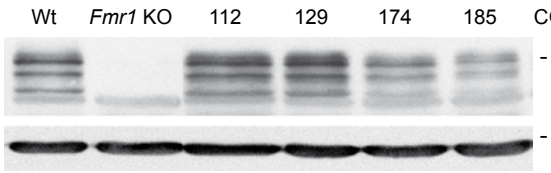

Figure 5.1 | Fmrp levels at different CGG-repeat lengths in 100-week old whole mouse brain lysates. An antibody against Gapdh was used as loading control. Fmrp levels decrease mildly as CGG-repeat length increases. Quantification of the amount of Fmrp in each sample relative to wt brain revealed 100% for 112 and 129 CGG-repeats and 80% and 70% for 174 and 185 CGGs respectively.

Corticosterone levels in $(CGG)_n$ mice

To study the regulation of the HPA axis in our mouse model, we first investigated the endpoint of this axis, namely corticosteroid levels. The predominant corticosteroid in mice is corticosterone. Moving the mice from the animal facility to the laboratory where they were sacrificed is a mild stressor. We waited at least 30 minutes before mice were sacrificed and since corticosterone response to stress occurs within approximately 30 minutes, we measured corticosterone levels in response to this mild stressor [35-37]. Mean corticosterone levels as measured in $(CGG)_n$ and wt mice at 25 and 100 weeks of age are depicted in figure 5.2. One-way ANOVA on log transformed corticosterone levels revealed that the four groups, based on genotype and age, were significantly different ($F_{df=3}$=10.675, p<0.001). Bonferroni corrected post hoc pair wise comparison revealed a statistically significant geometrical mean difference (GMD) between $(CGG)_n$ and wt mice at 100 weeks (GMD=285.4, p=0.002) and between $(CGG)_n$ mice at 100 weeks and $(CGG)_n$ mice at 25 weeks (GMD=338.4, p<0.001). No statistically significant differences were seen between $(CGG)_n$ and wt mice at 25 weeks (GMD=-102.1, p=0.44) or between wt mice at 25 weeks and wt mice at 100 weeks (GMD=-49.1, p=1). Scatter plots with repeat length plotted against corticosterone levels for each age did not reveal statistically significant correlations (data not shown).

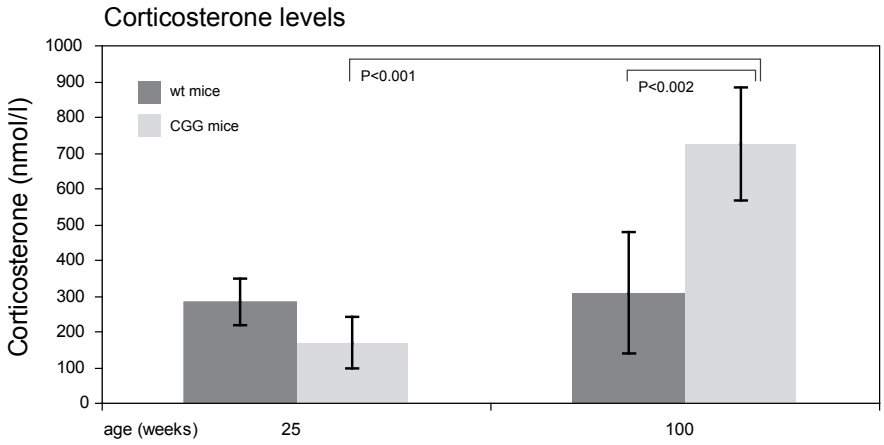

Figure 5.2 | Mean corticosterone levels as measured in CGG and wt mice at 25 and 100 weeks of age. Error bars represent 95% confidence intervals (mean ± 1.96 * st.dev). One-way ANOVA on logtransformed corticosterone levels revealed that the four groups, based on genotype and age, were significantly different ($F_{df=3}$=10.675, p<0.001). Bonferroni corrected post hoc pair wise comparison revealed a statistically significant geometrical mean difference (GMD) between CGG and wt mice at 100 weeks (GMD=285.4 nmol/L, p=0.002) and between CGG mice at 100 weeks and CGG mice at 25 weeks (GMD=338.4 nmol/L, p<0.001).

Ubiquitin-positive inclusions in HPA axis related organs

Immunohistochemistry on paraffin sections of the pituitary gland of 100-week old expanded $(CGG)_n$ mice showed intranuclear inclusions using an antibody directed against ubiquitin (figure 5.3). Highest numbers were present in the *pars intermedia* (table 5.1 and figure 5.3A and 5.3B), while inclusions were seen to a lesser extent in the *pars anterior* (table 5.1 and figure 5.3A). Inclusions were virtually absent in the *pars posterior* (table 5.1 and figure 5.3A). Immunolabelling for Fmrp expression in the pituitary gland correlated with the presence of the number of inclusions, that is, high Fmrp expression in the *pars intermedia* (figure 5.3C and 5.3D) and very low expression in both *pars anterior* and *pars posterior* (figure 5.3C).

Immunohistochemistry was also performed on pituitary gland sections of 25-week old $(CGG)_n$ mice. Ubiquitin-positive intranuclear inclusions could be detected, although in a much lower percentage of cells (table 5.1) compared to 100-week old animals. Also they were smaller and less consistently spherical (data not shown). Wild-type animals did not show inclusions at either age.

Cellular localisation of glucocorticoid receptors (GRs) in pituitary glands of 100-week old mice was not different between $(CGG)_n$ mice and wt mice (data not shown).

Next, immunostainings for ubiquitin in sections of 100-week old adrenal glands were performed, which showed significant numbers of inclusions in the parenchymal cells of the medulla (figure 5.4A) and parenchymal cells of the *zona fasciculata* (figure 5.4B and 5.C) of the adrenal cortex.

Parenchymal cells of the zona reticularis and the *zona glomerulosa* were virtually devoid of inclusions. The percentage of ubiquitin-positive intranuclear inclusions in the different layers of the adrenal gland was substantially less compared to the percentage in the *pars intermedia* of the pituitary gland (table 5.2).

		Pituitary gland		
		Pars anterior	*Pars intermedia*	*Pars posterior*
25 wks	**Wt**	0	0	0
	CGG	2 (1-3)	34 (25-45)	0
100 wks	**Wt**	0	0	0
	CGG	18 (4-38)	58 (44-75)	1 (0-2)

Table 5.1 | Mean percentages (range) of pituitary cells with ubiquitin-positive intranuclear inclusions in 25- and 100-week old wt and $(CGG)_n$ mice.

		Adrenal gland		
		Zona glomerulosa	*Zona fasciculata*	**Medulla**
25 wks	**Wt**	0	0	0
	CGG	1 (0-2)	2 (0-4)	6 (5-6)
100 wks	**Wt**	0	0	0
	CGG	1 (0-4)	4 (1-6)	16 (9-27)

Table 5.2 | Mean percentages (range) of adrenal cells with ubiquitin-positive intranuclear inclusions in 25- and 100-week old wt and $(CGG)_n$ mice.

Note that the intranuclear inclusions in the parenchymal cells of the *zona fasciculata* are more irregularly shaped, more elongated compared to the circular intranuclear inclusions found thus far in brain, pituitary gland and other layers of the adrenal gland. Also, in the *zona fasciculata* frequently small, round cytoplasmic inclusions were seen, occasionally more than one per cell (figure 5.4D). In adrenal gland of 25-week old $(CGG)_n$ animals, few inclusions were observed (table 5.2).

Neurons and astrocytes in the human FXTAS amygdala bear intranuclear inclusions and showed aberrant brain activation in a functional MRI study [27]. The amygdala helps control arginine vasopressin (AVP) and corticotrophin releasing hormone (CRH) secretion in the hypothalamus [32]. We observed the presence of intranuclear ubiquitin-positive inclusions throughout the amygdala of 100-week old $(CGG)_n$ mice. High percentages were seen in the posteromedial amygdalohippocampal nucleus (figure 5.5, 59% of neurons counted), the posteromedial cortical amygdaloid nucleus (57%)

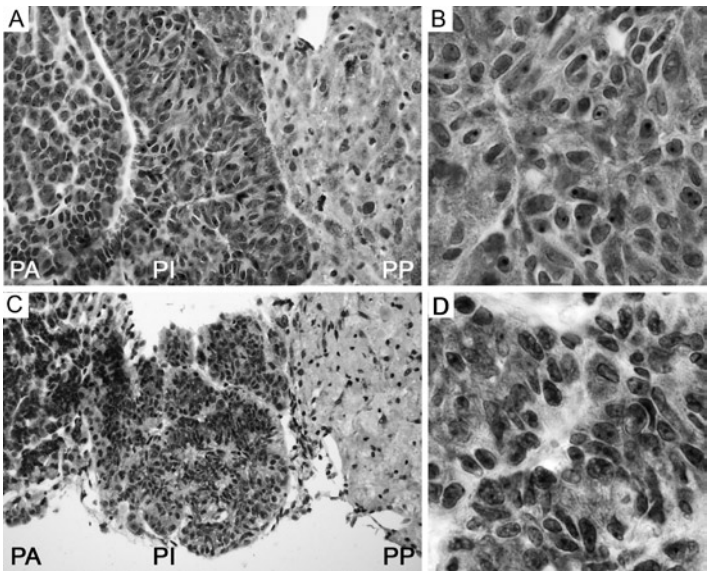

Figure 5.3 | Immunohistochemistry with antibodies against ubiquitin and Fmrp in pituitary gland of 100-week old CGG mice. **A**: Many intranuclear ubiquitin-positive inclusions (arrows) are seen in the *pars intermedia*, while hardly any were observed in the *pars posterior*. **B**: Ubiquitin-positive inclusions in the *pars intermedia*. **C**: Highest levels of Fmrp are observed in the *pars intermedia*. **D**: Fmrp expression in the *pars intermedia*. PA: *pars anterior*, PI: *pars intermedia*, PP: *pars posterior*. See page 163 onwards for colour version of this figure.

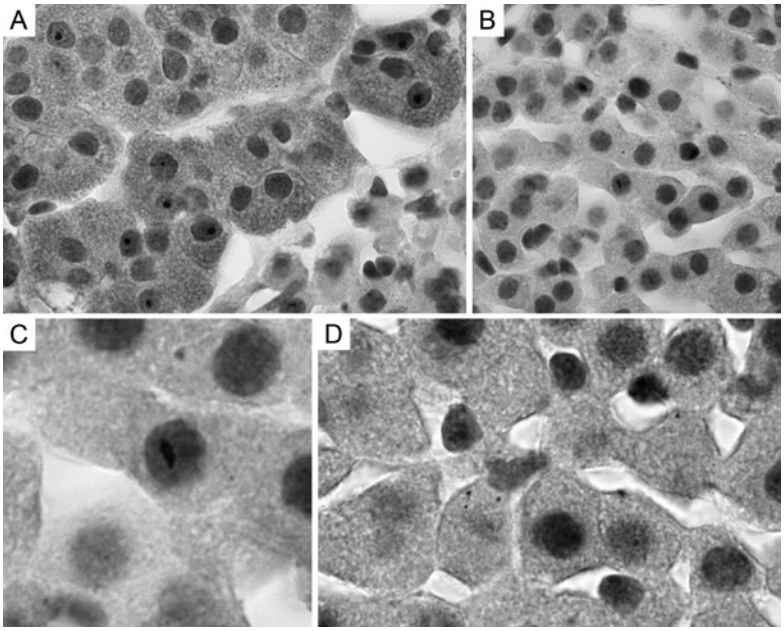

Figure 5.4 | Immunohistochemistry with an antibody against ubiquitin show ubiquitin-positive inclusions in adrenal gland of 100-week old CGG mice. **A**: Ubiquitin-positive intranuclear inclusions (arrows) in chromaffin cells of the adrenal gland. **B+C:** Elongated, irregularly shaped ubiquitin-positive intranuclear inclusions in the *zona fasciculata* of the adrenal gland. **D:** Cytoplasmic inclusions (arrow heads) in the *zona fasciculata* of the adrenal gland. See page 163 onwards for colour version of this figure.

and the anterior amygdaloid area (58%). Other regions of the amygdala only showed inclusions in up to 5% of neurons. In $(CGG)_n$ mice of 25 weeks of age, intranuclear inclusions were counted in on average 6% of neurons in the three regions specified for old mice.

Transcript levels in the pituitary gland

Fmr1 transcript levels were found to be 3.4-fold higher (SD=0.6) in pituitary glands of $(CGG)_n$ mice, as compared with wt mice, at 72 weeks of age.

Several feedback mechanisms exist for the HPA axis to prevent lasting high levels of corticosteroids. We analysed effects of corticosterone binding to its receptor, the GR, namely transcription levels of the glucocorticoid, Crh and vasopressin receptors and of the ACTH precursor molecule Pomc. Quantitative PCR revealed no statistically significant difference for any of the receptors tested, neither for *Pomc*, in $(CGG)_n$ mouse pituitary gland when compared with wt pituitary gland (data not shown).

Discussion | 5.4

Recently, observations have accumulated that PM carriers, both with and without FXTAS are more often affected by psychiatric problems than controls [17-20]. Although a link between the presence of inclusions in the hippocampus and the frequent occurrence of anxiety and depression in patients with FXTAS has been suggested [17], little is known about the origin of the psychopathology.

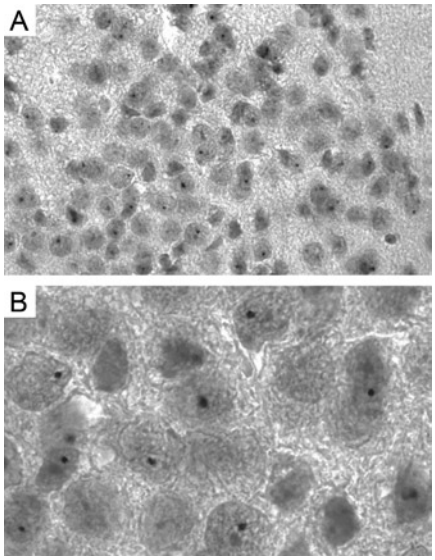

Figure 5.5 | Ubiquitin-positive intranuclear inclusions in the amygdala of a 100-week old CGG mouse. **A** en **B** show the posteromedial amygdalohippocampal nucleus, where B is a higher magnification of what is seen in A. See page 163 onwards for colour version of this figure.

In the $(CGG)_n$ mouse model, which shows genetic, biochemical, behavioural and neuropathological parallels to the human situation in FXTAS, we investigated whether altered HPA axis regulation could play a role in the development of these symptoms. The presence of ubiquitin-positive intranuclear inclusions in the pituitary and adrenal glands and the elevated corticosterone levels in 100-week old $(CGG)_n$ mice point towards disturbed HPA axis physiology. At 25 weeks of age $(CGG)_n$ mice did have ubiquitin-positive inclusions in pituitary gland, although these were smaller in size and number, and less spherical than in the 100-week old animals. In 25-week old $(CGG)_n$ mice also inclusions in the amygdala were observed. Interestingly, no inclusions have been observed in other brain regions at this age, including amygdala [29]. It seems that inclusions start to develop around this time point, with the pituitary gland and amygdala being the first sites of inclusion development followed by other regions in the brain at 40-50 weeks of age [29]. At 25 weeks of age corticosterone levels are not yet elevated in $(CGG)_n$ mice. *Fmr1* mRNA levels are elevated at all ages [30], including in pituitary gland. If inclusions in the amygdala play a role in the dysregulation of the HPA axis, then apparently a threshold exists for the amount of inclusions that can cause an effect. Intranuclear inclusions in the pituitary gland could impair cellular function, although they may be just a marker for the degree of protein dysregulation that is occurring in the cell.

The presence of inclusions in the *pars intermedia* is puzzling since this area contains one major endocrine cell type in mammals, the melanotrophs. These cells process the precursor molecule POMC and release β-endorphin and α-melanocyte stimulating hormone. This POMC processing is different from that in the *pars anterior*, where predominantly ACTH and β-endorphin are released. Also, in adult humans the *pars intermedia* has undergone involution [38]. Negative feedback of corticosteroids occurs via GRs in the hypothalamic paraventricular nucleus and the pituitary gland. Rapid feedback effects of glucocorticoids prepare an organism for a stressful situation [32,35]. Slower gene transcription-mediated systems come into action when corticosteroids bind to the cytoplasmic GR, present as a complex with other proteins among which hsp90 and hsp70. Ligand-binding disassembles the complex, upon which the GR can translocate to the nucleus. There it binds to negative glucocorticoid responsive elements, downregulating factors involved in corticosteroid synthesis [39-41]. Hence GR function would be worth investigating. However, since mice were mildly stressed before sacrifice, likely causing GR translocation, no localisation differences were observed.

We did not detect differences in pituitary gland receptor mRNA levels, involved in HPA axis negative feedback. The hypothalamus secretes AVP and CRH under the control of the hippocampus and

the amygdala [32]. CRH provides the predominant stimulus to the anterior pituitary gland to synthesise and secrete ACTH, whereas AVP strengthens the effect of CRH to secrete ACTH. Changes in hormone and receptor levels of the HPA axis are very specifically dependent on the type and duration of stressors [42]. GR is known to repress its own synthesis in a hormone-dependent manner [40]. Negative feedback at the level of the pituitary gland to block synthesis and secretion of ACTH by the corticotroph cells occurs by directly inhibiting synthesis of *Pomc* mRNA [35]. No such effect was found in pituitary glands of $(CGG)_n$ mice in this study. Not finding any transcriptional differences between $(CGG)_n$ and wt mice could be because mice were sacrificed before a change at the transcription level could have taken place, or the stressor could have been too mild.

In the adrenal gland, we found ubiquitin-positive inclusions not only in the chromaffin cells of the medulla, which are the main source of catecholamines, but also in the *zona glomerulosa* and *zona fasciculata* of the adrenal cortex. The *zona glomerulosa* is the site where mineralocorticoids are produced, while the *zona fasciculata* produces glucocorticoids [43]. It is as yet unclear how inclusions might affect the functioning of the adrenal gland. It is striking that the inclusions seen in the *zona fasciculata* were differently shaped from those seen thus far in brain, pituitary gland and the other cell layers of the adrenal gland. This might indicate that inclusions in the zona fasciculate develop in a different way and from different components compared to the ones studied previously.

A review summarising observations in a large group of patients with the premutation reported anxiety and depression in over 30% of patients with FXTAS and anxiety in almost 40% and depression in 9% of non-FXTAS PM carriers [19]. The authors suggested a relationship with the inclusions present in the hippocampus, which is the area with the highest inclusion density in the human FXTAS brain [17]. Another study described more psychopathology, including phobic anxiety and obsessive-compulsive symptoms in patients with FXTAS compared to published norms [19]. Interestingly, PM carriers without signs of FXTAS also showed more obsessive-compulsive symptoms. Moreover, there was a stronger correlation between psychopathology and *FMR1* mRNA levels in PM carriers who had not developed FXTAS, than in patients with FXTAS. It is not yet understood whether psychological symptoms are associated with the PM from a developmental perspective or whether they reflect prodromal symptoms of later onset FXTAS [19]. Although patients with FXTAS show higher scores of psychopathology on a neuropsychiatric inventory than controls [18], anxiety has also been observed in young PM carriers, who were free of FXTAS symptoms. Anxiety along with shyness and social deficits are common among young boys with the premutation compared to brothers who do not have the premutation [44]. This suggests that there is a developmental effect of the premutation that can lead to this type of psychopathology. As FXTAS develops these symptoms can be aggravated by the neuropsychological deficits that set in [18]. Another report described that seven out of fifteen male patients with FXTAS suffered from mood and/or anxiety disorders, and twelve out of fifteen were found to have neuropsychological problems [20]. Increased irritability, agitation/aggression and anxiety might suggest further HPA axis dysregulation.

Naturally, it will be of interest to measure cortisol levels in PM carriers with and without FXTAS. In humans, it is as yet unknown whether only symptomatic PM carriers develop inclusions or whether the PM itself leads to inclusion formation with another factor in turn causing clinical manifestations. All mice carrying the PM develop inclusions, so the mouse model cannot be used to show whether

individuals without inclusions will suffer from a similarly altered HPA axis regulation and associated psychopathology.

Given the broad range of cognitive and emotional disturbances seen in PM carriers or patients with FXTAS, it cannot be excluded that altered HPA physiology is secondary to impaired coping strategies in stressful situations. For example, the amygdala was suggested to be a potential site of dysfunction underlying social deficits, when PM carriers showed diminished brain activation in the amygdala and other brain areas that mediate social cognition while viewing fearful faces in a functional MRI study. In the same study, sympathetic activation as measured by skin conductance upon a mild social stressor, was also lower in PM carriers than in controls. Sympathetic outflow is normally conducted by the amygdala [27]. Interestingly, intranuclear inclusions are also present in neurons and astrocytes in the amygdala in humans [21]. Dysfunction of the amygdala through elevated *FMR1* mRNA levels and inclusion formation might play a role in the aetiology of psychological symptoms involving emotion and social cognition as well. The presence of inclusions had not been investigated in our previous studies, but now we demonstrate significant numbers of intranuclear neuronal inclusions in amygdala of $(CGG)_n$ mice of 100 weeks of age (figure 5.5). It is unclear why amygdala response is reduced in humans while corticosterone levels are elevated in $(CGG)_n$ mice. For human brain, it is as yet unknown when inclusions first develop, since no post mortem material has been available of relatively young patients. It should be noted that the mean age of subjects tested in the study by Hessl and co-workers [27] was lower (~43 years) than the age at which inclusions have been seen thus far [45]. Around 40 years of age in a human life might be comparable to about 25 weeks of age in mice, thus it could be that reduced limbic response and elevation of corticosteroid levels are different stages of the same syndrome.

Fragile X individuals [46,47], as well as *Fmr1* KO mice [48], have also been shown to have an exaggerated stress response. Children with the fragile X full mutation have elevated cortisol levels which are associated with severity of behavioural problems [46,47]. Both fragile X patients and *Fmr1* KO mice have a delayed return to baseline glucocorticoid levels, which was explained by the normal binding of GR mRNA to FMRP [49]. Fragile X individuals and *Fmr1* KO mice do not express FMRP, thus in the absence of FMRP inappropriate transport or translation of GR could lead to altered HPA responsiveness [48]. Mice with $(CGG)_n$ shorter than 170 trinucleotides have close to normal Fmrp levels (figure 5.1). In our study, corticosterone levels did not correlate with repeat length in the range tested (100-170 CGGs). Also, the 25-week old mice tested had higher $(CGG)_n$ lengths (170-200 CGGs) and lower Fmrp levels, but they did not have elevated corticosterone levels, indicating that lower Fmrp levels did not lead to inappropriate GR expression. Thus elevated stress responses develop differently in fragile X individuals and *Fmr1* KO mice as compared to $(CGG)_n$ mice. Although representing different molecular genetic mechanisms, these prior studies document the impact of *FMR1* gene mutation on the HPA axis. It should be noted that the decreased levels of Fmrp in animals with >170 CGGs do not represent a uniform decrease in cellular Fmrp throughout the brain. We have previously shown that some brain areas had very low Fmrp expression, while in other areas Fmrp levels were closer to normal [30].

Since we measured corticosterone levels only at one time point after a mild stressor, we cannot draw conclusions on the course of the corticosterone response over time. It is not yet known whether corticosterone levels in $(CGG)_n$ mice return to baseline levels in a normal or delayed fashion, or whether

peak stress levels are higher than in controls. Our future study will therefore focus on investigating corticosterone levels at different time points after a mild stressor.

It is intriguing that in different organs in the HPA axis ubiquitin-positive inclusions are observed, despite their different cell types. Within these organs, specific cells appear to be particularly prone to inclusion formation, for instance the chromaffin cells in the adrenal medulla. This, however, gives no clue as to why corticosterone levels are elevated in $(CGG)_n$ mice. The observation that there are much fewer and smaller intranuclear inclusions at 25 weeks than at 100 weeks of age and no difference in corticosterone levels at 25 weeks suggests that inclusions have a role in the origin of elevated corticosterone levels. Dysregulation of the HPA axis might arise at any of the organs involved. Although based on our current observations we cannot unravel the mechanism underlying the aberrant HPA axis physiology and its relation to the inclusions observed in $(CGG)_n$ mice, we do believe that this is an important observation in view of the emerging evidence of psychiatric symptoms in PM carriers. A combination of clinical and animal research is likely to lead to answering questions on the origin of the psychopathology observed in PM carriers, which may ultimately lead to novel therapeutic strategies. A first step into this direction is to determine basal salivary cortisol and cortisol stress response in PM carriers with and without FXTAS.

Acknowledgements |

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References |

1. Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS *et al.* 1991. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67:1047-1058.
2. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP *et al.* 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905-914.
3. Sherman SL. 2000. Premature Ovarian Failure among Fragile X Premutation Carriers: Parent-of-Origin Effect? Am J Hum Genet 67:11-13.
4. Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R *et al.* 2001. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 57:127-30.
5. Jacquemont S, Hagerman RJ, Leehey M, Grigsby J, Zhang L *et al.* 2003. Fragile X Premutation Tremor/Ataxia Syndrome: Molecular, Clinical, and Neuroimaging Correlates. Am J Hum Genet 72:869-78.
6. Hagerman RJ, Hagerman PJ. 2002. The fragile X premutation: into the phenotypic fold. Curr Opin Genet Dev 12:278-83.
7. Tassone F, Hagerman RJ, Chamberlain WD, Hagerman PJ. 2000. Transcription of the FMR1 gene in individuals with fragile X syndrome. Am J Med Genet 97:195-203.
8. Tassone F, Hagerman RJ, Taylor AK, Gane LW, Godfrey TE *et al.* 2000. Elevated levels of FMR1 mRNA in carrier males: A new mechanism of involvement in the Fragile-X syndrome. Am J Hum Genet 66:6-15.
9. Kenneson A, Zhang F, Hagedorn CH, Warren ST. 2001. Reduced FMRP and increased FMR1 transcription is proportionally associated with CGG repeat number in intermediate-length and premutation carriers. Hum Mol Genet 10:1449-1454.
10. Tassone F, Beilina A, Carosi C, Albertosi S, Bagni C *et al.* 2007. Elevated FMR1 mRNA in premutation carriers is due to increased transcription. RNA 13:555-62
11. Greco CM, Hagerman RJ, Tassone F, Chudley AE, Del Bigio MR *et al.* 2002. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. Brain 125:1760-1771.
12. Iwahashi CK, Yasui DH, An HJ, Greco CM, Tassone F *et al.* 2006. Protein composition of the intranuclear inclusions of FXTAS. Brain 129:256-71.
13. Tassone F, Iwahashi C, Hagerman PJ. 2004. FMR1 RNA withinthe intranuclear inclusions of fragile X-associated Tremor/Ataxia syndrome (FXTAS). RNA biology 1:103-105.
14. Jin P, Duan R, Qurashi A, Qin Y, Tian D *et al.* 2007. Pur alpha Binds to rCGG Repeats and Modulates Repeat-Mediated Neurodegeneration in a Drosophila Model of Fragile X Tremor/Ataxia Syndrome. Neuron 55:556-64.
15. Rosser TC, Johnson TR, Warren ST. 2002. A cerebellar FMR1 riboCGG binding protein. Am J Hum Genet 71:507.
16. Sofola OA, Jin P, Qin Y, Duan R, Liu H *et al.* 2007. RNA-Binding Proteins hnRNP A2/B1 and CUGBP1 Suppress Fragile X CGG Premutation Repeat-Induced Neurodegeneration in a Drosophila Model of FXTAS. Neuron 55:565-71.
17. Jacquemont S, Farzin F, Hall D, Leehey M, Tassone F *et al.* 2004. Aging in individuals with the FMR1 mutation. Am J Ment Retard 109:154-64.
18. Bacalman S, Farzin F, Bourgeois JA, Cogswell J, Goodlin-Jones BL *et al.* 2006. Psychiatric Phenotype of the Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) in Males: Newly Described Fronto-Subcortical Dementia. J Clin Psychiatry 67:87-94.
19. Hessl D, Tassone F, Loesch DZ, Berry-Kravis E, Leehey MA *et al.* 2005. Abnormal elevation of FMR1 mRNA is associated with psychological symptoms in individuals with the fragile X premutation. Am J Med Genet B Neuropsychiatr Genet 139B:115-121.
20. Bourgeois JA, Cogswell JB, Hessl D, Zhang L, Ono MY *et al.* 2007. Cognitive, anxiety and mood disorders in the fragile X-associated tremor/ataxia syndrome. Gen Hosp Psychiatry 29:349-56.
21. Louis E, Moskowitz C, Friez M, Amaya M, Vonsattel JP. 2006. Parkinsonism, dysautonomia, and intranuclear inclusions in a fragile X carrier: A clinical-pathological study. Mov Disord 27:193-201.
22. Greco CM, Soontrapornchai K, Wirojanan J, Gould JE, Hagerman PJ *et al.* 2007. Testicular and pituitary inclusion formation in fragile X associated tremor/ataxia syndrome. J Urol 177:1434-7.
23. Sullivan AK, Marcus M, Epstein MP, Allen EG, Anido AE *et al.* 2005. Association of FMR1 repeat size with ovarian dysfunction. Hum Reprod.20:402-12
24. Hundscheid RD, Braat DD, Kiemeney LA, Smits AP, Thomas CM. 2001. Increased serum FSH in female fragile X premutation carriers with either regular menstrual cycles or on oral contraceptives. Hum Reprod 16:457-462.
25. Welt CK, Smith PC, Taylor AE. 2004. Evidence of early ovarian aging in fragile x premutation carriers. J Clin Endocrinol Metab 89:4569-74.
26. MacNaughton J, Banah M, McCloud P, Hee J, Burger H. 1992. Age related changes in follicle stimulating hormone, luteinizing hormone, oestradiol and immunoreactive inhibin in women of reproductive age. Clin Endocrinol (Oxf) 36:339-45.

27. Hessl D, Rivera S, Koldewyn K, Cordeiro L, Adams J *et al.* 2006. Amygdala dysfunction in men with the fragile X premutation. Brain.
28. Bontekoe CJ, Bakker CE, Nieuwenhuizen IM, van Der Linde H, Lans H *et al.* 2001. Instability of a (CGG)(98) repeat in the Fmr1 promoter. Hum Mol Genet 10:1693-9.
29. Willemsen R, Hoogeveen-Westerveld M, Reis S, Holstege J, Severijnen L *et al.* 2003. The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. Hum Mol Genet 12:949-59.
30. Brouwer JR, Mientjes EJ, Bakker CE, Nieuwenhuizen IM, Severijnen LA *et al.* 2007. Elevated Fmr1 mRNA levels and reduced protein expression in a mouse model with an unmethylated Fragile X full mutation. Exp Cell Res 313:244-253.
31. Van Dam D, Errijgers V, Kooy RF, Willemsen R, Mientjes E *et al.* 2005. Cognitive decline, neuromotor and behavioural disturbances in a mouse model for Fragile-X-associated tremor/ataxia syndrome (FXTAS). Behavioural Brain Research 162:233-239.
32. Tronche F, Kellendonk C, Reichardt HM, Schutz G. 1998. Genetic dissection of glucocorticoid receptor function in mice. Curr Opin Genet Dev 8:532-8.
33. Gabel LA, Won S, Kawai H, McKinney M, Tartakoff AM *et al.* 2004. Visual Experience Regulates Transient Expression and Dendritic Localization of Fragile X Mental Retardation Protein. J Neurosci 24:10579-10583.
34. Bakker CE, de Diego Otero Y, Bontekoe C, Raghoe P, Luteijn T *et al.* 2000. Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse. Exp Cell Res 258:162-70.
35. Dallman MF. 2005. Fast glucocorticoid actions on brain: back to the future. Front Neuroendocrinol 26:103-8.
36. McGill BE, Bundle SF, Yaylaoglu MB, Carson JP, Thaller C *et al.* 2006. Enhanced anxiety and stress-induced corticosterone release are associated with increased Crh expression in a mouse model of Rett syndrome. Proc Natl Acad Sci U S A 103:18267-72.
37. Veenema AH, Meijer OC, de Kloet ER, Koolhaas JM, Bohus BG. 2003. Differences in basal and stress-induced HPA regulation of wild house mice selected for high and low aggression. Horm Behav 43:197-204.
38. Saland LC. 2001. The mammalian pituitary intermediate lobe: an update on innervation and regulation. Brain Res Bull 54:587-93.
39. Abbott CM, Proud CG. 2004. Translation factors: in sickness and in health. Trends Biochem Sci 29:25-31.
40. Webster JC, Cidlowski JA. 1999. Mechanisms of Glucocorticoid-receptor-mediated Repression of Gene Expression. Trends Endocrinol Metab 10:396-402.
41. Nishi M, Ogawa H, Ito T, Matsuda KI, Kawata M. 2001. Dynamic changes in subcellular localization of mineralocorticoid receptor in living cells: in comparison with glucocorticoid receptor using dual-color labeling with green fluorescent protein spectral variants. Mol Endocrinol 15:1077-92.
42. Aguilera G. 1994. Regulation of pituitary ACTH secretion during chronic stress. Front Neuroendocrinol 15:321-50.
43. Aguilera G. 1993. Factors controlling steroid biosynthesis in the *zona glomerulosa* of the adrenal. J Steroid Biochem Mol Biol 45:147-51.
44. Farzin F, Perry H, Hessl D, Loesch D, Cohen J *et al.* 2006. Autism spectrum disorders and attention-deficit/hyperactivity disorder in boys with the fragile X premutation. J Dev Behav Pediatr 27:S137-44.
45. Greco CM, Berman RF, Martin RM, Tassone F, Schwartz PH *et al.* 2006. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). Brain 129:243-255.
46. Hessl D, Glaser B, Dyer-Friedman J, Blasey C, Hastie T *et al.* 2002. Cortisol and behavior in fragile X syndrome. Psychoneuroendocrinology 27:855.
47. Hessl D, Glaser B, Dyer-Friedman J, Reiss AL. 2006. Social behavior and cortisol reactivity in children with fragile X syndrome. J Child Psychol Psychiatry 47:602-10.
48. Markham JA, Beckel-Mitchener AC, Estrada CM, Greenough WT. 2006. Corticosterone response to acute stress in a mouse model of Fragile X syndrome. Psychoneuroendocrinology. 31:781-5
49. Miyashiro KY, Beckel-Mitchener A, Purk TP, Becker KG, Barret T *et al.* 2003. RNA Cargoes Associating with FMRP Reveal Deficits in Cellular Functioning in Fmr1 Null Mice. Neuron 37:417-31.

CHAPTER 6

Correlation between CGG-repeat length and phenotype in FXTAS

Submitted

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Abstract |

The 5'UTR of the *FMR1* gene contains approximately 30 CGG-repeats ($(CGG)_n$) in the normal population, with a range of 5-55. This trinucleotide repeat is polymorphic and unstable upon transmission to the next generation. When repeat length exceeds 200 (full mutation), the *FMR1* gene generally undergoes methylation-mediated transcriptional silencing. The subsequent absence of the gene product FMRP results in mental retardation seen in fragile X patients. A repeat length of between 55 and 200 trinucleotides has been termed the premutation (PM). In particularly male elderly carriers of the PM are at risk of developing a progressive neurodegenerative disorder, called fragile X-associated tremor/ataxia syndrome (FXTAS). All PM carriers have elevated *FMR1* mRNA levels, in spite of slightly decreased FMRP levels. The presence of intranuclear ubiquitin-positive inclusions in many brain regions is a neuropathological hallmark of FXTAS. Efforts have been made to correlate clinical outcomes with molecular measures, although this has mostly been limited to $(CGG)_n$ length. However, such studies in humans are difficult due to the limited availability of tissue. Therefore, we have used the expanded $(CGG)_n$ knock-in mouse model of FXTAS to examine the relationship between the molecular and neuropathological parameters in brain, the central organ involved in the pathogenesis of FXTAS. In the present study *Fmr1* mRNA and Fmrp levels and the presence of intranuclear inclusions, the neuropathological hallmark of FXTAS, are described for different repeat lengths. In addition, our results suggest that in aged KI mice the presence of intranuclear inclusions may not depend on elevated *Fmr1* transcript levels.

Introduction | 6.1

The CGG-repeat ($(CGG)_n$) in the 5'UTR of the Fragile X mental retardation 1 (*FMR1*) gene is polymorphic. Normal individuals have alleles that are within the range of 5 to 44 CGGs [1]. Repeat lengths between 45 to 54 are considered intermediate alleles, which can show minor instability when transmitted to the next generation [2,3]. Over 200 CGGs has been named the full mutation (FM), since this usually leads to CpG methylation of the *FMR1* promoter and the $(CGG)_n$, which results in transcriptional silencing of the gene [4-6]. The consequent absence of the gene product FMRP is the cause of mental retardation in fragile X patients [7,8]. Unmethylated expansions of 55 to 200 CGGs are considered premutation (PM) alleles. Initially, it was thought that the only risk associated with the PM was expansion to an FM when a mother transmits the allele to her child. However, it was later recognised that carriers of the PM are at risk of developing a progressive neurodegenerative disorder called fragile X-associated tremor/ataxia syndrome (FXTAS) [9-11]. About 30% of male PM carriers over 50 years of age will develop FXTAS, although penetrance increases with age [12]. Few cases of female PM carriers presenting with FXTAS have been described [13,14]. Females are less severely affected, which can be explained by the presence of a second, normal allele. The process of X-inactivation determines how many PM alleles are on the active X chromosome, thereby influencing the clinical outcome [13,15]. About 20% of female carriers are at further risk of developing premature ovarian failure [16,17].

In FXTAS, patients usually present with progressive intention tremor and/or ataxia. As the disease progresses, symptoms worsen and a wider range of clinical involvement develops, including cognitive decline with impaired memory and executive function, autonomous dysfunction and peripheral neuropathy [11,18].

PM carriers have elevated *FMR1* mRNA levels in spite of normal or slightly reduced FMRP levels. The increase of *FMR1* mRNA levels seems to be correlated to the length of the $(CGG)_n$ [19-21]. Expanded $(CGG)_n$ tracts negatively influence translation of the *FMR1* mRNA, such that FMRP gradually decreases with increasing repeat length, despite increased levels of *FMR1* mRNA [19,20,22].

Since FXTAS is restricted to the PM range, as no aged FM carriers have been observed with signs of FXTAS, FMRP deficiency is unlikely the cause of the disease. This combined with the fact that *FMR1* mRNA levels are increased in PM carriers, led to the proposal that an RNA toxic gain-of-function mechanism underlies the symptoms seen in FXTAS patients [10,23]. In analogy to the well-described pathogenic RNA gain-of-function model for myotonic dystrophy (DM) (reviewed in [24]), this hypothesis predicts that certain proteins are sequestered to the expanded $(CGG)_n$-containing RNA, away from their normal function. Loss of the normal function of these $(CGG)_n$-binding proteins (BPs) could then lead to cellular toxicity or ultimately cell death [10,23].

Neurohistological studies on *post mortem* brains of PM carriers with FXTAS have revealed the presence of ubiquitin-positive intranuclear inclusions in neuronal and astrocytic cell types throughout the brain. Furthermore, Purkinje cell dropout and Bergmann gliosis were observed. Purkinje cells did not show intranuclear inclusions [25]. *FMR1* mRNA has also been detected in the intranuclear inclusions, further strengthening its involvement in the pathogenesis of FXTAS [26].

A mouse model originally developed in our group [27] to study instability of the *FMR1* $(CGG)_n$ is also an adequate model for FXTAS [28-30]. In this animal model, the endogenous mouse $(CGG)_8$ was

exchanged with a human $(CGG)_{98}$, which is in the PM range in humans. These expanded $(CGG)_n$ 'knock-in' ($(CGG)_n$) mice show moderate repeat instability upon both maternal and paternal transmission [27,28]. Neurohistological studies performed on $(CGG)_n$ mouse brains revealed ubiquitin-positive intranuclear inclusions in neurons. Number and size of inclusions increase with time, paralleling the progressive nature of the disease in human patients. Furthermore, the presence of inclusions in distinct brain regions and organs associated with the hypothalamo-pituitary-adrenal axis in $(CGG)_n$ mice can be linked to clinical features of FXTAS patients [29,30]. The mouse model also mimics human FXTAS in that *Fmr1* transcript levels are elevated [28,30].

Efforts have been made to correlate clinical outcomes to molecular measures, although this has mostly been limited to $(CGG)_n$ length [31-33]. Human studies using brain tissue are limited due to the availability of material. Therefore, this study is designed to use $(CGG)_n$ mice to establish the relationship between the molecular and neuropathological parameters in brain, the central organ involved in the pathogenesis of FXTAS. In the present study *Fmr1* mRNA and Fmrp levels and the presence of intranuclear inclusions, the neuropathological hallmark of FXTAS, are described for different repeat length categories. Our results in aged $(CGG)_n$ mice suggest that the sole presence of expanded $(CGG)_n$ *Fmr1* mRNA rather than elevation of *Fmr1* transcript levels is important for the neuropathology in FXTAS.

Methods and Materials | 6.2

Mice

The expanded $(CGG)_n$ mice used in this study have been described before [27-30]. Although this mouse model was created by exchanging the murine endogenous $(CGG)_8$ with a human $(CGG)_{98}$ [27], we now also report data on mice carrying $(CGG)_{70}$. These alleles arose in our colony through a contraction of the longer $(CGG)_n$. Both the expanded $(CGG)_n$ knock-in $(CGG)_n$ mice and the wild type (wt) mice with an endogenous $(CGG)_8$ were housed under standard conditions. All experiments were carried out with permission of the local ethics committee. $(CGG)_n$ lengths were determined for the whole colony, but only male mice were used for experiments. All mice had a mixed C57/BL6 and FVB/n genetic background. All mice used in this study were between 55 and 58 weeks old, unless specified otherwise.

Genotyping of the mice

DNA was extracted from mouse tail snips as previously described [28]. Determination of the $(CGG)_n$ length was performed by means of PCR using the Expand High Fidelity Plus PCR System (Roche Diagnostics), with forward primer 5'-CGGAGGCGCCGCTGCCAGG-3' and reverse primer 5'-TGCGGGCGCTCGAGGCCCAG-3. PCR products were visualised on a 6% polyacrylamide gel. As these primers are specific to the knock-in allele, a separate PCR is performed for the wt allele. The wt allele was detected using TaKaRa LA Taq polymerase (using GC buffer II), according to manufacturer's instructions (Takara Bio Inc), with 5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3' as forward primer and 5'-AGCCCCGCACTTCCACCACCAGCTCCTCCA-3' as reverse primer (detailed description in [28]). Based on their repeat length, mice were grouped into one of 5 repeat length categories, namely wt, 70CGG, 100-150CGG, 151-200CGG and >200CGG.

Immunohistochemistry

Half (sagittal) brains were fixed overnight in 3% paraformaldehyde. Tissues were embedded in paraffin according to standard protocols. Sections (7 µM) were deparaffinised followed by antigen retrieval using microwave treatment in 0.01 M sodium citrate solution. Endogenous peroxidase activity blocking and immunoincubation were performed as described before [34], using a polyclonal rabbit antibody against ubiquitin (Dako, ZO458) or a monoclonal 2F5-1 antibody specific for Fmrp [35]. Ubiquitin-positive intranuclear inclusion counts in colliculus inferior and dentate gyrus were quantified by counting the number of inclusion-bearing cells in a field of ~200 (colliculus inferior) or ~400 (dentate gyrus) neurons. At least four such fields were counted for each field of interest. Average inclusion counts and standard deviations were calculated for mice in each of the 5 repeat length categories.

Preparation of protein, RNA and DNA extracts

Half brains (sagittal) were homogenised in 500 µl HEPES-buffer (10 mM HEPES, 300 mM KCl, 3 mM $MgCl_2$, 100 µM $CaCl_2$, 0.45% Triton X-100 and 0.05% Tween-20, pH 7.6), with Complete protease inhibitor cocktail (Roche Diagnostics), 3 mM DTT (Invitrogen) and 20U RNAsin (Promega). After incubating the homogenates on ice for 30 minutes, 100 µl was taken for RNA isolation, using 1 ml of RNAbee (Tel-Test). 200 µl chloroform was then added and the mixture was centrifuged for 15 minutes at 13000 rpm at 4°C for phase separation. One volume of isopropanol was added to the aqueous phase to precipitate the RNA. The pellet was washed with 80% ethanol and dissolved in DEPC-treated MilliQ-H_2O. RNA concentration and purity was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

The remainder of the brain homogenates was centrifuged for 15 minutes at 13000 rpm at 4°C. Protein concentration of the supernatant was determined before use in SDS-PAGE. The pellet was used for DNA extraction, following the same protocol as described for the mouse tail snips.

Quantitative RT-PCR

Reverse transcriptase was performed on 1 µg RNA using iScript cDNA Synthesis Kit (BioRad) according to manufacturer's instructions. Q-PCR was performed on 0.1 µl of RT product. Primers used for Q-PCR were as follows: *Fmr1* transition exon 16/17 (5'-CCGAACAGATAATCGTCCACG-3' as forward primer and 5'-ACGCTGTCTGGCTTTTCCTTC-3' as reverse), *Fmr1* transition exon 7/8 (forward: 5'- TCTGCGCACCAAGTTGTCTC -3', reverse: 5'-CAGAGAAGGCACCAACTGCC-3'), *Gapdh* (internal reference) (forward: 5'-AAATCTTGAGGCAAGCTGCC-3', reverse: 5'-GGATAGGGCCTCTCTTGCTCA-3'). Efficiencies of the different primer sets were checked and found to be comparable. Ct values for *Gapdh* mRNA were subtracted from the Ct value of Fmr1 mRNA for each sample, which gives the ΔCt. Average ΔCt of wt mice was then subtracted from ΔCt of CGG mice, which is designated as the ΔΔCt. The value $2^{-\Delta\Delta Ct}$ then gives the fold change. Average *Fmr1* mRNA levels for each of the repeat length categories were calculated. Differences between the repeat length categories were investigated with one-way ANOVA with fold change *Fmr1* mRNA as dependent variable and repeat length category as the grouping factor. Dunnett's post hoc test was performed to compare all expanded repeat categories against the wt animals and to test for significantly elevated *Fmr1* mRNA levels.

In addition, the existence of a correlation between $(CGG)_n$ length and *Fmr1* mRNA levels was investigated by calculation of the Spearman's rho, both for all repeat lengths taken together, and for each repeat category separately.

Western blotting

We loaded 150 µg of protein onto an 8% SDS-PAGE gel, which was then electroblotted onto a nitrocellulose membrane. The membrane was incubated overnight at 4°C with the monoclonal 2F5-1 antibody specific for Fmrp [35] and a monoclonal antibody against Gapdh (Chemicon), which served as a loading control. The next day the membrane was incubated with a goat-anti-mouse secondary antibody with 800 nm conjugate. After washing, the blot was scanned using the Odyssey™ Infrared Imager. Quantification of integrated intensities of the fluorescent signals was performed using the Odyssey™ 2.1 software. All three Fmrp isoforms were included in the calculations.

The relative Fmrp levels in the $(CGG)_n$ animals were calculated using the average of the Fmrp levels measured in wt animals as a reference. One-way ANOVA was performed with relative Fmrp levels as the dependent variable and repeat length category as the grouping factor. Dunnett's post hoc test was performed to compare all expanded repeat categories against the wt animals and to test for significantly decreased Fmrp levels.

Spearman's rho was calculated to reveal the existence of a correlation between $(CGG)_n$ length and Fmrp levels.

Methylation status

Shortly after birth, tail DNA was checked for the presence of methylated CpGs in the *Fmr1* promoter region. We subjected 1 µg of DNA to bisulphite conversion, using the Qiagen Epitect kit, according to manufacturer's instructions. 1.5 µl of converted DNA was then used for two PCR reactions, where one reaction detects methylated alleles and the other PCR detects unmethylated alleles. For the methylated allele, we used the forward primer 5'- GTTTAAATAGGTTTTACGTTAGTGTC-3', and the reverse primer 5'-CGTCCGTTTACTTCACTACCCG-3', followed by a semi-nested PCR on 3 µl of PCR product using forward primer 5'- GAAGAGGTTTTTAGTTTTCGCGGC-3' and reverse primer 5'-CTCAAACGCGACCCCTCACCG-3'. The unmethylated primer was amplified with forward primer 5'-GTTTAAATAGGTTTTATGTTAGTGTT-3' and reverse primer 5'-CATCCATTTACTTCACTACCCA-3', followed by a semi-nested PCR on 3 µl of PCR product using forward primer 5'-GAAGAGGTTTTTAGTTTTTGTGGT-3' and reverse primer 5'-CTCAAACACAACCCCTCACCA-3'. The PCR mixtures contained 10x PCR buffer, W-1, 4% DMSO, 0.5 µM of both the forward and reverse primer, 1 unit Taq Polymerase (all Invitrogen) and 2 mM $MgCl_2$ for the methylated allele and 1.5 mM $MgCl_2$ for the unmethylated allele. The PCR program was as follows: 5 minutes denaturation at 95°C, 35 cycles of 10 seconds at 93°C, 20 seconds annealing at 55°C and 30 seconds at 72°C, followed by a final elongation step of 10 minutes at 72°C. PCR products were visualised on a 2% agarose gel. The same methylation analysis was performed on DNA isolated from brain.

Results | 6.3

A threshold for $(CGG)_n$ length exist for inclusion formation

When some mice in our colony showed a contraction to about $(CGG)_{70,}$ we examined how this shorter repeat length affected inclusion formation. Immunohistochemistry for ubiquitin in 72-week old animals with $(CGG)_{70}$ and $(CGG)_{\sim 100}$ shows that inclusions were present neither in the colliculus inferior (figure 6.1), nor in other brain areas (data not shown) of $(CGG)_{70}$ animals. In contrast, the colliculus inferior of animals with $(CGG)_{\sim 100}$ showed many inclusions (figure 6.1 and figure 6.3). Thus, a threshold $(CGG)_n$ length exists for the development of inclusions. Fmrp levels were comparable between $(CGG)_{70}$ and wt animals (figure 6.1 and figure 6.5).

Elevated *Fmr1* mRNA levels are only seen in lower range PM alleles

With the availability of a range of repeat lengths we extended our findings about the *Fmr1* mRNA levels to a broader range of repeat lengths using more mice [28,30]. Figure 6.2 shows average fold changes of *Fmr1* mRNA from brain in the different repeat categories with error bars indicating standard deviations. In these experiments, 13 Wt, 4 $(CGG)_{70}$, 15 $(CGG)_{100\text{-}150}$, 8 $(CGG)_{151\text{-}200}$ and 14 $(CGG)_{>200}$ animals were

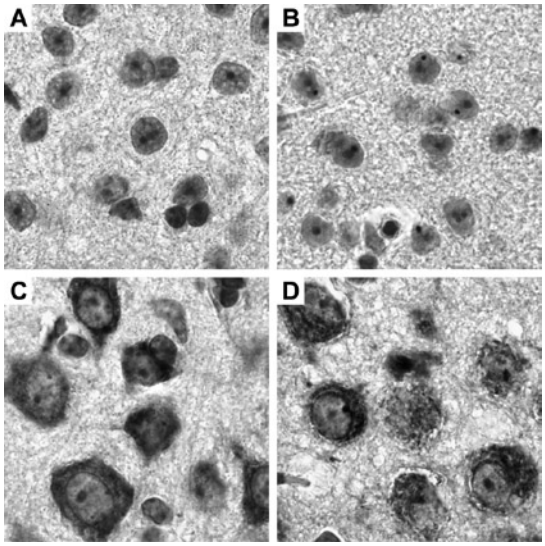

Figure 6.1 | No ubiquitin-positive inclusions are seen in the colliculus inferior of 72-week old mice with $(CGG)_{70}$ (A), while mice with $(CGG)_{106}$ do show ubiquitin-positive inclusions (**B**) brown, round intranuclear staining). Immunohistochemistry for Fmrp in cortex of $(CGG)_{70}$ (**C**) and wt (**D**) animals gives a similar staining pattern. See page 163 onwards for colour version of this figure.

used. One-way ANOVA revealed a statistically significant overall difference between the categories (F=6.47, df=4, p<0.001). To determine which individual categories differed, Dunnett's post hoc test was performed. This revealed that only animals with 100-150 CGGs differ significantly from wt animals (fold change mean difference= 1.60, SE=0.36, p<0.001, statistical parameters of other categories not shown).

No statistically significant correlation was found between $(CGG)_n$ length and *Fmr1* mRNA levels when all categories were taken together (Spearman's rho=0.22, p=0.11), neither when repeat categories were analysed separately (data not shown).No statistically significant correlation was found between $(CGG)_n$ length and *Fmr1* mRNA levels when all categories were taken together (Spearman's rho=0.22, p=0.11), neither when repeat categories were analysed separately (data not shown).

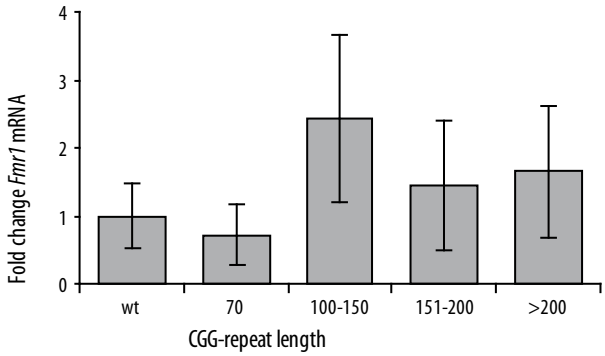

Figure 6.2 | Average fold change of *Fmr1* mRNA levels in the different $(CGG)_n$ length categories. Only $(CGG)_{100-150}$ animals had statistically significantly elevated levels (mean difference=1.60, SE=0.36, p<0.001), as compared with wt.

Not all $(CGG)_n$ animals have inclusions in the dentate gyrus

In our previous studies in which we looked for the presence of inclusions in our expanded $(CGG)_n$ animals, we did not see inclusions in the dentate gyrus at 55 and 72 weeks of age (106 CGGs) [30]. When examining mice with a wider range of $(CGG)_n$ lengths, we now see that some mice have many inclusions, while

others do not have inclusions in this brain region (figure 6.3). The presence or absence of inclusions does not appear to be dependent on $(CGG)_n$ length (data not shown), indicating that the dentate gyrus may be less consistent in forming inclusions than other brain regions such as the colliculus inferior.

A threshold for $(CGG)_n$ length exists above which few inclusions are present

FXTAS is proposed to be the result of an RNA gain-of-function mechanism [10], and inclusions are associated with the development of the disease [25]. Therefore, we were curious to see the proportion of inclusion-bearing cells in our mice, which do ($(CGG)_{100\text{-}150}$) or do not ($(CGG)_{>150}$) have significantly elevated *Fmr1* mRNA levels. Percentages of neurons with inclusions in the colliculus inferior and the dentate gyrus are shown in figure 6.3 (7 $(CGG)_{100\text{-}150}$, 4 $(CGG)_{151\text{-}200}$ and 6 $(CGG)_{>200}$ animals were used). Mice with repeat lengths exceeding 200 CGGs showed very little inclusion formation (figure 6.3 and 6.4). One-way ANOVA revealed a statistically significant difference between the three inclusion-bearing repeat length categories in both the colliculus inferior ($F=8.16$, $df=2$, $p<0.01$) and the dentate gyrus ($F=6.13$, $df=2$, $p=0.02$). Bonferroni-corrected post hoc pair-wise comparisons revealed that inclusion counts did not differ statistically significantly between $(CGG)_{100\text{-}150}$ and $(CGG)_{151\text{-}200}$ in the colliculus inferior (mean difference=0.10, SE=0.15, p=1) and the dentate gyrus (mean difference=0.08, SE=0.12, p=0.12). However, inclusion formation in the $(CGG)_{>200}$ mice differed statistically significantly from $(CGG)_{100\text{-}150}$ in the colliculus inferior (mean difference=-0.42, SE=0.11, $p<0.01$) and the dentate gyrus (mean difference=-0.30, SE=0.09, p=0.02), but not from $(CGG)_{151\text{-}200}$ (colliculus inferior: mean difference=-0.33, SE=0.15, p=0.15, dentate gyrus: mean difference=-0.22, SE=0.12, p=0.31). Not surprisingly, Spearman's rho revealed a statistically significant correlation for $(CGG)_n$ repeat length and the proportion of inclusion-bearing neurons in the colliculus inferior (rho=-0.90, $p<0.001$) and the dentate gyrus (rho=-0.78, p=0.001).

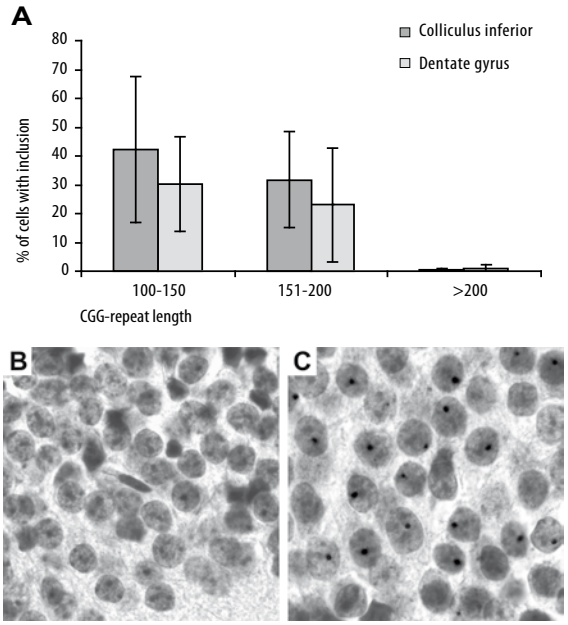

Figure 6.3 | A: Average percentages of inclusion-bearing cells in the colliculus inferior and dentate gyrus in the different $(CGG)_n$ length categories. Error bars show standard deviations. **B**: Dentate gyrus of an animal with $(CGG)_{106}$ that does not show intranuclear inclusions, while a mouse with $(CGG)_{130}$ (**C**) does. See page 163 onwards for colour version of this figure.

Although percentages vary in different regions, an abundance of inclusions is seen in $(CGG)_n$ mice with 100 to 200 CGGs repeats in most brain regions. The observation that only few inclusions can be seen in the brain areas described here (colliculus inferior and the dentate gyrus) in mice with >200 CGGs is representative for all other brain areas examined (data not shown).

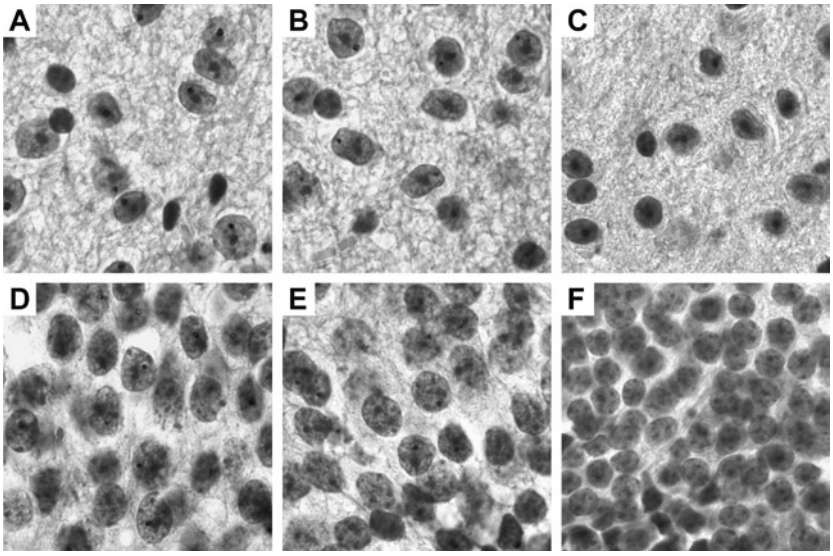

Figure 6.4 | Ubiquitin-positive inclusions are present in colliculus inferior (A-C) and dentate gyrus (D-F) of mice with $(CGG)_{130}$ and $(CGG)_{180}$, but not in mice with $(CGG)_{>200}$. See page 163 onwards for colour version of this figure.

Fmrp levels are reduced in the upper PM range

Measurement of Fmrp levels by SDS-PAGE followed by Western blotting revealed decreased Fmrp levels in expanded $(CGG)_n$ mice. A total of 3 Wt, 1 $(CGG)_{70}$, 10 $(CGG)_{100\text{-}150}$, 5 $(CGG)_{151\text{-}200}$ and 9 $(CGG)_{>200}$ animals were used for these studies. Since only 1 brain of an animal with $(CGG)_{70}$ was available for protein measurement, this sample was not included in the statistical analyses. One-way ANOVA revealed that there was a statistically significant difference between $(CGG)_n$ categories ($F=7.02$, $df=3$, $p<0.01$). A Dunnett's post hoc test was performed to compare Fmrp levels between mice in each repeat category with wt animals. Animals with $(CGG)_{100\text{-}150}$ did not show significantly altered Fmrp levels compared to wt mice (%Frmp mean difference= -0.19, SE=0.11, p=0.11). Mice with expanded alleles of $(CGG)_{151\text{-}200}$ (%Frmp mean difference= -0.40, SE=0.13, $p<0.01$) or $(CGG)_{>200}$ (%Frmp mean difference= -0.44, SE=0.12, $p<0.01$) did have statistically significantly lower Fmrp levels than wt mice.

A significant correlation between CGG repeat length and Fmrp level was also found (Spearman's rho= -0.58, $p<0.01$).

As shown before [28] and confirmed by immunohistochemistry in the present study (data not shown) this decrease in Fmrp is not found in all cells of the brain. For example, while most brain areas showed greatly reduced levels, levels of Fmrp in other regions such as the hippocampal CA2 and CA3 region were relatively unchanged from wt.

Correlations of other parameters

All data (without indication of the variability of the data) are summarised in figure 6.6. Spearman's two-tailed bivariate correlations between all possible combinations of *Fmr1* mRNA, Fmrp and inclusion counts in both regions quantified (i.e., colliculus inferior and dentate gyrus) only revealed a statistically

significant correlation coefficient (rho=0.61, p=0.03) for the relation between Fmrp levels and the proportion of inclusion-bearing cells in the colliculus inferior (other correlation analyses not shown).

No methylation is seen in the Fmr1 promoter region

No methylation was detected in any of the DNA samples isolated from tail DNA shortly after birth (data not shown). Nor was methylation seen in DNA isolated from brain at the time of sacrifice at approximataely 55-58 weeks of age (data not shown).

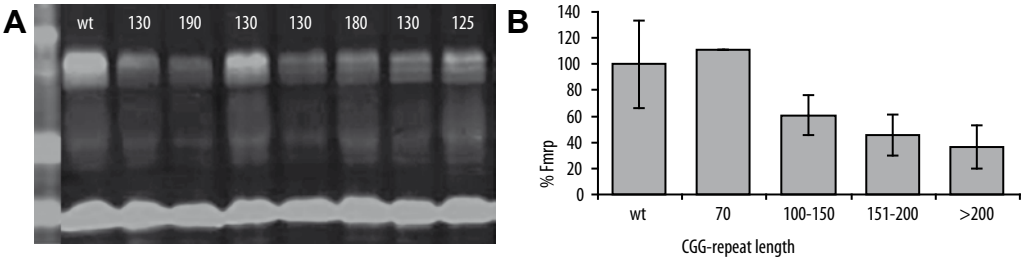

Figure 6.5 | Fmrp levels in mouse brain of the different $(CGG)_n$ length categories. A shows an example of a Western Blot, visualised with the 2F5 antibody against Fmrp, Gapdh was used as a loading control. Approximate $(CGG)_n$ lengths are indicated. B shows average Fmrp levels relative to the average wt level. Error bars represent standard deviations. Note that $(CGG)_{70}$ has no error bar, as this bar represents one sample. Animals with $(CGG)_{>150}$ have statistically significantly lowered Fmrp levels. See text for details on statistical analyses. See page 163 onwards for colour version of this figure.

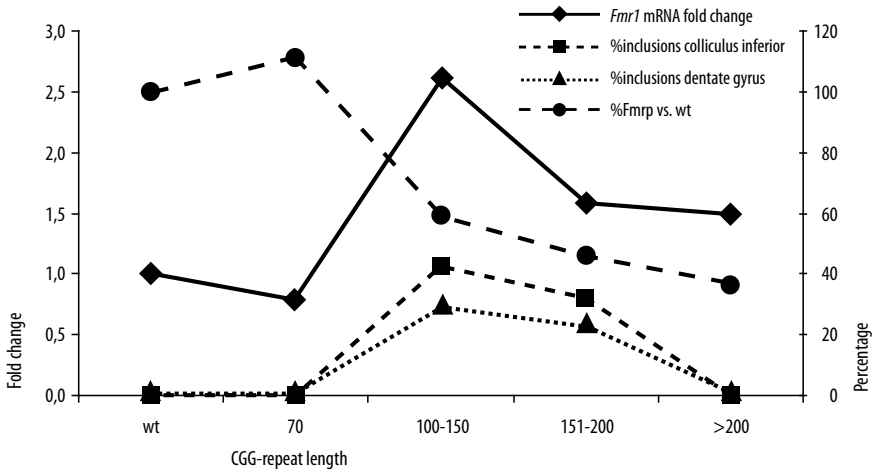

Figure 6.6 | Summary of the molecular correlates. Fold change Fmr1 mRNA (left y-axis), % Fmrp and % of inclusion-bearing neurons in colliculus inferior and dentate gyrus (right y-axis) are given. Please refer to the individual graphs for an indication of the variability of the observed data. See page 163 onwards for colour version of this figure.

Somatic stability in brain

Repeat length analysis in DNA extracted from total brain lysate only showed a single PCR product. Thus, no heterogeneity for repeat length was apparent in the brains of $(CGG)_n$ mice, although we cannot exclude the possibility that with more sensitive methods of visualisation some heterogeneity might be detectable. In addition, comparison with repeat length in tail DNA obtained at 10 days postnatal

revealed very minor repeat length differences (data not shown). Thus, we did not find evidence that somatic instability occurs over the course of life in our mouse model.

Discussion | 6.4

This study was designed to establish the relationships between molecular and neuropathological parameters in an expanded $(CGG)_n$ knock-in mouse model. The results show a high percentage of neurons with intranuclear inclusions in mice with 100 to 200 CGGs, while mice with greater than 200 CGGs show few such inclusions. In addition, only mice with $(CGG)_{100\text{-}150}$ have significantly elevated *Fmr1* mRNA levels, and Fmrp levels are significantly reduced when $(CGG)_n$ length exceeds 150. Our studies furthermore indicate that a lower $(CGG)_n$ length threshold exists, below which no inclusions are formed, as well as a higher threshold, above which few inclusions are seen.

It is striking that mice with 151-200 CGGs have many inclusions, in spite of relatively normal *Fmr1* mRNA levels. This might imply that it is the presence of mutant $(CGG)_n$ RNA that determines the occurrence of cellular toxicity (i.e., inclusions), rather than the precise level of $(CGG)_n$ mRNA. Since there is slightly more Fmrp expressed in mice with 151-200 CGGs as compared with >200 CGGs, it could also be that a minimal amount of Fmrp is necessary for inclusion formation, which is still present in mice with $(CGG)_{151\text{-}200}$. In mice with over 200 CGGs, Fmrp levels might have dropped just below this level, thereby preventing inclusion formation. However, the possibility can not yet be excluded that repeat tracts with over 200 CGGs adopt different tertiary structures than do shorter repeats, thereby influencing the binding of CGG binding proteins (CGG-BPs) and possibly preventing the formation of inclusions.

While Fmrp may be important for inclusion formation, it is noteworthy that Fmrp has not yet been detected in inclusions in mice [30] and in humans [36]. Furthermore, inclusions could be induced in a human neuronal cell model expressing a $(CGG)_n$ fused to a *Gfp* reporter [37], showing that inclusions can be formed, without the need for the $(CGG)_n$ to be in the context of *Fmr1*. These cells did express endogenous Fmrp levels [37]. In order to investigate the role of Fmrp in the formation of inclusions, breedings of female mice homozygous for $(CGG)_{>200}$ with male *Fmr1* KO mice heterozygous for a yeast artificial chromosome (YAC) containing the entire human *FMR1* gene and some flanking sequences [38,39] will be set up. This will allow comparison of inclusion formation in the $(CGG)_{>200}$ offspring in the presence (YAC +/-) or absence (YAC -/-) of FMRP expression.

It is possible that a minimal abundance of CGG triplets is needed, either due to elevated transcript levels or the length of the repeat tract, or a combination thereof, to cause cellular pathology. Such an effect of 'CGG molarity' in the development of cellular toxicity in FXTAS has been hypothesised in the toxic RNA gain-of-function model [23]. In light of the toxic RNA gain-of-function model, it could be that a longer repeat tract provides more binding sites for $(CGG)_n$-BPs, such that it exerts toxicity similar to higher levels of a shorter repeat. With regard to the protein sequestration model for DM, it has been found that Muscleblind-like protein 1 (MBNL1) binding is proportional to $(CUG)_n$ length [40]. Sequestration of $(CUG)_n$-BPs away from their normal cellular functions, including splice regulation, has been proposed to be the mechanism underlying DM [40-42]. Interestingly, MBNL1 has also been found in intranuclear inclusions in human FXTAS brain [36], suggesting that a similar pathogenic mechanism may occur in FXTAS. However, no downstream splicing defects have thus far been revealed in FXTAS. In addition, we

have not detected Mbnl1 in the inclusions present in our $(CGG)_n$ mouse model (unpublished results).

Our studies in animals carrying a $(CGG)_{70}$ allele may provide insight on the observation that shorter PM alleles (<70 CGGs) may pose a lower risk of developing FXTAS for the carrier than do longer repeats [43]. Although some cases with repeat lengths below 70 CGGs have been described [44,45], clinical presentation was atypical and thus these findings may be coincidental [43]. It is therefore interesting that mice with $(CGG)_{70}$ neither show inclusions, nor elevated *Fmr1* mRNA, suggesting that there is a minimum repeat length necessary for the development of disease in mice.

Some correlations between molecular and clinical measures in FXTAS patients have been described. For instance, $(CGG)_n$ length strongly correlates with age of death [31]. $(CGG)_n$ length has also been reported to correlate negatively with age of onset of action tremor and ataxia. Age of onset of these motor symptoms was not correlated to *FMR1* mRNA or FMRP levels in blood. For FMRP levels, this is not surprising, as they are close to normal in FXTAS patients. The lack of correlation with *FMR1* transcript levels might be explained by the fact that mRNA levels are measured in blood, whereas clinical symptoms derive from brain pathology [32]. Furthermore, $(CGG)_n$ has been correlated with increased cognitive and functional impairment [33]. In addition, $(CGG)_n$ length in humans has been found to be highly correlated to the number of intranuclear inclusions in both neurons and astrocytes, suggesting that it might serve as a powerful predictor of neuropathological involvement [31].

The presence of inclusions is associated with disease [31]. The size and number of inclusions in murine brain increase with time, reflective of the progressive nature of the disease. In addition, functions of some inclusion-bearing brain regions in expanded $(CGG)_n$ mice can be linked to clinical symptoms in patients with FXTAS. Both findings are suggestive of a role for inclusions in the disease process [30]. It is, however, as yet unknown how inclusions cause cellular dysfunction, and eventually neurodegeneration and clinical pathophysiology. Their immunoreactivity to antibodies against ubiquitin and components of the proteasome, Hsp70 and αB-crystallin suggests involvement of the proteasomal protein degradation pathway [25,30]. In mouse brain, an antibody directed against only poly-ubiquitinated proteins, as well as against mono- and polyubiquitinated proteins, detected inclusions [30]. However, in isolated inclusions from human FXTAS brain, there was little evidence that the proteins present in the inclusions are poly-ubiquitinated, which is normally the signal that causes a protein to be degraded by the proteasome. Thus, impaired proteasomal degradation does not seem to play a central role in the formation of the inclusions [36]. It appears more likely that expanded $(CGG)_n$ *FMR1* mRNA acts as a nucleation centre for other proteins, similar to the nuclear RNA foci seen in DM [23].

In a previous study we reported elevated *Fmr1* mRNA levels in mice with over 150 CGGs [28], which is in contrast with our current findings. At the time of that study, availability of mice with such long repeat tracts was limited, so few animals could be investigated. Therefore, along with considering the large variability seen for all repeat categories as well as for the wt animals, we believe that our previous findings likely represented a coincidental, biased selection of animals for study. Our conclusion still stands that in mouse brain there is no linear relationship between $(CGG)_n$ length and *Fmr1* mRNA levels, unlike what has been reported in the blood of human PM carriers. In humans, there is no evidence for a drop in *FMR1* mRNA levels above a certain unmethylated $(CGG)_n$ length [16,19,20]. In another expanded $(CGG)_n$ mouse model developed by Entezam and colleagues, a linear increase in *Fmr1* mRNA with

increasing $(CGG)_n$ length was reported [46]. However, the number of animals that was used per repeat length was not reported. It is therefore unclear whether those findings were also influenced by high inter-animal variation. Also, t-tests were performed to compare the different repeat lengths, without the mention of correction for multiple comparisons.

The Fmrp levels measured in this study are consistent with those reported in our previous study [28], such that decreased levels were only seen when $(CGG)_n$ exceeded 150. Again, variability was large. In the other mouse model for FXTAS Fmrp levels had already decreased at $(CGG)_{130}$, but again the number of mice used in that study was not reported [46].

The high variability found in *Fmr1* transcript levels in KI mouse brain, might explain the fact that not all PM carriers develop FXTAS. Most studies on *FMR1* expression have been done in blood of PM carriers with or without FXTAS, and both show elevated levels on average. However, increases of differing magnitude in *FMR1* mRNA have been described [19,20,22]. The generally higher fold change of *FMR1* mRNA in human PM carriers versus normal controls, in comparison with lower 2-3 fold changes measured in our mice can be explained by the fact that we isolate RNA from whole brain lysate, whereas RNA in human studies is extracted from blood. Thus far, brain *FMR1* mRNA levels have been quantified in one FXTAS patient. Absolute *FMR1* mRNA levels were found to be higher in brain than in peripheral blood leukocytes, although fold increases as compared to normal controls were more pronounced in RNA isolated from blood. Furthermore, differential *FMR1* expression was seen in different brain regions, despite the fact that neurons in each region showed the same repeat length [47]. Thus our results in $(CGG)_n$ mice might represent an average of higher and lower regional expression levels throughout the brain. It would be interesting to measure *Fmr1* mRNA and Fmrp levels in different mouse brain areas and relate this to the proportion of inclusion-bearing neurons. However, it would be very challenging to reliably dissect the small mouse brain.

In postmitotic neurons in a mouse model for Huntington's disease, higher $(CAG)_n$ instability was observed in brain regions that show high expression of the mutant allele [48]. Thus, if *Fmr1* mRNA expression varies among different brain regions in our $(CGG)_n$ mice, it would be interesting to investigate somatic instability in those specific regions. However, to date there is no evidence that $(CGG)_n$ length heterogeneity exists in human FXTAS brain [47]. In the present study very minor $(CGG)_n$ instability was seen when comparing brain DNA obtained at death with DNA obtained from tail shortly after birth. Naturally, this comparison cannot be made in patients with FXTAS. No evidence for a broader pattern of $(CGG)_n$ lengths was seen in brain DNA as compared with tail DNA, thus repeat length heterogeneity does not seem to be a major occurrence.

The elevated *FMR1* mRNA levels have been attributed to increased transcription, rather than to increased stability of the *FMR1* transcripts [49,50]. The cause of increased transcription is unknown, although it could be that the expanded $(CGG)_n$ causes the chromatin to adopt a more open conformation, which could facilitate transcription. In addition, the $(CGG)_n$ length has been shown to influence which transcription start site is used, with the use of more upstream start sites corresponding to increased *FMR1* transcription [51]. It has been proposed that a feedback system might exist such that lower FMRP levels cause increased *FMR1* transcription [20,52]. However, cellular studies show increased expression of reporter constructs containing the *FMR1* 5'UTR containing various $(CGG)_n$ lengths, in the absence of

FMRP. This suggests that an intrinsic quality of the *FMR1* 5'UTR and/or the $(CGG)_n$ might be the cause of the elevated transcription. In addition, since the *FMR1* 5'UTR was driven by the CMV immediate early promoter in these experiments, the increased transcription must be independent of the *FMR1* promoter. Thus, although it should be noted that this study was performed under conditions of overexpression of the construct, at least part of the elevated transcript levels seen in PM carriers may be a direct *cis*-acting effect of the $(CGG)_n$, rather than a compensatory response to reduced FMRP levels [53]. This possibility might be reflected by the lack of correlation between *Fmr1* transcript levels and Fmrp levels in this study.

Current evidence suggests that *FMR1* transcripts are not retained in the nucleus, making this an unlikely reason for reduced translation [50]. The $(CGG)_n$ has been found to impede the 40s ribosomal unit, leading to suppression of translation [54]. Indeed, FMRP levels are mostly reduced in the upper PM range in humans [19,50], as well as in mice.

In humans, CGG expansions above 200 generally lead to methylation of the repeat and silencing of the gene [4-6]. This has not been observed in expanded $(CGG)_n$ mouse models [28,46]. In general, it has been proposed that mouse models for trinucleotide repeat disorders need longer repeats in order to see major instability than do human repeats [55]. The same might be true for methylation of the gene and/or phenotypes associated with the repeats.

The finding that some animals show inclusions in the dentate gyrus, while others do not, irrespective of $(CGG)_n$ length, implies that modifying factors play a role in the formation of inclusions. We cannot exclude that the same effect occurs in other brain regions, as we have not investigated all brain areas extensively. As our breedings are mostly set up to obtain certain repeat lengths, animals have a mixed genetic background. As it is known that genetic background has an influence on behavioural phenotype in different strains of *Fmr1* KO mice [56], a similar influence of the genetic background on inclusion formation in specific regions could have taken place in our mice.

In summary, this study indicates that there is both a lower repeat length threshold, below which no inclusions form, and an upper threshold above which hardly any inclusions develop. There does not appear to be a linear correlation between $(CGG)_n$ and *Fmr1* mRNA levels in brain or the presence of inclusions. Fmrp levels do decrease with increasing repeat length, specifically after the $(CGG)_n$ exceeds 150. Thus, if inclusions are responsible for the clinical outcome, the current results suggest that merely the presence of expanded $(CGG)_n$ *Fmr1* mRNA, rather than the precise level of expression, possibly in combination with a minimal level of remaining Fmrp, determines the occurrence of inclusion neuropathology. Behavioural studies in mice of differing $(CGG)_n$ repeat lengths will be necessary to establish whether an aberrant phenotype is primarily related to the length of the $(CGG)_n$ expansion or to *Fmr1* expression levels.

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References |

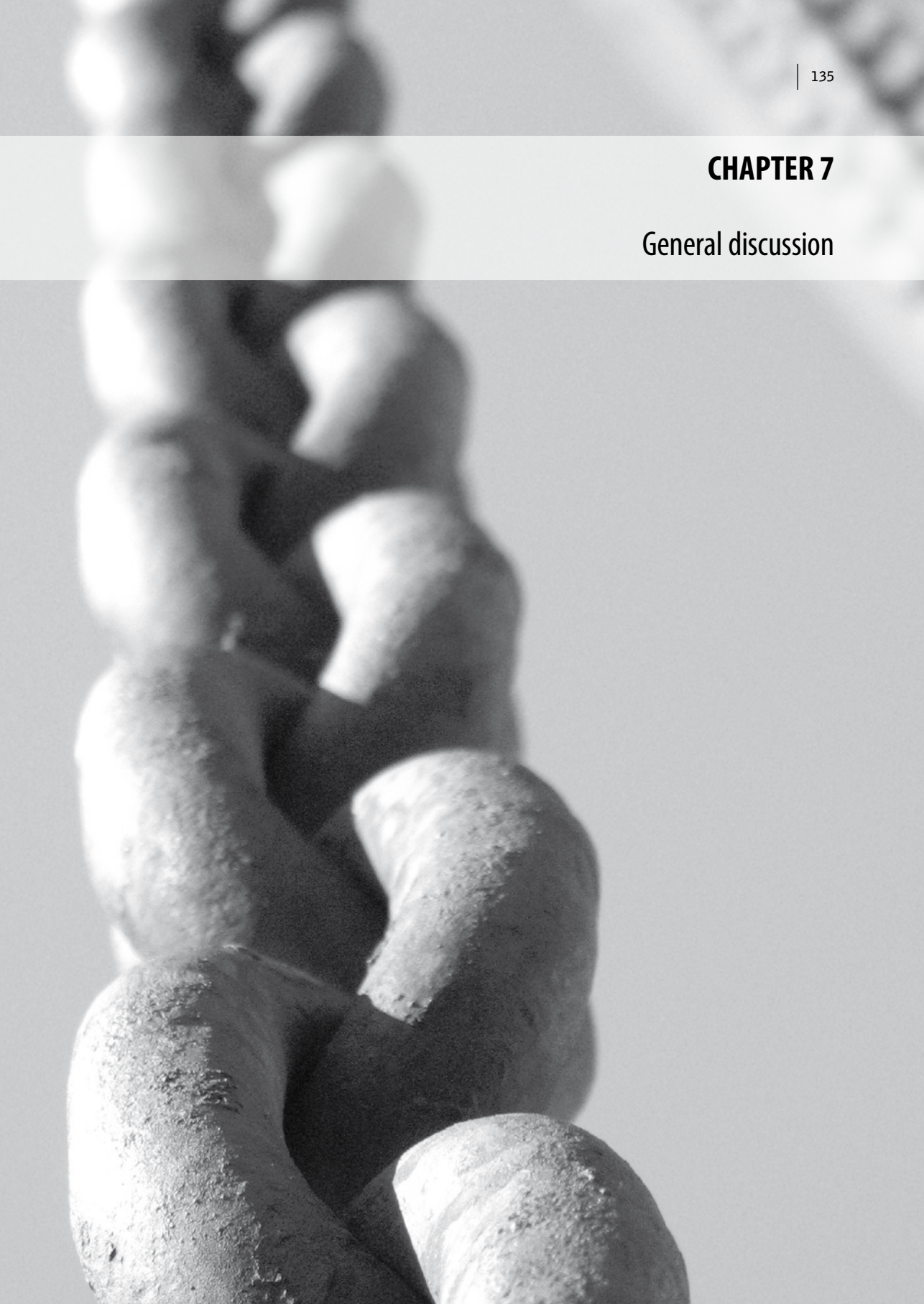
1. Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS *et al.* 1991. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67:1047-1058.
2. Zhong N, Ju WN, Pietrofesa J, Wang DW, Dobkin C *et al.* 1996. Fragile X "gray zone" alleles: AGG patterns, expansion risks, and associated haplotypes. Am J Med Genet 64:261-265.
3. Nolin SL, Lewis FA, Ye LL, Houck GE, Glicksman AE *et al.* 1996. Familial transmission of the FMR1 CGG repeat. Am J Hum Genet 59:1252-1261.
4. Oberlé I, Rousseau F, Heitz D, Kretz C, Devys D *et al.* 1991. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. Science 252:1097-1102.
5. Sutcliffe JS, Nelson DL, Zhang F, Pieretti M, Caskey CT *et al.* 1992. DNA methylation represses FMR-1 transcription in fragile X syndrome. Hum Mol Genet 1:397-400.
6. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP *et al.* 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905-914.
7. Pieretti M, Zhang FP, Fu YH, Warren ST, Oostra BA *et al.* 1991. Absence of expression of the FMR-1 gene in fragile X syndrome. Cell 66:817-822.
8. Verheij C, Bakker CE, de Graaff E, Keulemans J, Willemsen R *et al.* 1993. Characterization and localization of the FMR-1 gene product associated with fragile X syndrome. Nature 363:722-724.
9. Hagerman RJ, Hagerman PJ. 2002. The fragile X premutation: into the phenotypic fold. Curr Opin Genet Dev 12:278-283.
10. Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R *et al.* 2001. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 57:127-130.
11. Jacquemont S, Hagerman RJ, Leehey M, Grigsby J, Zhang L *et al.* 2003. Fragile X Premutation Tremor/Ataxia Syndrome: Molecular, Clinical, and Neuroimaging Correlates. Am J Hum Genet 72:869-878.
12. Jacquemont S, Hagerman RJ, Leehey MA, Hall DA, Levine RA *et al.* 2004. Penetrance of the fragile x-associated tremor/ataxia syndrome in a premutation carrier population. JAMA 291:460-469.
13. Hagerman RJ, Leavitt BR, Farzin F, Jacquemont S, Greco CM *et al.* 2004. Fragile-X-Associated Tremor/Ataxia Syndrome (FXTAS) in Females with the FMR1 Premutation. Am J Hum Genet 74:1051-1056.
14. Zuhlke C, Budnik A, Gehlken U, Dalski A, Purmann S *et al.* 2004. FMR1 premutation as a rare cause of late onset ataxia. Evidence for FXTAS in female carriers. J Neurol 251:1418-1419.
15. Berry-Kravis E, Potanos K, Weinberg D, Zhou L, Goetz CG. 2004. Fragile X-associated tremor/ataxia syndrome in sisters related to X-inactivation. Ann Neurol 57:144-147.
16. Allen EG, He W, Yadav-Shah M, Sherman SL. 2004. A study of the distributional characteristics of FMR1 transcript levels in 238 individuals. Hum Genet 114:439-447.
17. Sherman SL. 2000. Premature ovarian failure in the fragile X syndrome. Am J Med Genet 97:189-194.
18. Berry-Kravis E, Abrams L, Coffey SM, Hall DA, Greco C *et al.* 2007. Fragile X-associated tremor/ataxia syndrome: Clinical features, genetics, and testing guidelines. Mov Disord 14:2014-2030.
19. Kenneson A, Zhang F, Hagedorn CH, Warren ST. 2001. Reduced FMRP and increased FMR1 transcription is proportionally associated with CGG repeat number in intermediate-length and premutation carriers. Hum Mol Genet 10:1449-1454.
20. Tassone F, Hagerman RJ, Taylor AK, Gane LW, Godfrey TE *et al.* 2000. Elevated levels of FMR1 mRNA in carrier males: A new mechanism of involvement in the Fragile-X syndrome. Am J Hum Genet 66:6-15.
21. Tassone F, Hagerman RJ, Taylor AK, Mills JB, Harris SW *et al.* 2000. Clinical involvement and protein expression in individuals with the FMR1 premutation. Am J Med Genet 91:144-152.
22. Primerano B, Tassone F, Hagerman RJ, Hagerman P, Amaldi F *et al.* 2002. Reduced FMR1 mRNA translation efficiency in Fragile X patients with premutations. RNA 8:1-7.
23. Hagerman PJ, Hagerman RJ. 2004. The Fragile-X Premutation: A Maturing Perspective. Am J Hum Genet 74:805-816.
24. Ranum LP, Day JW. 2004. Pathogenic RNA repeats: an expanding role in genetic disease. Trends Genet 20:506-512.
25. Greco CM, Hagerman RJ, Tassone F, Chudley AE, Del Bigio MR *et al.* 2002. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. Brain 125:1760-1771.
26. Tassone F, Iwahashi C, Hagerman PJ. 2004. FMR1 RNA withinthe intranuclear inclusions of fragile X-associated Tremor/Ataxia syndrome (FXTAS). RNA biology 1:103-105.
27. Bontekoe CJ, Bakker CE, Nieuwenhuizen IM, van Der Linde H, Lans H *et al.* 2001. Instability of a (CGG)(98) repeat in the Fmr1 promoter. Hum Mol Genet 10:1693-1699.

28. Brouwer JR, Mientjes EJ, Bakker CE, Nieuwenhuizen IM, Severijnen LA *et al.* 2007. Elevated Fmr1 mRNA levels and reduced protein expression in a mouse model with an unmethylated Fragile X full mutation. Exp Cell Res 313:244-253.
29. Brouwer JR, Severijnen E, de Jong FH, Hessl D, Hagerman RJ *et al.* 2008. Altered hypothalamus-pituitary-adrenal gland axis regulation in the expanded CGG-repeat mouse model for fragile X-associated tremor/ataxia syndrome. Psychoneuroendocrinology.
30. Willemsen R, Hoogeveen-Westerveld M, Reis S, Holstege J, Severijnen L *et al.* 2003. The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. Hum Mol Genet 12:949-959.
31. Greco CM, Berman RF, Martin RM, Tassone F, Schwartz PH *et al.* 2006. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). Brain 129:243-255.
32. Tassone F, Adams J, Berry-Kravis EM, Cohen SS, Brusco A *et al.* 2007. CGG repeat length correlates with age of onset of motor signs of the fragile X-associated tremor/ataxia syndrome (FXTAS). Am J Med Genet B Neuropsychiatr Genet 144B:566-569.
33. Grigsby J, Brega AG, Jacquemont S, Loesch DZ, Leehey MA *et al.* 2006. Impairment in the cognitive functioning of men with fragile X-associated tremor/ataxia syndrome (FXTAS). J Neurol Sci 248:227-233.
34. Bakker CE, de Diego Otero Y, Bontekoe C, Raghoe P, Luteijn T *et al.* 2000. Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse. Exp Cell Res 258:162-170.
35. Gabel LA, Won S, Kawai H, McKinney M, Tartakoff AM *et al.* 2004. Visual Experience Regulates Transient Expression and Dendritic Localization of Fragile X Mental Retardation Protein. J Neurosci 24:10579-10583.
36. Iwahashi CK, Yasui DH, An HJ, Greco CM, Tassone F *et al.* 2006. Protein composition of the intranuclear inclusions of FXTAS. Brain 129:256-271.
37. Arocena DG, Iwahashi CK, Won N, Beilina A, Ludwig AL *et al.* 2005. Induction of inclusion formation and disruption of lamin A/C structure by premutation CGG-repeat RNA in human cultured neural cells. Hum Mol Genet 14:3661-3671.
38. Peier AM, McIlwain KL, Kenneson A, Warren ST, Paylor R *et al.* 2000. (Over)correction of FMR1 deficiency with YAC transgenics: behavioral and physical features. Hum Mol Genet 9:1145-1159.
39. Musumeci SA, Calabrese G, Bonaccorso CM, D'Antoni S, Brouwer JR *et al.* 2007. Audiogenic seizure susceptibility is reduced in fragile X knockout mice after introduction of FMR1 transgenes. Exp Neurol 203:233-240.
40. Timchenko LT, Miller JW, Timchenko NA, DeVore DR, Datar KV *et al.* 1996. Identification of a (CUG)n triplet repeat RNA-binding protein and its expression in myotonic dystrophy. Nucleic Acids Res 24:4407-4414.
41. Miller JW, Urbinati CR, Teng-Umnuay P, Stenberg MG, Byrne BJ *et al.* 2000. Recruitment of human muscleblind proteins to (CUG)(n) expansions associated with myotonic dystrophy. Embo J 19:4439-4448.
42. Fardaei M, Larkin K, Brook JD, Hamshere MG. 2001. In vivo co-localisation of MBNL protein with DMPK expanded-repeat transcripts. Nucleic Acids Res 29:2766-2771.
43. Jacquemont S, Leehey MA, Hagerman RJ, Beckett LA, Hagerman PJ. 2006. Size bias of fragile X premutation alleles in late-onset movement disorders. J Med Genet 43:804-809.
44. Kamm C, Healy DG, Quinn NP, Wullner U, Moller JC *et al.* 2005. The fragile X tremor ataxia syndrome in the differential diagnosis of multiple system atrophy: data from the EMSA Study Group. Brain 128:1855-1860.
45. Macpherson J, Waghorn A, Hammans S, Jacobs P. 2003. Observation of an excess of fragile-X premutations in a population of males referred with spinocerebellar ataxia. Hum Genet 112:619-620.
46. Entezam A, Biacsi R, Orrison B, Saha T, Hoffman GE *et al.* 2007. Regional FMRP deficits and large repeat expansions into the full mutation range in a new Fragile X premutation mouse model. Gene 395:125-134.
47. Tassone F, Hagerman RJ, Garcia-Arocena D, Khandjian EW, Greco CM *et al.* 2004. Intranuclear inclusions in neural cells with premutation alleles in fragile X associated tremor/ataxia syndrome. J Med Genet 41:E43.
48. Gonitel R, Moffitt H, Sathasivam K, Woodman B, Detloff PJ *et al.* 2008. DNA instability in postmitotic neurons. Proc Natl Acad Sci U S A 105:3467-3472.
49. Tassone F, Hagerman RJ, Chamberlain WD, Hagerman PJ. 2000. Transcription of the FMR1 gene in individuals with fragile X syndrome. Am J Med Genet 97:195-203.
50. Tassone F, Beilina A, Carosi C, Albertosi S, Bagni C *et al.* 2007. Elevated FMR1 mRNA in premutation carriers is due to increased transcription. RNA 13:555-562.
51. Beilina A, Tassone F, Schwartz PH, Sahota P, Hagerman PJ. 2004. Redistribution of transcription start sites within the FMR1 promoter region with expansion of the downstream CGG-repeat element. Hum Mol Genet 13:543-549.
52. Oostra BA, Willemsen R. 2003. A fragile balance: FMR1 expression levels. Hum Mol Genet 12 Suppl 2:R249-257.
53. Chen LS, Tassone F, Sahota P, Hagerman PJ. 2003. The (CGG)n repeat element within the 5'untranslated region of the FMR1 message provides both positive and negative cis effects on in vivo translation of a downstream reporter. Hum Mol Genet 12:3067-3074.

54. Feng Y, Zhang FP, Lokey LK, Chastain JL, Lakkis L *et al.* 1995. Translational suppression by trinucleotide repeat expansion at FMR1. Science 268:731-734.
55. Gourdon G, Radvanyi F, Lia AS, Duros C, Blanche M *et al.* 1997. Moderate intergenerational and somatic instability of a 55-CTG repeat in transgenic mice. Nature Genet 15:190-192.
56. Spencer CM, Serysheva E, Yuva-Paylor LA, Oostra BA, Nelson DL *et al.* 2006. Exaggerated behavioral phenotypes in Fmr1/Fxr2 double knockout mice reveal a functional genetic interaction between Fragile X-related proteins. Hum Mol Genet 15:1884-1894.

CHAPTER 7

General discussion



Although the clinical consequences for carriers of *FMR1* FM alleles had long been established [1-3], only few reports were initially published on mild clinical involvement in carriers of the PM, predominantly in females (reviewed in [4]). Intelligence was normal [5,6], but emotional problems such as attention deficit hyperactivity disorder and anxiety were observed [7,8]. Premature menopause and a higher rate of twinning had been recognised among female PM carriers [9,10]. Male PM carriers were also characterised; a subgroup was found to have cognitive impairment [11,12]. These findings prompted Tassone and colleagues to look into the consequences of premutations at a molecular level. They found that PM carriers have elevated *FMR1* mRNA levels [13].

The authors hypothesised that elevated transcript levels, in combination with slightly decreased FMRP levels, represented a defective translational efficiency, which might be responsible for the clinical involvement in the PM range [13]. Soon after the recognition of the molecular phenotype, a new neurodegenerative syndrome was appreciated in a subgroup of elderly men with the *FMR1* premutation [14-17], which was designated fragile X-associated tremor/ataxia syndrome (FXTAS) [18]. This syndrome was postulated to result from a toxic RNA gain-of-function mechanism [4,14,15,18-20], similar to the pathogenesis model described for myotonic dystrophy [21].

The toxic RNA gain-of-function pathogenesis model for FXTAS | 7.1

The combination of several observations has lead to the hypothesis that FXTAS is the result of a toxic RNA gain-of-function mechanism. First, PM carriers have elevated *FMR1* mRNA levels, while FMRP levels are only mildly reduced [13,22]. No signs of FXTAS have been observed among elderly FM carriers, thus, the slight protein deficiency in PM carriers is not likely the cause of FXTAS [14,18,19]. Also the presence of *FMR1* transcripts in intranuclear inclusions suggests a role for the mRNA in the pathogenesis ([23], summarised in [4]).

In support of the toxic RNA pathogenesis model for FXTAS, long, transcribed but untranslated $(CGG)_n$ were found to be toxic to human cells. The repeat-containing transcripts alter the expression of a wide variety of genes, suggestive of an effect on a variety of biological processes [24]. In a neural cell model, expression of the *FMR1* 5'UTR containing the expanded $(CGG)_n$, fused to a reporter gene, as RNA, was capable of inducing inclusion formation [25], showing that inclusion formation was possible without FMRP synthesis. A neurodegenerative eye phenotype could be induced upon expression of $(CGG)_n$-containing constructs in a *Drosophila* model. Higher expression levels of $(CGG)_{60}$-containing RNA were necessary than of $(CGG)_{90}$ RNA to induce a similar neurodegenerative phenotype [26]. This indicates that both $(CGG)_n$ length and expression level determine RNA toxicity in the *Drosophila* model for FXTAS.

$(CGG)_n$-binding proteins | 7.1.1

The capacity of some $(CGG)_n$-binding proteins ($(CGG)_n$-BPs: Pur α, hnRNP A2/B1, (CUG)-BP1), as well as co-expression of the *FMR2* $(GCC)_n$ to rescue the neurodegenerative phenotype in *Drosophila* further underscores the central role of the expanded $(CGG)_n$ RNA in the pathogenesis of FXTAS [27-29].

Some of these $(CGG)_n$-BPs have also been identified in inclusions in human FXTAS brain, such as hnRNP A2/B1 [30] and Pur α [27]. Also MBNL1, known to bind $(CUG)_n$-containing transcripts and to play a

central role in the pathogenesis of myotonic dystrophy, was present in the inclusions in human FXTAS brain [30]. In myotonic dystrophy, it is now well established that sequestering these $(CUG)_n$-BPs away from their normal function leads to dysregulated splicing, of which the effects can sometimes directly be linked to the clinical outcome (summarised in [31]). In FXTAS, no downstream effects of the sequestering of certain proteins into the inclusions, such as altered splicing regulation, have been described to date.

It has been speculated that hnRNP A2 is involved in the development of FXTAS, as it was present in intranuclear inclusions and found to bind directly to the 5'UTR of *FMR1* mRNA. hnRNP A2 mediates transport of mRNAs, including Myelin Basic Protein (MBP) mRNA in oligodendroglial cells. MBP is a major component of the myelin sheath, and was also found to be present in neuronal and glial inclusions. hnRNP A2 function might be affected in FXTAS. Interestingly, white matter disease, with loss of axons and myelin, is a central neuropathological feature in FXTAS. Partial sequestration of hnRNP A2 might thus affect MBP mRNA transport and could adversely affect axon myelination in FXTAS [30].

We made an attempt at visualising cellular localisation of *Fmr1* mRNA in mouse brain, by means of *in situ* hybridisation. Naturally, we were interested to see whether *Fmr1* mRNA is also present in inclusions in mouse brain. Unfortunately this failed due to the low abundance of these transcripts and/or the inability to design good probes for this gene. Immunohistochemistry using antibodies against possible $(CGG)_n$-binding proteins on brain sections of $(CGG)_n$ and wt mice could reveal altered cellular localisation. This might shed light on proteins that are sequestered to the $(CGG)_n$ RNA and drawn away from their normal function, possibly into the intranuclear inclusions. Another approach to answer the question which proteins interact with the $(CGG)_n$ RNA is to cross-link proteins to RNA in $(CGG)_n$ mouse brain cell suspensions and then to specifically pull down the *Fmr1* mRNA. The $(CGG)_n$ RNA-interacting proteins can then for example be investigated using immunogenic visualisation by SDS-PAGE followed by Western blotting for likely targets or protein identification by mass spectrometry to find new binding partners.

hnRNP A2/B1 showed a much more prominent interaction with $(CGG)_n$ RNA in the cytoplasmic fraction than in the nuclear fraction of mouse cerebellar lysates [29]. Thus, cytoplasmic interaction of $(CGG)_n$-BP with $(CGG)_n$ RNA should also be considered, although no model has been proposed which could link cytoplasmic protein-RNA interactions with presence of this protein in the intranuclear inclusions.

Inclusion formation in the brain | 7.1.2

It is currently unknown how mutant RNA leads to inclusion formation. Chapter 6 describes that *Fmr1* levels are elevated in mice with 100 to 150 CGGs, but return to normal when $(CGG)_n$ exceeds 150. Inclusions are ubiquitous in animals with up to 200 CGGs. Fmrp levels are significantly reduced in mice with over 150 CGGs. Thus, in light of the toxic RNA gain-of-function hypothesis, it is interesting to note that mice with 151-200 CGGs do have many inclusions, despite normal *Fmr1* mRNA levels. Based on these findings it could be hypothesised that not necessarily the precise amount of *Fmr1* mRNA determines cellular toxicity, but that the mere presence of mutant RNA might be responsible for the formation of inclusions. Willemsen and colleagues already proposed before that the *Fmr1* gene is likely to play a direct role in the development of inclusions, either by $(CGG)_n$ expansion itself or elevated transcript levels [32].

It can, however, not be excluded that a minimal amount of Fmrp is needed for formation of inclusions, which is still present in mice with $(CGG)_{151-200.}$ Above 200 CGGs, the Fmrp level might drop below this threshold, protecting the mice from inclusion formation. Nevertheless, this role for Fmrp is difficult to explain considering that no Fmrp has been detected in inclusions in mice [32] or men [30]. In order to determine whether Fmrp is necessary for inclusion formation, breedings of female mice homozygous for $(CGG)_{>200}$ with male *Fmr1* KO mice heterozygous for a yeast artificial chromosome (YAC) containing the entire human *FMR1* gene and some flanking sequences [33,34] will be set up. Since the YAC transgene introduces FMRP expression, this will allow comparison of inclusion formation in the $(CGG)_{>200}$ offspring in the presence (YAC +/-) or absence (YAC -/-) of FMRP expression. Alternatively, $(CGG)_{>200}$ might adopt different secondary structures, such that CGG-BPs cannot bind the repeat, possibly thereby preventing inclusion formation.

Chapter 4 discusses cellular pathology in fibroblast lines derived from embryonic and adult $(CGG)_{>200}$ mice. *Fmr1* mRNA levels are elevated as compared with wt mice in $(CGG)_n$ embryonic fibroblasts, but not in adult fibroblasts. Although more pronounced in the embryonic fibroblasts, both $(CGG)_n$ cell lines show signs of cellular stress. This also suggests that the presence of expanded $(CGG)_n$, although not at high levels, is sufficient to cause cellular toxicity. Intranuclear inclusions were not observed, likely due to the high proliferation rate of fibroblast lines.

It is unclear what this means for carriers of repeat lengths in the high PM range. Few PM individuals with $(CGG)_{151-200}$ have been described, but all had elevated *FMR1* mRNA levels in blood [22,35-37]. It is interesting to note that to date no patients with FXTAS have been seen in carriers of repeats longer than 150 CGGs (R. Hagerman, personal communication). This could mean that carriers with PM alleles in this range hardly exist, because shorter PM alleles almost always expand directly into the FM range. Alternatively, they might exist, but they might not develop any clinical signs, and therefore do not consult clinicians. They would, however, likely be discovered in screening efforts in fragile X families. Interestingly, although prevalence of POF increases in female PM carriers with $(CGG)_n$ with up to 100 CGGs, it is noteworthy that above this length, the risk for ovarian dysfunction remains stable or perhaps decreases [38]. Very recently, also the risk of getting lifetime major depressive disorder (MDD) was found to exhibit a non-linear relationship with $(CGG)_n$ length in PM mothers with at least one child with fragile X. Although preliminary, this study suggests that PM females with longer $(CGG)_n$ are at lower risk of developing MDD than are carriers of mid-range $(CGG)_n$ [39].

It remains to be solved whether inclusions are a by-product of cellular toxicity, or the origin of the cellular dysfunction. It has been proposed that $(CGG)_n$ mRNA may act as a nucleation centre for other proteins [4]. Indeed, *FMR1* mRNA is present in inclusions [23]. Over 20 proteins have been identified in isolated inclusions. There is no dominant protein species present; all proteins represent less than 10% of the total protein content of the isolated inclusions [30]. A role for intermediate filaments (IFs) and αB-crystallin, both present in the inclusions [30], has been suggested in the formation of inclusions. Increased αB-crystallin immunoreactivity is seen in several neurodegenerative disorders. αB-crystallin is a small heat shock protein, thought to be involved in normal dynamics of cytoskeletal proteins, as well as in prevention of protein aggregation during cellular stress [40]. Also IFs are seen in inclusions in FXTAS and aggregates of other neurodegenerative diseases. Thus, aggregate formation may reflect a failed attempt

of αB-crystallin to repair the stress-induced disrupted IF cytoskeletal architecture. In FXTAS, in particular lamin A/C appears be involved [25,30]. In expanded $(CGG)_n$ mice this heat shock protein reactivity and disrupted lamin A/C architecture have not been detected.

It is unclear whether the components are trapped into the inclusions via a specific process, or whether this may be the result of a somewhat random process. It could be that not all inclusions have the same composition. Most of the proteins in the inclusions have been identified by tandem mass spectrometry analysis [30]. Although a sensitive method, it does not give information about the composition of individual inclusions. More immunocytochemical experiments with antibodies against the identified proteins might answer the question whether all inclusions contain the same constituents. This in turn could give an indication whether inclusion formation is a determined or a random process, after an initial nucleation event.

We have investigated the presence of proteins in inclusions in murine brain [32] and compared with those that have been identified in inclusions in human FXTAS brain. We did not find Pur-α, hnRNP A2/B1, αB-crystallin or lamin A/C in inclusions in the expanded $(CGG)_n$ mice. Neither was a prominent difference in distribution or immunoreactivity in expanded $(CGG)_n$ mice observed in comparison with wt mice. The differences in protein composition of the inclusions between human and mice could derive from fundamental differences in the disease progress between the two species, or they could reflect the possible random trapping of proteins in the inclusions.

Intranuclear inclusion formation is not limited to disease development in FXTAS. For instance, all polyglutamine diseases caused by an expanded $(CAG)_n$ in a coding sequence, are associated with neuronal intranuclear aggregates [41-43]. Mouse models suggest that the inclusions are clearly related to disease pathology [44]. However, it is unclear whether the formation of inclusions is a prerequisite for toxicity [45]. Interestingly, evidence from a neuronal model for Huntington's disease suggests that inclusions may represent a protective mechanisms against cytotoxicity, by sequestering away soluble, more toxic forms of a protein [46-48]. It should, however, be noted that polyglutamine-induced diseases, including Huntington, result from the presence of a mutant protein, the effects of which might differ greatly from mutant RNA.

Knowing how inclusions are formed and what they are composed of will hopefully shed light on the origin of cellular toxicity, as well as how this leads to neurodegeneration. Future studies should and will furthermore continue to focus on $(CGG)_n$ RNA-binding proteins, to find downstream effects, which may explain the pathogenic mechanisms underlying the symptoms seen in FXTAS. Although two different expanded $(CGG)_n$ knock-in mouse lines are available that exhibit at least most of the symptoms observed in humans with FXTAS, they do not model human FXTAS in all aspects, including absence of inclusions in mouse astrocytes and absence of a significant degenerative neuropathology. Therefore new transgenic mouse models need to be developed with conditional and inducible $(CGG)_n$ expression in neurons and/or astrocytes, which will allow testing for sufficiency and necessity (which cell types) and timing of reversibility of neuropathology when stopping expanded $(CGG)_n$ expression (critical period). These new transgenic mice probably will result in earlier onset and increased severity of the FXTAS phenotype in mice and model FXTAS more accurately.

Inclusions in organs other than the brain | 7.1.3

When considering the presence of inclusions in organs other than the brain, their role in relation to disease possibly becomes even more puzzling. Chapter 5 describes altered hypothalamo-pituitary -adrenal (HPA) axis regulation in aged expanded $(CGG)_n$ mice, with elevated corticosterone levels as the most prominent finding. Furthermore, inclusions were detected in the pituitary and adrenal gland. Although these organs are directly linked to the HPA axis, the precise cell types in which inclusions are found are puzzling in relation to HPA axis physiology. In mice, inclusions in the pituitary gland were mostly seen in the pars intermedia. The pars anterior, however, is the region where the corticotrophs secrete adrenocorticotrophin hormone (ACTH). The pars intermedia has undergone involution in adult humans, so it is unclear what this observation in mice means for the human situation. Similarly, in the adrenal gland, inclusions are also seen in tissue layers that are not responsible for the production of corticosterone.

In human FXTAS tissue, inclusions were found in the anterior and posterior lobes of the pituitary gland [49,50]. Although some publications suggests that PM carriers suffer from more psychopathological symptoms than do controls [39,51-55], of which the origin might lie in elevated stress hormone levels, it is unclear how inclusions might affect cell function and thereby HPA axis physiology.

An attempt has been made to study the HPA axis negative feedback loop, which should keep corticosterone levels within the physiological range, or cause it return to normal levels after a stress-induced elevation. We wanted to know whether corticosterone levels in aged $(CGG)_n$ mice had a slower return to pre-stressor levels, or whether they might react inappropriately to a mild stressor, as this might hint at which level in the HPA axis the altered physiology originates. We analysed corticosterone levels in serum obtained at different time points after moving the animals from the animal facility to the lab, which can be considered a mild stressor. Measuring corticosterone levels at 0, 15, 30 or 90 minutes after the stressor in both $(CGG)_n$ and wt mice (on average 6 per genotype per time point) did not allow us to draw a conclusion on the HPA feedback system. Individual variation was too large, despite standardising the experimental set up as much as possible. Adrenal weight was also measured, as prolonged exposure to ACTH stimulation causes adrenal hypertrophy. No significant difference was seen between $(CGG)_n$ and wt mice, likely due to large variation.

The pituitary gland is of particular interest, as this is also a central organ in the hypothalamo-pituitary-gonadal (HPG) axis. Female PM carriers have elevated follicle stimulating hormone (FSH) levels [49,56,57], which is indicative of decreasing ovarian reserve. This can be linked to the risk of developing premature ovarian insufficiency in female PM carriers. Testosterone measurements have been conducted in three FXTAS patients, revealing lowered levels or complete deficiency. Testes of two other men have been studied, and showed non-specific atrophic changes and inclusions in Leydig cells, the testosterone producing cells and in myoid cells of the tubular walls. These testicular inclusions had an appearance identical to those reported in brain [49]. Immunohistochemical studies in murine testis and ovary did not reveal this. It could be that the pituitary gland is dysfunctional at a more general neuroendocrine level, which has an effect on both the HPA and the HPG axes. The basis for this phenomenon remains to be established.

It is furthermore interesting that inclusions were detectable in mouse amygdala and pituitary gland at 25 weeks of age, before they appeared in brain. The amygdala projects to and helps to control secretion of HPA-related hormones in the hypothalamus (chapter 4, [58]). FSH levels are increased in PM women before ages at which inclusions have been seen in human FXTAS brain [19]. Also, young adult male PM carriers were shown to have amygdala dysfunction in a functional MRI study [59]. Thus, altered pituitary gland physiology, or possibly broader changes involving brain regions that project to the HPA axis, might be responsible for symptoms seen in male and female carriers, before the possible onset of FXTAS.

Non-mammalian models for FXTAS | 7.2

In the process of gaining more insight into the pathogenic mechanisms underlying FXTAS, studying human material is of limited use. Molecular measures can be quantified in blood, but the most interesting organ in this disease, the brain, is only available after the patient has passed away, thus, at the final stage of the disease. Attempts are made to find alternatives, such as skin fibroblast cell lines, which mimic cellular changes seen in *post mortem* brain material, which could serve as a marker for the disease progress (described in chapter 4).

Cellular models | 7.2.1

Cell lines could also be valuable in unravelling aspects of the mechanisms underlying cellular toxicity, as they are easier to manipulate than complex organisms such as mouse models. Although fibroblasts have the advantage that they can directly be taken from patients or mice, a neural cell type might be more informative, as it has more resemblance to cells in the brain.

We have made attempts to develop a cellular model by stably expressing an expanded $(CGG)_n$ (30CGG or 100CGG) fused to a *GFP* reporter gene in rat pheochromocytoma (PC12) cells, which can adapt a neuron-like phenotype upon differentiation with neuronal growth factor. This cell line was established to study factors that might play a role in the formation of inclusions. Unfortunately, although inclusions did form in a minority of cells, cells did not remain healthy and attached to the cell culture dish long enough, for it to be a workable model. Hence, we are limited to the mouse model. Naturally, working with a mouse model cannot be seen as a limitation, as mice are very important in approaching the complexity of human disease. In addition, the fact that FXTAS only manifests itself in aged individuals underscores the advantage of using mice as a model organism. We did, however, generate adult skin and embryonic fibroblast lines from mice that carry $(CGG)_{>200}$, as described in chapter 4.

Drosophila melanogaster model | 7.2.2

Jin and colleagues developed a *Drosophila* model in which they can induce ectopic expression of different $(CGG)_n$ lengths at various expression levels [26]. Moderate expression of a tract of 60 CGGs hardly causes any phenotype in the eye, whereas 90 CGGs lead to rough eye, loss of pigmentation and disturbed architecture. Strong expression of either repeat length caused a more severe phenotype, with an even stronger effect for the $(CGG)_{90}$, including major cell death. The neurodegenerative eye phenotype worsened with age. It was a late onset phenomenon rather than a developmental defect. Inclusions have been found in retina, both in nuclei and cytoplasm, positive for ubiquitin, hsp70 and proteasome.

By using this model to investigate the effects of overexpression of several proteins, including heat shock proteins and $(CGG)_n$-BPs, a proof-of-principle was provided that the RNA-induced phenotype can be rescued [26]. For instance, overexpression of Hsp70 [26], CUGBP1, hnRNP A2/B1 [29], Pur α [27] was found to be capable of suppressing the CGG RNA-induced degeneration.

The expanded $(CGG)_n$ mouse model for FXTAS | 7.3

Although the expanded $(CGG)_n$ knock-in mouse was originally generated to study mechanisms of $(CGG)_n$ instability [60], it turned out to be a suitable model to study FXTAS as well [32]. Not only elevated *Fmr1* mRNA levels, decreased Fmrp levels and instability of the $(CGG)_n$ upon transmission to the next generation mimic the situation in humans. Also the presence of intranuclear inclusions, the neuropathological hallmark of FXTAS, greatly adds to the value of this mouse model. Inclusions increase in size and number with age, paralleling the progressive nature of FXTAS (chapter 3 and 6, [32,61]). In addition, behavioural studies revealed mild learning disabilities and motor problems in aged $(CGG)_n$ mice [62]. Thus, the expanded $(CGG)_n$ knock-in mouse model is a valuable tool to study the molecular mechanisms underlying FXTAS, as well as the onset and progression of the disease.

However, some differences exist between the human and murine situation. As described in chapter 3, the $(CGG)_n$ in our mice has expanded to over 200 trinucleotides, which is in the full mutation range in humans. In humans this generally leads to methylation-mediated silencing of *FMR1*, but in our knock-in mice this has not occurred yet. Another *Fmr1* expanded $(CGG)_n$ knock-in mouse model also yielded mice carrying alleles with over 200 CGGs that remained unmethylated [63]. It has been proposed that the threshold for large increases in trinucleotide repeat length is higher in transgenic mice [64]. Thus, as gene silencing in humans is preceded by a large 'jump' in repeat length, and since this kind of large expansions have not been seen frequently in our mice, the threshold for expansion-induced gene silencing might also be higher.

It is interesting to note that the phenotype in the expanded $(CGG)_n$ mice is much less severe than it is in humans. A recent study that compared documented phenotypes of null mutations in humans and mice, showed that over 20% of mutations that in humans cause death before puberty ('gene essentiality'), showed 'nonessentiality' for the mouse orthologue, meaning that mice survived up to reproductive age and were fertile [65].

Aged $(CGG)_n$ mice only showed mild learning and motor impairment in comparison with age-matched wt mice [62]. Neuropathologically, human FXTAS brain shows more signs of neurodegeneration, as well as significant astrocytic involvement. Unlike in humans [19,66], mice do not have astrocytic inclusions. This could be due to the much lower expression or total absence of *Fmr1* mRNA in mouse astrocytes [32]. These differences might mean that astrocyte disease is important in the development of human disease, as has been suggested before [66].

Studies in a transgenic mouse model for SCA7 showed dramatic Purkinje cell degeneration, even though the expanded $(CAG)_n$-*Ataxin7* construct was not expressed in Purkinje cells. This was interpreted as that polyglutamine-induced dysfunction in neighbouring cells contributes to neurodegeneration in Purkinje cells [67]. As the cell bodies of Bergmann glia radial fibers surround Purkinje neurons and

the dendritic processes, these cells were investigated for having a role in Purkinje degeneration. A transgenic mouse model that expresses an expanded $(CAG)_n$-SCA7 in Bergmann glia developed motor coordination problems, as well as Purkinje cell pathology. Bergmann glia are responsible for the removal of glutamate from Purkinje cell synaptic regions. GLAST is a glutamate transporter that is only expressed in Bergmann glia. GLAST expression, as well as glutamate uptake, was markedly reduced in presymptomatic expanded $(CAG)_n$-SCA7 mice. These findings showed that expression of expanded $(CAG)_n$-*Ataxin7* in Bergmann glia is sufficient to cause Purkinje cell degeneration, through interfering with Bergmann glia-mediated glutamate uptake. Prolonged glutamatergic stimulation leads to glutamate excitotoxicity [68]. Evidence is slowly accumulating that altered glutamate uptake may also contribute to other cerebellar disorders, such as SCA1, SCA5 and Huntington. Different glutamate transporters might disturb glutamate transport similarly, thereby causing different cerebellar and polyglutamine degenerative disorders [69-71]. Similar glutamate excitotoxicity might underlie Purkinje cell degeneration in FXTAS. It would be interesting to see whether driving expanded $(CGG)_n$ expression in Bergmann glia aggravates the neurodegenerative phenotype in mice. Thus, also studying differences of disease development between mice and humans can provide important insight into disease mechanisms.

Individual variation in the manifestation of FXTAS | 7.4

The core features of FXTAS are action tremor and cerebellar gait ataxia, but it can be accompanied by a wide range of other symptoms. Inter-individual variability of clinical involvement, as well as severity of the syndrome is large [72,73]. All PM carriers have elevated *FMR1* mRNA levels in blood, irrespective of whether they have FXTAS [13,22]. It will be important to unravel factors that determine whether somebody will develop FXTAS or not, in addition to factors that influence the onset and severity of the clinical picture. It cannot be excluded that every PM carrier would develop FXTAS, if only he (or she) would become old enough. An MRI study showed subtle volumetric differences between PM carriers without FXTAS and controls, which might be early indicators of developing FXTAS at a later stage, which is associated with more prominent brain atrophy [74]. However, PM carriers older than 80 years have been known that were devoid of any signs of FXTAS [75].

The effect of genotype differences for the serotonin transporter polymorphism has been described to affect aggressive and stereotypic behaviour in boys with fragile X [76]. Thus, although FXTAS, like fragile X syndrome, is commonly considered to be a single gene disorder, it might similarly be affected by the influence of modifying genes. Also the effect of aging or environmental factors on the severity of the phenotype cannot be excluded.

It is also still unknown whether inclusions form a direct link to the disease; the presence of inclusions is associated with FXTAS [19,66], but it remains to be elucidated whether inclusions are a prerequisite for the symptoms to manifest themselves, or alternatively, whether FXTAS can develop without inclusion formation. Thus, a clear causal relationship as found for the role of nuclear foci in myotonic dystrophy [31,77] remains to be established. Furthermore, it is interesting to know whether PM carriers that did not show signs of FXTAS also have inclusions. Over time, more brains of PM carriers with or without FXTAS will become available for research. This might reveal a basis for individual variation in the severity of the clinical outcome in patients with FXTAS. Presence of inclusions in certain brain

regions could play a role, but also size and quantity, or specific composition of the inclusions might affect individual clinical outcome. Also, studying more brains might shed light on to what extent inclusions can explain other symptoms associated with the PM. Several studies have reported clinical involvement in PM carriers, other than FXTAS. For instance, emotional problems, such as anxiety and depression, as well as mild cognitive impairment and autism spectrum disorder have been described ([4,39,54,78,79]). It is still unknown whether these symptoms reflect prodromal symptoms, or a milder form of RNA pathogenesis or a consequence of mildly reduced FMRP levels.

Most alleles in the general population are interspersed with an AGG, about every $(CGG)_9$ [80-82]. The expansion process of the $(CGG)_n$ is believed to begin with loss of the most 3' AGG interruption [80,83]. Most PM alleles only have one AGG at the 5' end of the $(CGG)_n$, or none at all [80,82,84]. Interruptions determine the likelihood of the repeat to fold into hairpins, as well as the stability of these hairpins. This can lead to instability of the repeat, as hairpins may cause slippage of the replication fork [80,85]. It may also affect binding of $(CGG)_n$-BPs, thereby possibly affecting the extent to which cellular dysfunction develops. Napierala and colleagues postulated that interruption-dependent structure variants of the *FMR1* mRNA affect the phenotype in PM carriers. Indeed they found that AGG interruptions shorten the effective length of a pure $(CGG)_n$ hairpin, and hypothesised that this may protect the PM carrier from developing FXTAS. Their proposal that AGG interruption status can influence the correlation between repeat length and clinical outcome may, however, particularly be important for short PM alleles [86]. Thus, it will be interesting to check AGG interruption patterns in a large cohort of PM carriers, with or without FXTAS, to reveal whether a correlation exist with clinical outcome.

Interestingly, the expanded $(CGG)_n$ in our knock-in mouse model does not have any AGG interruptions. If the above is true, one would expect less individual variation in the clinical or neuropathological outcome. Indeed, all mice develop inclusions. However, it cannot be ruled out that also PM carriers who do not develop FXTAS have inclusions.

$(CGG)_n$ instability | 7.5

All known trinucleotide repeat disorders result from $(CNG)_n$ expansions, where N can be any nucleotide. Although the different repeats share some aspects of repeat instability (described in chapter 2), there might also be some fundamental differences. The specific sequence determines the propensity to form secondary structures such as hairpins and the stability thereof [87-89]. The hairpin-like structures formed by single stranded $(CNG)_n$ consist of both Watson-Crick and mismatched base pairs. The stability of these imperfect hairpins is dependent on the sequence, due to the free energy contribution of the mismatched base pairs. Hairpins made of $(CGG)_n$ are most stable, $(CTG)_n$ is a bit less stable, followed by $(CAG)_n$ and $(CCG)_n$ with similar stability [87].

Although 64 (4^3) triplet compositions are possible, large expansions have only been observed in two trinucleotide groups, namely (CAG)·(CTG) and (CGG)·(CCG), which are the repeats responsible for the majority of trinucleotide repeat disorders. This sequence selectivity cannot solely be explained by GC-content or the frequency of occurrence in the genome. (CAG)·(CTG) and (CGG)·(CCG) both contain purine and pyrimidine bases. (CCA)·(TGG), another GC-rich group that is overrepresented in the genome, is similar in that respect, but to date no disease has been found to be associated with

this repeat [90]. Both a correct sequence and a correct repeat length need to be present in order to form hairpins of the threshold energy needed for the large expansions associated with human disease. These criteria are only met for (CAG)·(CTG) and (CGG)·(CCG) [87].

Analysis of $(CGG)_n$ with or without AGG interruptions showed that the presence of an interruption greatly affects the free energy of a hairpin. Alleles with AGG interruptions form hairpins with energies below the threshold for expansion. Loss of an interruption by a single point mutation causes formation of a hairpin with an energy much greater than the threshold for expansion, allowing expansion of the repeat. This is another clear indication that the exact sequence determines the energy of a hairpin and thereby its stability [87]. However, a more recent melting study showed that an AGG interruption did not have a significantly different thermodynamic stability at near physiological MG^{2+} concentrations. However, this study found evidence for the presence of a G·G non-Watson-Crick base pair in a $(CGG)_n$ duplex RNA, that increases the free energy of the hairpin [91].

Another aspect that might cause the $(CGG)_n$ to behave differently from other trinucleotide repeats, is its X-chromosomal location. Although some instability was seen upon transgenically expressing a YAC carrying the *FMR1* with a PM-sized $(CGG)_n$ with a substantial amount of flanking region [92], only large $(CGG)_n$ instability was seen after expanded $(CGG)_n$ knock-in mouse models were generated (chapter 3, [61,63]). This suggests that factors causing repeat instability exist, related to the X-chromosome, that could not be mimicked when the YAC carrying the $(CGG)_n$ with flanking regions was randomly integrated and thus autosomally expressed. It is unknown whether meiosis plays a role herein.

Furthermore, the $(CGG)_n$ is different from some other $(CNG)_n$ in that it does not show substantial somatic instability. It is as yet unknown whether this is the result of the same mechanism as which causes intergenerational instability. In addition, timing of instability could vary among different trinucleotide repeats. For instance, although most evidence points at a prezygotic origin of the *FMR1* $(CGG)_n$ instability (outlined in chapter 2), in DM1, intergenerational instability appears to result not only from $(CTG)_n$ instability in the germ line of the transmitting father, but another instability event occurring shortly after fertilisation also appears to take place [93].

These factors likely contribute to the observation that, despite similarities, different trinucleotide repeats do behave differently (discussed in more detail in chapter 2). One should keep this in mind when wanting to extrapolate observations on repeat behaviour of other trinucleotides to $(CGG)_n$ instability.

7.6 | $(CGG)_n$ transmission and gene silencing in the mouse model

Both humans and $(CGG)_n$ mice show a bias towards $(CGG)_n$ expansions, when transmitting PM alleles to the next generation. While in humans, risk of expansion appears to be greater when the expanded $(CGG)_n$ allele is of maternal origin and large increases into the FM range are exclusively seen when the repeat allele is maternally transmitted [94], no difference in direction or magnitude of the instability between maternal or paternal transmission was seen in mice (chapter 3, [61]). Gender of offspring has no effect on the risk of expansion to an FM in humans [95], neither on direction or magnitude of instability in $(CGG)_n$ mice (chapter 3, [61]). It should be noted, that in mice, only occasional large expansions have been

observed. The resulting $(CGG)_n$ lengths would be considered FMs in humans, however, thus far they remain unmethylated in mice (chapter 3, [61,63]). Thus, as yet, the expanded $(CGG)_n$ knock-in mice are not an appropriate model to study gene silencing.

Although the current hypothesis is that expansions to an FM occur prezygotically, followed by selection for PM alleles in sperm, the fact that expansion to an FM only occurs upon maternal transmission might also suggest that men lack specific factors that allow this expansion event, or it may mean that men possess other factors that prevent this expansion from taking place. Both scenarios could also be true for our mouse model.

Only time will tell whether methylation-mediated silencing of the *Fmr1* gene will happen in mice. It is possible that mice are not equipped with the same methylases and histone deacetylases that are responsible for the silencing of *FMR1* in humans. It is clear that, if inactivation of *Fmr1* will happen in mice, it will do so at a higher threshold $(CGG)_n$ length than in humans. It has indeed been proposed before that the threshold for large expansions is higher in mice than in men [64]. Such large jumps in repeat length with major phenotypic consequences have been observed in a transgenic mouse model for DM1 [96], but this is no guarantee that the same could happen with $(CGG)_n$ in *Fmr1*. In case our mice will undergo a transition to the FM situation, such that *Fmr1* gets inactivated, the order of events in this transition will be the focus of our attention. Studying the gonads of the transmitting parents as well as early embryos will shed light on the timing of expansion and the accompanying gene silencing events. It will be interesting to elucidate whether the same methyl binding proteins recognise the methylated CpGs and subsequently recruit proteins that deacetylate and affect methylation status of certain histone tails, as in humans (outlined in more detail in chapter 1). Ultimately, this might provide a model in which ways to modify *Fmr1* gene activity can be tested. Also without gene silencing taking place, studies in our mice might also provide information on the moment of expansion, by looking at germ cells and the early embryo. This, however, is currently highly challenging, given the small repeat length changes that we see between different generations.

Up till now, we have only checked for methylation of the *Fmr1* promoter region as a screening method to reveal gene silencing. A recent study in human embryonic stem cells carrying an FM suggests that chromatin changes precede methylation of the promoter region and that methylation might contribute to maintenance rather than to the induction of the transcriptionally inactive state. Reduction of H3 tail acetylation and H3K9 becoming methylated, both associated with reduced gene activity, were observed in combination with a hypomethylated *FMR1* 5'UTR [97]. Until our mice have proven to be an appropriate model to study epigenetic changes in the *Fmr1* silencing process, these embryonic stem cells might provide a good alternative. However, the teratomas generated by inducing differentiation of the stem cells have a limited life span, so they cannot provide as much information as a living, complex organism would.

To our knowledge, these histone modifications or chromatin changes have not been studied in carriers with high end PM alleles, thus it is not known whether some of these changes might already occur before *FMR1* gets fully inactivated. It could be proposed that the lack of elevated *Fmr1* mRNA levels in $(CGG)_n$ mice with >150 CGGs represent a transition phase in which histone tail modifications

lead to partial chromatin condensation, without inducing methylation of the *Fmr1* promoter region. It might therefore be informative to investigate histone tail modifications in mice with $(CGG)_{151-200}$ and $(CGG)_{>200}$.

Two brothers, grandfathers of boys with fragile X, who carry an unmethylated FM allele and do not show the fragile X phenotype [98], might also represent such a transition state, as they escaped methylation, despite having a $(CGG)_n$ in the FM range. Although these brothers are mosaic for a PM in blood leukocytes, analysis of a cell line derived from one of these high functioning males, revealed a $(CGG)_{\sim400}$, which was unmethylated. This cell line has deacetylated histones and methylated lysine 9 of histone 3, as is normally found in fragile X cell lines, while also showing high levels of methylated lysine 4 of histone 3, which is correlated with active transcription in normal controls. Indeed *FMR1* mRNA is detected at normal levels in this cell line. FMRP is also detected, although at about 20-30% compared with a normal cell line. Thus, the FM length $(CGG)_n$ reduces translational efficiency [99]. Molecular epigenetic characterisation of cell lines of two newly identified individuals without an apparent phenotype but with unmethylated full mutations, revealed similar characteristics as the cell line with an unmethylated $(CGG)_{\sim400}$ described above. It was concluded that DNA methylation is the critical step in the silencing of *FMR1*. The suggestion that a common mechanism exists for these rare unmethylated FM alleles, might be important in view of therapeutic strategies. It would be worthwhile to investigate options to convert methylated into unmethylated alleles, to restore *FMR1* expression [100].

A combination of research in these cells with unmethylated FM alleles, the FM human embryonic stem cell line and hopefully in due time the mouse model that shows $(CGG)_n$ expansion-induced *Fmr1* silencing will likely complete the puzzle of epigenetic events involved in inactivation of *FMR1*.

Concluding remarks and future directions | 7.7

The work in this thesis has attempted to unravel part of the mechanisms underlying FXTAS, by using the expanded $(CGG)_n$ knock-in mouse model. Transmission of the $(CGG)_n$ in mice has been studied and molecular changes accompanying $(CGG)_n$ expansion have been characterised. Studies in $(CGG)_n$ mice suggest that inclusions are indeed related to the expanded $(CGG)_n$ RNA and links to the clinical outcome in humans can be drawn. Preliminary findings of cellular stress in human FXTAS brain and cell lines might shed light on the nature of the cellular dysfunction induced by the $(CGG)_n$ RNA. The main question remains however; namely how the $(CGG)_n$ RNA leads to formation of inclusions and how cellular dysfunction originates, ultimately leading to neurodegeneration and clinical problems. Future studies should focus on the identity and role of $(CGG)_n$-BP, and the cellular consequences of their binding. If these proteins are indeed sequestered away from their normal function, as the toxic RNA gain-of-function model predicts, their function might provide important links between the mutant RNA and clinical symptoms.

The mouse model will remain valuable since the onset as well as the course of disease can be studied. Not only symptoms associated with FXTAS, but also prodromal symptoms should be the focus of attention, although the latter might be challenging to study in mice, due to their subtle nature. Since

the fibroblast cell lines derived from patients and expanded $(CGG)_n$ mice mimic signs of cellular stress seen in FXTAS brain, further characterisation of these cells might also proof valuable in recognising early signs of cellular dysfunction.

The $(CGG)_n$ mice will furthermore be valuable in attempts at reversing the neurodegenerative phenotype. Conditional transgenic mice can provide important information, when *Fmr1* expanded $(CGG)_n$ transgene expression can be directed to certain tissues or turned off or on in certain time windows. Therapeutic strategies will likely focus on diminishing $(CGG)_n$ mRNA levels. The studies conducted in *Drosophila* are promising in this light, as reversing the negative effect of the expanded $(CGG)_n$ RNA by co-expressing an anti-sense repeat or by overexpressing the proteins sequestered to the repeat RNA, rescued the neurodegenerative phenotype. Hence, strategies involving the siRNA pathway might prove beneficial in the future. In addition, when more $(CGG)_n$-BPs will be identified, this might provide new therapeutic targets.

This thesis has not focused on the effect of the expanded $(CGG)_n$ in female mice, in relation to the risk of developing premature ovarian insufficiency. Preliminary data suggest that female $(CGG)_n$ mice indeed have lower AMH levels, representing a smaller follicle pool. Additional studies will likely confirm that expanded $(CGG)_n$ mice are also a valuable model for premature ovarian insufficiency seen in female PM carriers. This research might also shed light on the link between the presence of inclusions in brain or other organs and clinical consequences.

References |

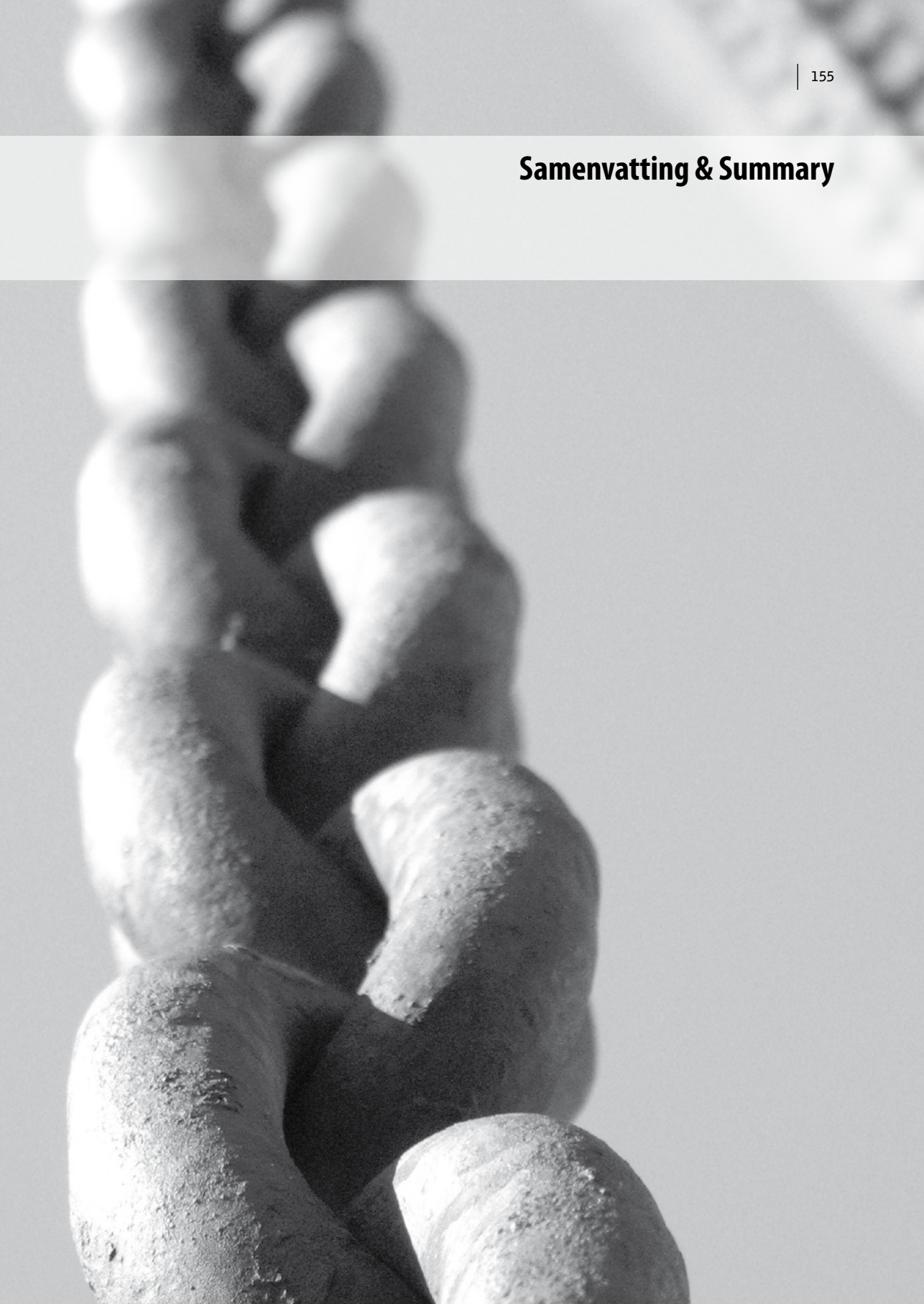
1. Oberlé I, Rousseau F, Heitz D, Kretz C, Devys D *et al.* 1991. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. Science 252:1097-1102.
2. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP *et al.* 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905-914.
3. Richards RI, Holman K, Kozman H, Kremer E, Lynch M *et al.* 1991. Fragile X syndrome: genetic localisation by linkage mapping of two microsatellite repeats FRAXAC1 and FRAXAC2 which immediately flank the fragile site. J Med Genet 28:818-823.
4. Hagerman PJ, Hagerman RJ. 2004. The Fragile-X Premutation: A Maturing Perspective. Am J Hum Genet 74:805-816.
5. Mazzocco MM, Pennington BF, Hagerman RJ. 1993. The neurocognitive phenotype of female carriers of fragile X: additional evidence for specificity. J Dev Behav Pediatr 14:328-335.
6. Rousseau F, Heitz D, Tarleton J, Macpherson J, Malmgren H *et al.* 1994. 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 55:225-237.
7. Franke P, Leboyer M, Gansicke M, Weiffenbach O, Biancalana V *et al.* 1998. Genotype-phenotype relationship in female carriers of the premutation and full mutation of FMR-1. Psychiatry Res 80:113-127.
8. Franke P, Maier W, Hautzinger M, Weiffenbach O, Gansicke M *et al.* 1996. Fragile-X carrier females: evidence for a distinct psychopathological phenotype? Am J Med Genet 64:334-339.
9. Murray A, Webb J, Grimley S, Conway G, Jacobs P. 1998. Studies of FRAXA and FRAXE in women with premature ovarian failure. J Med Genet 35:637-640.
10. Turner G, Robinson H, Wake S, Martin N. 1994. Dizygous twinning and premature menopause in fragile X syndrome. Lancet 344:1500.
11. Aziz M, Stathopulu E, Callias M, Taylor C, Turk J *et al.* 2003. Clinical features of boys with fragile X premutations and intermediate alleles. Am J Med Genet 121:119-127.
12. Hagerman RJ, Staley LW, Oconner R, Lugenbeel K, Nelson D *et al.* 1996. Learning-disabled males with a fragile X CGG expansion in the upper premutation size range. Pediatrics 97:122-126.
13. Tassone F, Hagerman RJ, Taylor AK, Gane LW, Godfrey TE *et al.* 2000. Elevated levels of FMR1 mRNA in carrier males: A new mechanism of involvement in the Fragile-X syndrome. Am J Hum Genet 66:6-15.
14. Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R *et al.* 2001. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 57:127-130.
15. Berry-Kravis E, Lewin F, Wuu J, Leehey M, Hagerman R *et al.* 2003. Tremor and ataxia in fragile X premutation carriers: Blinded videotape study. Ann Neurol 53:616-623.
16. Hagerman PJ, Greco CM, Hagerman RJ. 2003. A cerebellar tremor/ataxia syndrome among fragile X premutation carriers. Cytogenet Genome Res 100:206-212.
17. Leehey MA, Munhoz RP, Lang AE, Brunberg JA, Grigsby J *et al.* 2003. The fragile X premutation presenting as essential tremor. Arch Neurol 60:117-121.
18. Jacquemont S, Hagerman RJ, Leehey M, Grigsby J, Zhang L *et al.* 2003. Fragile X Premutation Tremor/Ataxia Syndrome: Molecular, Clinical, and Neuroimaging Correlates. Am J Hum Genet 72:869-878.
19. Greco CM, Hagerman RJ, Tassone F, Chudley AE, Del Bigio MR *et al.* 2002. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. Brain 125:1760-1771.
20. Oostra BA, Willemsen R. 2003. A fragile balance: FMR1 expression levels. Hum Mol Genet 12 Suppl 2:R249-257.
21. Mankodi A, Logigian E, Callahan L, McClain C, White R *et al.* 2000. Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat. Science 289:1769-1773.
22. Kenneson A, Zhang F, Hagedorn CH, Warren ST. 2001. Reduced FMRP and increased FMR1 transcription is proportionally associated with CGG repeat number in intermediate-length and premutation carriers. Hum Mol Genet 10:1449-1454.
23. Tassone F, Iwahashi C, Hagerman PJ. 2004. FMR1 RNA withinthe intranuclear inclusions of fragile X-associated Tremor/Ataxia syndrome (FXTAS). RNA biology 1:103-105.
24. Handa V, Goldwater D, Stiles D, Cam M, Poy G *et al.* 2005. Long CGG-repeat tracts are toxic to human cells: Implications for carriers of Fragile X premutation alleles. FEBS Lett 579:2702-2708.
25. Arocena DG, Iwahashi CK, Won N, Beilina A, Ludwig AL *et al.* 2005. Induction of inclusion formation and disruption of lamin A/C structure by premutation CGG-repeat RNA in human cultured neural cells. Hum Mol Genet 14:3661-3671.
26. Jin P, Zarnescu DC, Zhang F, Pearson CE, Lucchesi JC *et al.* 2003. RNA-Mediated Neurodegeneration Caused by the Fragile X Premutation rCGG Repeats in Drosophila. Neuron 39:739-747.

27. Jin P, Duan R, Qurashi A, Qin Y, Tian D *et al.* 2007. Pur alpha Binds to rCGG Repeats and Modulates Repeat-Mediated Neurodegeneration in a Drosophila Model of Fragile X Tremor/Ataxia Syndrome. Neuron 55:556-564.
28. Sofola OA, Jin P, Botas J, Nelson DL. 2007. Argonaute-2 dependent rescue of a Drosophila model of FXTAS by FRAXE premutation repeat. Hum Mol Genet.
29. Sofola OA, Jin P, Qin Y, Duan R, Liu H *et al.* 2007. RNA-Binding Proteins hnRNP A2/B1 and CUGBP1 Suppress Fragile X CGG Premutation Repeat-Induced Neurodegeneration in a Drosophila Model of FXTAS. Neuron 55:565-571.
30. Iwahashi CK, Yasui DH, An HJ, Greco CM, Tassone F *et al.* 2006. Protein composition of the intranuclear inclusions of FXTAS. Brain 129:256-271.
31. Mankodi A, Takahashi MP, Jiang H, Beck CL, Bowers WJ *et al.* 2002. Expanded CUG repeats trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability of skeletal muscle in myotonic dystrophy. Mol Cell 10:35-44.
32. Willemsen R, Hoogeveen-Westerveld M, Reis S, Holstege J, Severijnen L *et al.* 2003. The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. Hum Mol Genet 12:949-959.
33. Peier AM, McIlwain KL, Kenneson A, Warren ST, Paylor R *et al.* 2000. (Over)correction of FMR1 deficiency with YAC transgenics: behavioral and physical features. Hum Mol Genet 9:1145-1159.
34. Musumeci SA, Calabrese G, Bonaccorso CM, D'Antoni S, Brouwer JR *et al.* 2007. Audiogenic seizure susceptibility is reduced in fragile X knockout mice after introduction of FMR1 transgenes. Exp Neurol 203:233-240.
35. Primerano B, Tassone F, Hagerman RJ, Hagerman P, Amaldi F *et al.* 2002. Reduced FMR1 mRNA translation efficiency in Fragile X patients with premutations. RNA 8:1-7.
36. Tassone F, Hagerman PJ. 2003. Expression of the FMR1 gene. Cytogenet Genome Res 100:124-128.
37. Tassone F, Beilina A, Carosi C, Albertosi S, Bagni C *et al.* 2007. Elevated FMR1 mRNA in premutation carriers is due to increased transcription. RNA.
38. Allen EG, He W, Yadav-Shah M, Sherman SL. 2004. A study of the distributional characteristics of FMR1 transcript levels in 238 individuals. Hum Genet 114:439-447.
39. Roberts JE, Bailey DB, Jr., Mankowski J, Ford A, Sideris J *et al.* 2008. Mood and anxiety disorders in females with the FMR1 premutation. Am J Med Genet B Neuropsychiatr Genet.
40. Head MW, Goldman JE. 2000. Small heat shock proteins, the cytoskeleton, and inclusion body formation. Neuropathol Appl Neurobiol 26:304-312.
41. Davies SW, Turmaine M, Cozens BA, DiFiglia M, Sharp AH *et al.* 1997. Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90:537-548.
42. DiFiglia M, Sapp E, Chase KO, Davies SW, Bates GP *et al.* 1997. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277:1990-1993.
43. Paulson HL, Perez MK, Trottier Y, Trojanowski JQ, Subramony SH *et al.* 1997. Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. Neuron 19:333-344.
44. Li H, Li SH, Cheng AL, Mangiarini L, Bates GP *et al.* 1999. Ultrastructural localization and progressive formation of neuropil aggregates in Huntington's disease transgenic mice. Hum Mol Genet 8:1227-1236.
45. Saudou F, Finkbeiner S, Devys D, Greenberg ME. 1998. Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell 95:55-66.
46. Kopito RR. 2000. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol 10:524-530.
47. Arrasate M, Mitra S, Schweitzer ES, Segal MR, Finkbeiner S. 2004. Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature 431:805-810.
48. Zoghbi HY, Botas J. 2002. Mouse and fly models of neurodegeneration. Trends Genet 18:463-471.
49. Greco CM, Soontrapornchai K, Wirojanan J, Gould JE, Hagerman PJ *et al.* 2007. Testicular and pituitary inclusion formation in fragile X associated tremor/ataxia syndrome. J Urol 177:1434-1437.
50. Louis E, Moskowitz C, Friez M, Amaya M, Vonsattel JP. 2006. Parkinsonism, dysautonomia, and intranuclear inclusions in a fragile X carrier: A clinical-pathological study. Mov Disord 27:193-201.
51. Bacalman S, Farzin F, Bourgeois JA, Cogswell J, Goodlin-Jones BL *et al.* 2006. Psychiatric Phenotype of the Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) in Males: Newly Described Fronto-Subcortical Dementia. J Clin Psychiatry 67:87-94.
52. Hessl D, Tassone F, Loesch DZ, Berry-Kravis E, Leehey MA *et al.* 2005. Abnormal elevation of FMR1 mRNA is associated with psychological symptoms in individuals with the fragile X premutation. Am J Med Genet B Neuropsychiatr Genet 139B:115-121.
53. Bourgeois JA, Cogswell JB, Hessl D, Zhang L, Ono MY *et al.* 2007. Cognitive, anxiety and mood disorders in the fragile X-associated tremor/ataxia syndrome. Gen Hosp Psychiatry 29:349-356.

54. Hunter JE, Allen EG, Abramowitz A, Rusin M, Leslie M *et al.* 2008. Investigation of Phenotypes Associated with Mood and Anxiety Among Male and Female Fragile X Premutation Carriers. Behav Genet.
55. Rodriguez-Revenga L, Madrigal I, Alegret M, Santos M, Mila M. 2008. Evidence of depressive symptoms in fragile-X syndrome premutated females. Psychiatric genetics 18:153-155.
56. Hundscheid RD, Braat DD, Kiemeney LA, Smits AP, Thomas CM. 2001. Increased serum FSH in female fragile X premutation carriers with either regular menstrual cycles or on oral contraceptives. Hum Reprod 16:457-462.
57. Sullivan AK, Marcus M, Epstein MP, Allen EG, Anido AE *et al.* 2004. Association of FMR1 repeat size with ovarian dysfunction. Hum Reprod.
58. Brouwer JR, Severijnen E, de Jong FH, Hessl D, Hagerman RJ *et al.* 2008. Altered hypothalamus-pituitary-adrenal gland axis regulation in the expanded CGG-repeat mouse model for fragile X-associated tremor/ataxia syndrome. Psychoneuroendocrinology.
59. Hessl D, Rivera S, Koldewyn K, Cordeiro L, Adams J *et al.* 2006. Amygdala dysfunction in men with the fragile X premutation. Brain.
60. Bontekoe CJ, Bakker CE, Nieuwenhuizen IM, van Der Linde H, Lans H *et al.* 2001. Instability of a (CGG)(98) repeat in the Fmr1 promoter. Hum Mol Genet 10:1693-1699.
61. Brouwer JR, Mientjes EJ, Bakker CE, Nieuwenhuizen IM, Severijnen LA *et al.* 2007. Elevated Fmr1 mRNA levels and reduced protein expression in a mouse model with an unmethylated Fragile X full mutation. Exp Cell Res 313:244-253.
62. Van Dam D, Errijgers V, Kooy RF, Willemsen R, Mientjes E *et al.* 2005. Cognitive decline, neuromotor and behavioural disturbances in a mouse model for Fragile-X-associated tremor/ataxia syndrome (FXTAS). Behavioural Brain Research 162:233-239.
63. Entezam A, Biacsi R, Orrison B, Saha T, Hoffman GE *et al.* 2007. Regional FMRP deficits and large repeat expansions into the full mutation range in a new Fragile X premutation mouse model. Gene.
64. Gourdon G, Radvanyi F, Lia AS, Duros C, Blanche M *et al.* 1997. Moderate intergenerational and somatic instability of a 55-CTG repeat in transgenic mice. Nature Genet 15:190-192.
65. Liao BY, Zhang J. 2008. Null mutations in human and mouse orthologs frequently result in different phenotypes. Proc Natl Acad Sci U S A 105:6987-6992.
66. Greco CM, Berman RF, Martin RM, Tassone F, Schwartz PH *et al.* 2006. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). Brain 129:243-255.
67. Garden GA, Libby RT, Fu YH, Kinoshita Y, Huang J *et al.* 2002. Polyglutamine-expanded ataxin-7 promotes non-cell-autonomous purkinje cell degeneration and displays proteolytic cleavage in ataxic transgenic mice. J Neurosci 22:4897-4905.
68. Custer SK, Garden GA, Gill N, Rueb U, Libby RT *et al.* 2006. Bergmann glia expression of polyglutamine-expanded ataxin-7 produces neurodegeneration by impairing glutamate transport. Nat Neurosci 9:1302-1311.
69. Ikeda Y, Dick KA, Weatherspoon MR, Gincel D, Armbrust KR *et al.* 2006. Spectrin mutations cause spinocerebellar ataxia type 5. Nat Genet 38:184-190.
70. Serra HG, Byam CE, Lande JD, Tousey SK, Zoghbi HY *et al.* 2004. Gene profiling links SCA1 pathophysiology to glutamate signaling in Purkinje cells of transgenic mice. Hum Mol Genet 13:2535-2543.
71. Shin JY, Fang ZH, Yu ZX, Wang CE, Li SH *et al.* 2005. Expression of mutant huntingtin in glial cells contributes to neuronal excitotoxicity. J Cell Biol 171:1001-1012.
72. Berry-Kravis E, Abrams L, Coffey SM, Hall DA, Greco C *et al.* 2007. Fragile X-associated tremor/ataxia syndrome: Clinical features, genetics, and testing guidelines. Mov Disord 14:2014-2030.
73. Peters N, Kamm C, Asmus F, Holinski-Feder E, Kraft E *et al.* 2006. Intrafamilial variability in fragile X-associated tremor/ataxia syndrome. Mov Disord 21:98-102.
74. Cohen S, Masyn K, Adams J, Hessl D, Rivera S *et al.* 2006. Molecular and imaging correlates of the fragile X-associated tremor/ataxia syndrome. Neurology 67:1426-1431.
75. Jacquemont S, Hagerman RJ, Leehey MA, Hall DA, Levine RA *et al.* 2004. Penetrance of the fragile x-associated tremor/ataxia syndrome in a premutation carrier population. JAMA 291:460-469.
76. Hessl D, Tassone F, Cordeiro L, Koldewyn K, McCormick C *et al.* 2007. Brief Report: Aggression and Stereotypic Behavior in Males with Fragile X Syndrome-Moderating Secondary Genes in a "Single Gene" Disorder. J Autism Dev Disord.
77. Ranum LP, Day JW. 2004. Pathogenic RNA repeats: an expanding role in genetic disease. Trends Genet 20:506-512.
78. Cornish KM, Li L, Kogan CS, Jacquemont S, Turk J *et al.* 2008. Age-dependent cognitive changes in carriers of the fragile X syndrome. Cortex 44:628-636.
79. Farzin F, Perry H, Hessl D, Loesch D, Cohen J *et al.* 2006. Autism spectrum disorders and attention-deficit/hyperactivity disorder in boys with the fragile X premutation. J Dev Behav Pediatr 27:S137-144.

80. Eichler EE, Holden J, Popovich BW, Reiss AL, Snow K *et al.* 1994. Length of uninterrupted CGG repeats determines instability in the FMR1 gene. Nature Genet 8:88-94.
81. Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS *et al.* 1991. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67:1047-1058.
82. Zhong N, Yang WH, Dobkin C, Brown WT. 1995. Fragile X gene instability: Anchoring AGGs and linked microsatellites. Am J Hum Genet 57:351-361.
83. Kunst CB, Warren ST. 1994. Cryptic and polar variation of the fragile X repeat could result in predisposing normal alleles. Cell 77:853-861.
84. Snow K, Tester DJ, Kruckeberg KE, Schaid DJ, Thibodeau SN. 1994. Sequence analysis of the fragile X trinucleotide repeat: Implications for the origin of the fragile X mutation. Hum Mol Genet 3:1543-1551.
85. Napierala M, Michalowski D, de Mezer M, Krzyzosiak WJ. 2005. Facile FMR1 mRNA structure regulation by interruptions in CGG repeats. Nucleic Acids Res 33:451-463.
86. Napierala M, Bacolla A, Wells RD. 2005. Increased negative superhelical density in vivo enhances the genetic instability of triplet repeat sequences. J Biol Chem 280:37366-37376.
87. Gacy AM, Goellner G, Juranic N, Macura S, McMurray CT. 1995. Trinucleotide repeats that expand in human disease form hairpin structures *in vitro*. Cell 81:533-540.
88. Miret JJ, Pessoa-Brandao L, Lahue RS. 1998. Orientation-dependent and sequence-specific expansions of CTG/CAG trinucleotide repeats in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 95:12438-12443.
89. Mitas M. 1997. Trinucleotide repeats associated with human disease. Nucleic Acids Res 25:2245-2254.
90. Han J, Hsu CC, Zhu Z, Longshore JW, Finley WH. 1994. Over-representation of the disease associated (CAG) and (CGG) repeats in the human genome. Nucleic Acids Res 22:1735-1740.
91. Zumwalt M, Ludwig A, Hagerman PJ, Dieckmann T. 2007. Secondary Structure and Dynamics of the r(CGG) Repeat in the mRNA of the Fragile X Mental Retardation 1 (FMR1) Gene. RNA Biol 4:93-100.
92. Peier A, Nelson D. 2002. Instability of a premutation-sized CGG repeat in FMR1 YAC transgenic mice. Genomics 80:423-432.
93. Savouret C, Brisson E, Essers J, Kanaar R, Pastink A *et al.* 2003. CTG repeat instability and size variation timing in DNA repair-deficient mice. Embo J 22:2264-2273.
94. Nolin SL, Lewis FA, Ye LL, Houck GE, Glicksman AE *et al.* 1996. Familial transmission of the FMR1 CGG repeat. Am J Hum Genet 59:1252-1261.
95. Nolin SL, Brown WT, Glicksman A, Houck Jr GE, Gargano AD *et al.* 2003. Expansion of the Fragile X CGG Repeat in Females with Premutation or Intermediate Alleles. Am J Hum Genet 72:454-464.
96. Gomes-Pereira M, Foiry L, Nicole A, Huguet A, Junien C *et al.* 2007. CTG Trinucleotide Repeat "Big Jumps": Large Expansions, Small Mice. PLoS Genet 3:e52.
97. Eiges R, Urbach A, Malcov M, Frumkin T, Schwartz T *et al.* 2007. Developmental Study of Fragile X Syndrome Using Human Embryonic Stem Cells Derived from Preimplantation Genetically Diagnosed Embryos. Cell Stem Cell 1:568-577.
98. Smeets H, Smits A, Verheij CE, Theelen J, Willemsen R *et al.* 1995. Normal phenotype in two brothers with a full FMR1 mutation. Hum Mol Genet 4:2103-2108.
99. Pietrobono R, Tabolacci E, Zalfa F, Zito I, Terracciano A *et al.* 2005. Molecular dissection of the events leading to inactivation of the FMR1 gene. Hum Mol Genet 14:267-277.
100. Tabolacci E, Moscato U, Zalfa F, Bagni C, Chiurazzi P *et al.* 2008. Epigenetic analysis reveals a euchromatic configuration in the FMR1 unmethylated full mutations. Eur J Hum Genet.

Samenvatting & Summary



Samenvatting |

Het *FMR1* gen, gelegen op het X chromosoom, heeft een aaneenschakeling van CGG trinucleotiden in een DNA regio die wel als RNA wordt afgeschreven, maar niet naar eiwit wordt vertaald. In 1991 werd ontdekt dat deze CGG herhaling ($(CGG)_n$) in lengte kan variëren, doordat hij instabiel is bij overerving. Een lengte van meer dan 200 CGGs bleek verantwoordelijk voor het fragiele X syndroom, dat zich met name uit in mentale retardatie. Deze $(CGG)_n$ is een van de eerste instabiele, verlengde trinucleotide-herhalingen die geïdentificeerd zijn als genetische oorzaak van een neurologische aandoening. In de normale populatie is de $(CGG)_n$ in het *FMR1* gen tussen de 5 en 55 CGGs lang. Wanneer de $(CGG)_n$ langer is dan 200 trinucleotiden (volledige mutatie: FM), wordt deze doorgaans gemethyleerd, evenals de promoter regio van het *FMR1* gen. Dit resulteert in een inactivatie van het gen, waardoor het eiwit FMRP niet wordt gemaakt. In uitlopers van neuronen reguleert FMRP het transport en de vertaling van bepaalde mRNA moleculen naar eiwitten. FMRP beïnvloedt in deze dendrieten de plasticiteit van de synaps: de ruimte tussen twee neuronen waar de signaaloverdracht plaatsvindt. Hierdoor speelt het een belangrijke rol bij leerprocessen en geheugen. De afwezigheid van FMRP in neuronen resulteert in de verstandelijke handicap bij fragiele X patiënten.

Lang werd gedacht dat mensen met een $(CGG)_n$ lengte van tussen de 55 en 200 (premutatie: PM) geen risico op klinische problemen lopen, behalve de mogelijkheid dat bij vrouwen de $(CGG)_n$ lengte bij overdracht naar de volgende generatie kan verlengen tot een FM, waardoor het fragiele X syndroom optreedt bij het nageslacht. In 2001 werden echter de eerste PM dragers beschreven die op latere leeftijd een neurologisch syndroom hadden ontwikkeld. Dit progressieve, neurodegeneratieve syndroom werd fragiele X-geassocieerd tremor/ataxie syndroom (FXTAS) genoemd, naar de twee belangrijkste symptomen waarin het syndroom zich uit. Inmiddels zijn veel meer patiënten met deze aandoening beschreven. Het blijkt dat het syndroom een zeer grote individuele variabiliteit kent wat betreft ernst, verloop en verscheidenheid aan symptomen. FXTAS patiënten kunnen naast eerder genoemde symptomen ook onder meer Parkinsonisme ontwikkelen, alsmede problemen bij het uitvoeren van dagelijkse handelingen en op termijn ook cognitieve achteruitgang.

Alle PM dragers hebben verhoogde *FMR1* mRNA niveaus. Omdat zij ongeveer normale hoeveelheden eiwit hebben én omdat in oudere FM dragers geen symptomen van FXTAS zijn gezien, is het niet aannemelijk dat een gebrek aan FMRP de oorzaak is van het ontwikkelen van FXTAS. De hypothese is daarom dat de aanwezigheid van teveel *FMR1* mRNA, dat een verlengde $(CGG)_n$ bevat, voor problemen zorgt in cellen in het brein, hetgeen uiteindelijk voor de klinische symptomen zorgt. Een indicatie hiervoor is onder andere de detectie van *FMR1* mRNA in de eiwitinsluitsels ('inclusies') die in celkernen gezien worden in diverse regios van de hersenen van overleden FXTAS patiënten.

Er is nog veel onduidelijk over de ontstaanswijze van de klinische symptomen bij FXTAS patiënten. Tevens is onbekend waarom sommige dragers van de PM wel FXTAS ontwikkelen en anderen niet. Onderzoek naar het onderliggende mechanisme van dit syndroom is in mensen beperkt tot *post mortem* hersenmateriaal, dus in het eindstadium van de ziekte. Om het ontstaan en het verloop van de ziekte te kunnen bestuderen, kan een muismodel een waardevol hulpmiddel zijn. In ons lab is een genetisch gemodificeerde muis ontwikkeld, die als model dient voor FXTAS. De $(CGG)_8$ die een normale muis in zijn *Fmr1* gen heeft, is vervangen door een humane $(CGG)_{98}$. Dit muismodel werd ontwikkeld

om de instabiliteit van de $(CGG)_n$ te kunnen bestuderen. Toen FXTAS voor het eerst beschreven werd, bleek de verlengde $(CGG)_n$ muis ook een geschikt model te zijn voor dit syndroom, aangezien het naast de $(CGG)_n$ instabiliteit, ook de verhoogde *Fmr1* mRNA niveaus en de vorming van inclusies nabootst.

Het doel van dit onderzoek was middels dit muismodel meer inzicht te verkrijgen over het ontstaansmechanisme van FXTAS.

Hoofdstuk 1 van dit proefschrift geeft een algemene inleiding op de eigenschappen van het *FMR1* gen, FMRP en de verschillende syndromen die daaraan gerelateerd zijn. De bevindingen en hypotheses over de onderliggende pathogenese worden beschreven, alsmede bestaande diermodellen om dit te onderzoeken.

Hoofdstuk 2 gaat uitgebreid in op verscheidene neurodegeneratieve ziekten, die allen veroorzaakt worden door een verlengde trinucleotideherhaling. Zowel de overeenkomsten als de verschillen tussen deze ziekten worden belicht. Verschillende mechanismen die ten grondslag liggen aan deze aandoeningen worden besproken, evenals het moment en de wijze waarop de verlenging van de trinucleotideherhalingen zou kunnen plaatsvinden. Diverse factoren die van invloed blijken te zijn op instabiliteit van trinucleotideherhalingen worden beschreven.

Hoofdstuk 3 laat zien dat, hoewel de $(CGG)_n$ in ons muismodel inmiddels is verlengd tot meer dan 200 trinucleotiden, er geen methylatie en dus inactivatie van het *Fmr1* gen optreedt, wat in mensen bij deze lengte gewoonlijk wel gebeurt. De muis produceert nog steeds Fmrp, hoewel deze eiwitniveaus wel aanzienlijk lager zijn dan bij muizen met een kortere $(CGG)_n$. Het patroon van overerving van de $(CGG)_n$ blijkt niet verschillend te zijn voor de overdracht door de vader of de moeder, hoewel bij mensen expansie naar een volledige mutatie alleen wordt gezien wanneer een moeder haar X-chromosoom doorgeeft.

Hoofdstuk 4 gaat in op de veranderingen die in de cel plaatsvinden, als gevolg van expressie van verlengde $(CGG)_n$ *FMR1* mRNA. Een verstoring van de lamine-architectuur en een stress-geïnduceerde activatie van 'heat shock' eiwitten worden gezien in zowel *post mortem* hersenmateriaal en huidfibroblasten-cellijnen van FXTAS patiënten, als ook in fibroblasten-cellijnen van verlengde $(CGG)_n$ muizen. Dit impliceert dat cellijnen in de toekomst mogelijk ook nuttige informatie kunnen geven over de verstoring van de celfunctie, aangezien zij bepaalde veranderingen in het brein nabootsen, maar relatief eenvoudig te verkrijgen zijn terwijl de patiënt nog in leven is.

Hoofdstuk 5 beschrijft veranderingen in het functioneren van de hypothalamus-hypofyse-bijnier as (de 'stress-as') die werden gezien in de $(CGG)_n$ muizen. Er zijn aanwijzingen dat PM dragers meer psychiatrische problemen als depressie, angst en agressie vertonen, welke veroorzaakt zouden kunnen worden door verhoogde stresshormoonniveaus. $(CGG)_n$ muizen bleken inderdaad significant verhoogde stresshormoonniveaus te hebben in vergelijking tot normale muizen. Organen betrokken bij de stress-as laten ook inclusies zien, hetgeen ook als indicatie kan worden opgevat dat de regulatie van de stress-as anders is dan in normale muizen. Het is echter nog onduidelijk hoe verhoogde mRNA niveaus, die de verlengde $(CGG)_n$, bevatten zouden kunnen leiden tot de beschreven effecten.

Hoofdstuk 6 beschrijft de onderlinge verbanden tussen $(CGG)_n$ lengte, *Fmr1* mRNA niveaus, Fmrp niveaus en de hoeveelheid inclusies in bepaalde hersengebieden in muizen met verschillende $(CGG)_n$ lengtes. Als inclusies direct gerelateerd zijn aan de klinische gevolgen, suggereren de beschreven

resultaten mogelijk dat niet zozeer de precieze hoeveelheid *Fmr1* mRNA belangrijk is voor de vorming van inclusies en daarmee cellulaire problemen en uiteindelijk klinische symptomen, maar dat de aanwezigheid van het verlengde $(CGG)_n$ mRNA op zich reeds voldoende is. De resultaten laten ook zien dat er zowel een onder- als een bovengrens van $(CGG)_n$ lengte bestaat voor de vorming van inclusies.

Hoofdstuk 7 vormt een algemene discussie waarin de resultaten van dit promotieonderzoek en de huidige stand van zaken in dit onderzoeksveld worden besproken. Ondanks dat de $(CGG)_n$ lengte de humane drempel van *FMR1* gen inactivatie heeft overschreden, is het *Fmr1* gen in de muis nog actief. Dus is de verlengde $(CGG)_n$ muis momenteel nog geen goed model om de gebeurtenissen rondom deze inactivatie te bestuderen. Het is echter wel een geschikt model om het ontstaan en het verloop van de neuropathologische, biochemische en gedragsmatige kenmerken van FXTAS te bestuderen. Het is nog onduidelijk waarom sommige PM dragers FXTAS ontwikkelen en anderen niet. Ook is het onbekend hoe en waarom inclusies ontwikkelen en wat zij betekenen in de pathogenese; zijn ze de bron of slechts een bijproduct van de cellulaire dysfunctie? Toekomstig onderzoek zal zich ook moeten richten op mogelijke eiwitten die een interactie aangaan met $(CGG)_n$ mRNA, welke volgens het hypothetisch model weggevangen worden van hun normale functie en zodoende mogelijk verantwoordelijk zijn voor een verstoorde celfunctie. Aangezien bij patiënten de *FMR1* mRNA niveaus gemeten zijn in bloed, is het niet bekend of deze ook verhoogd zijn in brein. Alleen als verhoogde mRNA niveaus cruciaal zijn voor ontwikkeling van neurotoxiciteit, is het relevant om de mogelijkheden tot verlaging van de *FMR1* mRNA niveaus te onderzoeken, om zo mogelijkheden voor therapeutische interventie te vinden.

Summary

The *FMR1* gene, located on the X chromosome, harbours a CGG-trinucleotide repeat in a DNA region that is transcribed into RNA, but is not translated into protein. In 1991 it was discovered that this CGG repeat ($(CGG)_n$) can vary in length, since it is unstable upon transmission to the next generation. A length exceeding 200 CGGs turned out to be responsible for fragile X syndrome, which presents itself as mental retardation. This $(CGG)_n$ is one of the first trinucleotide repeats identified to be the genetic cause of a neurological disorder. In humans, the $(CGG)_n$ in the *FMR1* gene is between 5 and 55 CGGs long in the normal population. Both the *FMR1* promoter region and the $(CGG)_n$ become methylated when the $(CGG)_n$ exceeds 200 trinucleotides (full mutation: FM),. This results in inactivation of the gene, thus preventing the protein product FMRP from being synthesised. FMRP regulates transport and translation of certain mRNA molecules in neural processes. By affecting the plasticity of synapses (the space between two neurons where signal transduction takes place) in these dendrites, FMRP plays an important role in learning and memory processes. The absence of FMRP in fragile X patients therefore results in mental retardation.

It was long thought that people with a $(CGG)_n$ length of between 55 and 200 (premutation: PM were not at risk of clinical consequences, except for the possibility of expansion of the $(CGG)_n$ length to an FM, upon transmission to the next generation, which would cause the child to have fragile X syndrome. In 2001 however, some aged PM carriers were described to have developed a neurological syndrome. This progressive, neurodegenerative disorder was called fragile X-associated tremor/ataxia syndrome (FXTAS), after the two most prominent clinical symptoms with which FXTAS patients usually present. In the meantime, many more patients have been described and it is now known that this syndrome shows major interindividual variability with regard to the severity and the range of clinical involvement. FXTAS patients likely develop Parkinsonism, executive defects and in a later stage also cognitive decline, among many other possible symptoms.

All PM carriers have elevated *FMR1* mRNA levels. Because their FMRP levels are close to normal, and since no signs of FXTAS have been observed in elderly FM carriers, it is unlikely that an FMRP deficiency is the cause of developing FXTAS. Thus, the working hypothesis is that the presence of elevated *FMR1* mRNA, containing an expanded $(CGG)_n$, can cause problems in brain cells, ultimately leading to clinical symptoms. An indication that this might be the case, comes from the observation that *FMR1* mRNA has been detected in the intranuclear protein inclusions seen in many regions of *post mortem* brains of FXTAS patients .

The origin of the clinical symptoms in FXTAS remains to be elucidated. In addition, it is unknown why some PM carriers develop FXTAS, while others do not. Research into the underlying mechanism of this syndrome in humans is limited to *post mortem* brain material, thus, the end stage of the disease. To investigate the onset and development of the disorder, a mouse model could be a valuable tool. A genetically modified mouse has been generated in our lab, which serves as a model for FXTAS. The endogenous mouse *Fmr1* $(CGG)_8$ was exchanged with a human $(CGG)_{98}$. This mouse model was originally developed to study $(CGG)_n$ instability and possibly silencing of the *Fmr1* when the $(CGG)_n$ would reach sufficient length. However, when FXTAS was first described, the expanded $(CGG)_n$ mice

turned out to be a good model for this syndrome as well. Not only $(CGG)_n$ instability, but also elevated *Fmr1* mRNA levels and inclusion formation mimic the human situation.

The aim of this research was to use the expanded $(CGG)_n$ mouse model to unravel aspects of the mechanisms underlying FXTAS.

Chapter 1 of this thesis gives a general introduction about the characteristics of the *FMR1* gene and the syndromes associated with it. Observations and hypotheses on the underlying pathogenic mechanisms are discussed, as well as existing animal models used to study this.

Chapter 2 extensively describes various neurodegenerative disorders, which are all caused by expanded trinucleotide repeats. Similarities, as well as differences between these diseases are discussed. The different mechanisms underlying the disorders, as well as the timing and the molecular mechanism of the trinucleotide repeat expansions are considered. Several factors that have been shown to influence trinucleotide repeat instability are touched on.

Chapter 3 shows that in our mice no methylation, thus no *Fmr1* gene silencing has taken place, although the $(CGG)_n$ expanded to over 200 trinucleotides. This length would generally induce gene inactivation in humans. $(CGG)_{>200}$ mice still produce Fmrp, albeit at lower levels than in mice with shorter $(CGG)_n$. The $(CGG)_n$ transmission pattern is the same for transmission through the father or the mother, although in humans more instability is seen upon transmission of the maternal X chromosome.

Chapter 4 looks into the cellular changes, as a result of expression of an expanded $(CGG)_n$. Disturbed lamin-architecture and stress-induced activation of heat shock proteins is seen in *post mortem* brain material and skin fibroblast cell lines of FXTAS patients, as well as fibroblast lines obtained from expanded $(CGG)_n$ mice. This suggests that cell lines may provide useful information about the cellular dysfunction, since they mimic certain changes seen in brain. Naturally, they are more easily accessible and available while the patient is still alive.

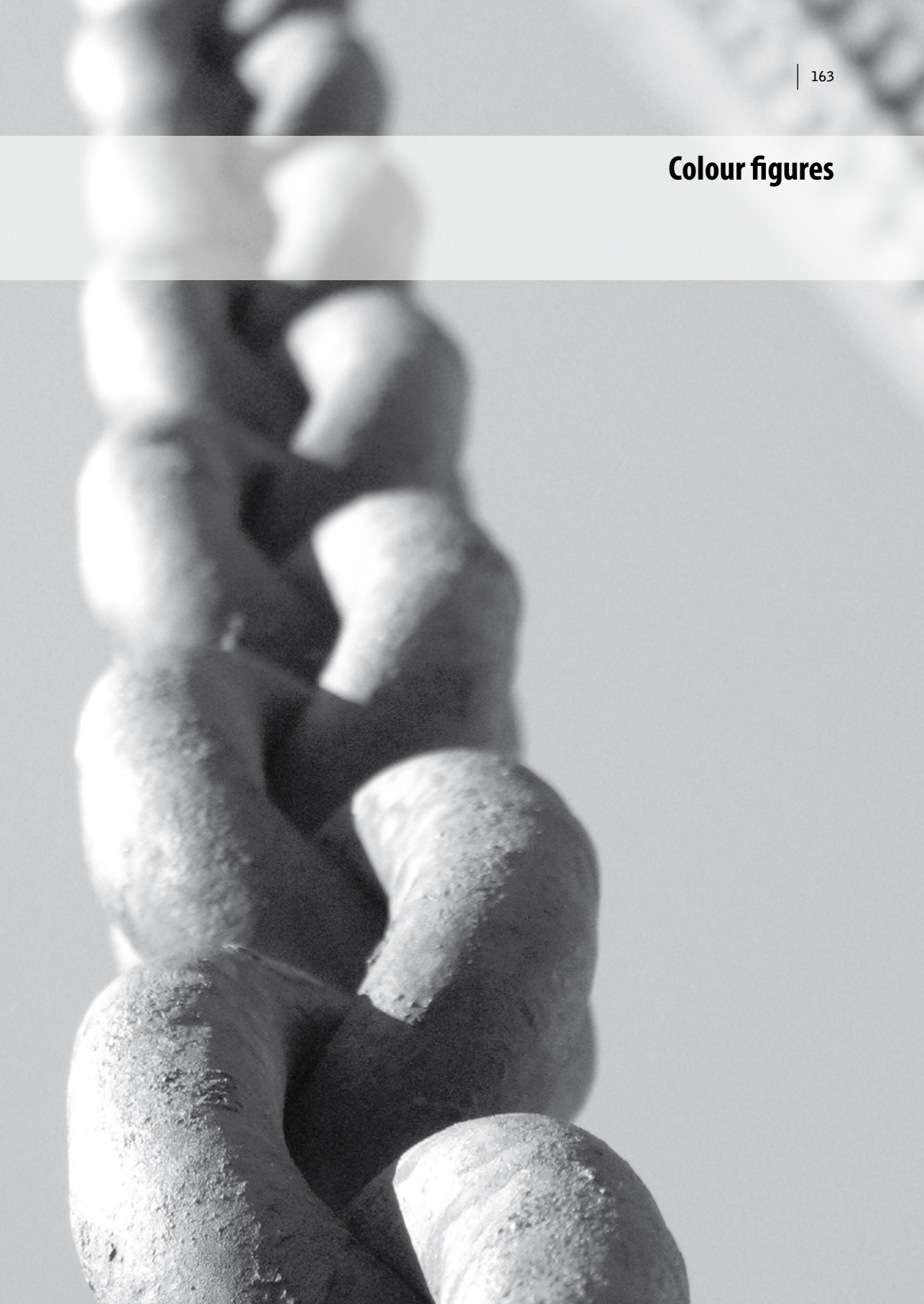
Chapter 5 describes altered functioning of the hypothalamo-pituitary-adrenal axis ('stress axis') in $(CGG)_n$ mice. Evidence exists that PM carriers develop more psychiatric problems such as anxiety, aggression and depression, which could be caused by elevated stress hormone levels. $(CGG)_n$ mice indeed showed higher stress hormone levels as compared with normal mice. Organs involved in the stress axis also bear inclusions, which might be interpreted that stress axis regulation is aberrant in $(CGG)_n$ mice. It remains to be elucidated how elevated expanded $(CGG)_n$ mRNA levels could lead to the effects described.

Chapter 6 describes correlations between $(CGG)_n$ length, *Fmr1* mRNA levels, Fmrp levels and the proportion of inclusion-bearing cells in $(CGG)_n$ mice with different $(CGG)_n$ lengths. If inclusions can directly be linked to clinical outcome, the results possibly suggest that not so much the precise amount of *Fmr1* mRNA is important for inclusion formation, but instead the mere presence of the expanded $(CGG)_n$ mRNA . The results furthermore suggest that a lower as well as an upper threshold $(CGG)_n$ length exists for inclusions formation.

Chapter 7 is an overall discussion of the results presented in this thesis and the current status of this research field. Since *Fmr1* gene silencing has not taken place yet, despite having reached the human threshold $(CGG)_n$ length for this event, the expanded $(CGG)_n$ mouse is currently not an

appropriate model to study the events associated with *Fmr1* inactivation. It is, however, a suitable model to study the onset and the course of the biochemical, neuropathological and behavioural parameters. It is unclear why some PM carriers develop FXTAS, while others do not. Furthermore, it is unknown how and why inclusions are formed and what they mean in the pathogenesis of FXTAS: are they a by-product or the source of cellular dysfunction, or do they even exert a protective effect? Future research should focus on proteins that might interact with expanded $(CGG)_n$ mRNA. According to the hypothetical pathogenesis model, such proteins are sequestered away from their normal function, thereby causing disturbed cellular function. In addition, the possibilities of decreasing *FMR1* mRNA levels should be explored, since this may prove promising in light of therapeutic intervention strategies.

Colour figures



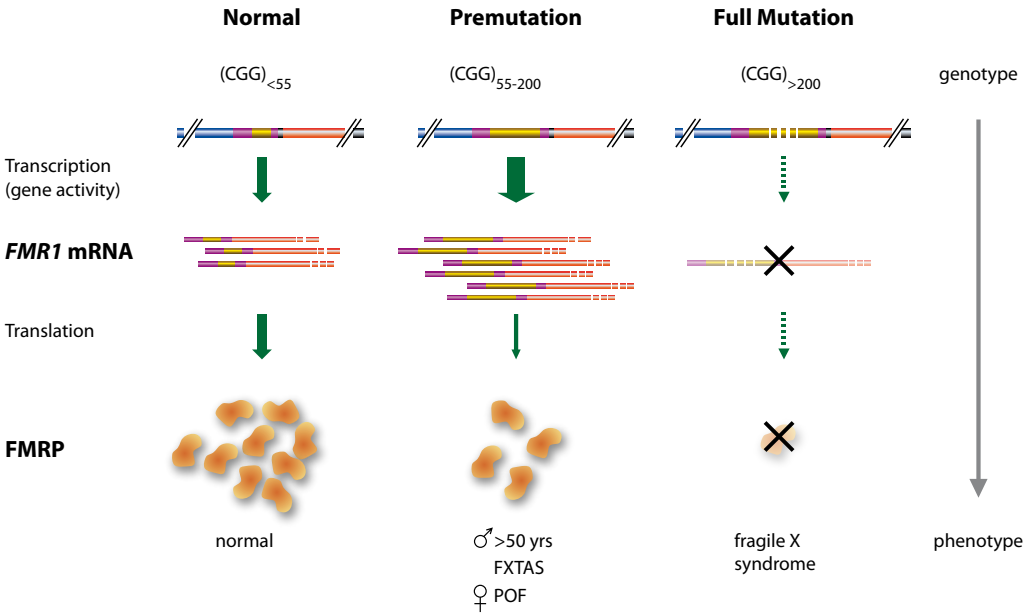


Figure 1.1 | $(CGG)_n$ length and *FMR1* expression and clinical outcome. This figure shows the consequences of the different repeat length categories on transcription, translation and clinical phenotype. (Adapted from [76] with permission of the authors.)

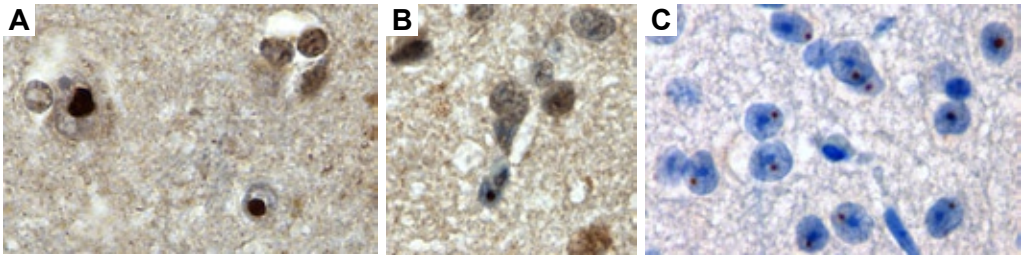


Figure 1.2 | Ubiquitin-positive intranuclear inclusions, the neuropathological hallmark of FXTAS in A: human neurons, B: human astrocytes, C: murine neurons. No inclusions have been seen in murine astrocytes.

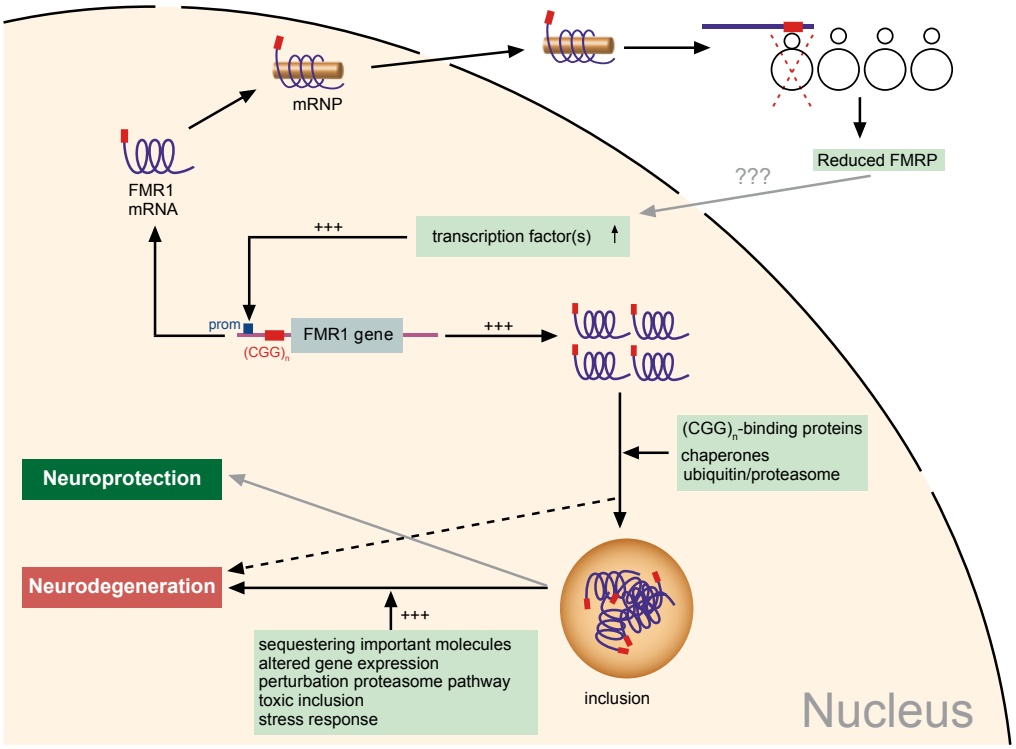


Figure 1.3 | A schematic representation of **the RNA gain-of-function mechanism proposed for the pathogenesis of FXTAS**. The *FMR1* gene is transcribed in the nucleus and transported to the ribosomes. The expanded $(CGG)_n$ present in the 5'UTR of the *FMR1* gene hampers translation, leading to lower FMRP levels. Through an as yet unknown mechanism, transcription is upregulated, leading to increased *FMR1* mRNA levels. In an attempt to get rid of the excess of *FMR1* mRNA, the cell might attract chaperones or elements of the ubiquitin/proteasome system. Also $(CGG)_n$-binding proteins might be recruited. These processes could lead to formation of intranuclear inclusions. Sequestration of proteins into the inclusions might prevent them from exerting their normal function, thereby disturbing cellular function, which in the end might cause neurodegeneration. Also, it cannot be excluded that neuroprotection takes place, such that cells that are capable of capturing the toxic transcripts in the inclusions are the cells that survive.

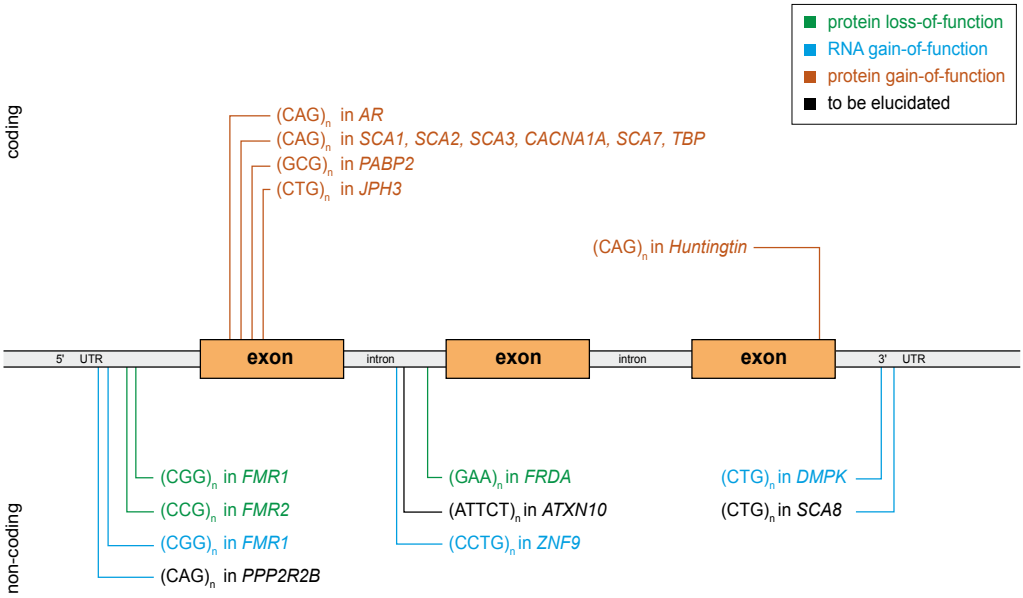


Figure 2.1 | Location of repeats within genes in relation to pathogenic mechanism. This schematic gene shows all microsatellite repeats described in this chapter, with their location within the gene. The colours indicate which pathogenic mechanism is thought to underlie the associated diseases. All repeats depicted above the gene are located in a coding sequence of the gene, while all repeats below are in non-coding regions. Please refer to the text and table 2.1 for further information regarding these repeats, genes and associated disorders.

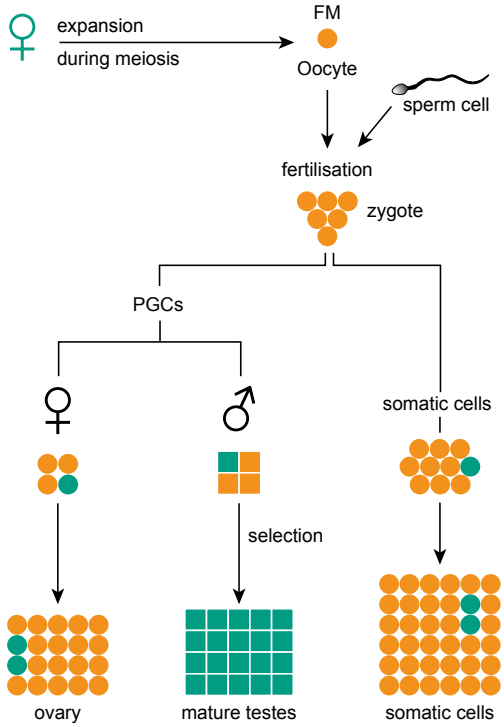


Figure 2.2 | The prezygotic model of expansion of the fragile X mutation. This model assumes that an expansion of a maternal PM to an FM takes place during meiosis. The fertilised oocyte carries an FM allele. After separation from the embryo proper, the primordial germ cells (PGCs) have an FM. Some alleles will contract to PMs. To explain why FMs are only transmitted through females, some selection must exist agains FMs in the male germ line during spermatogenesis. In the mature testes, PM alleles predominate. In somatic cells and the female germ line, this selection did not take place. Cells with a PM are shown ingreen and cells with an FM are depicted in orange

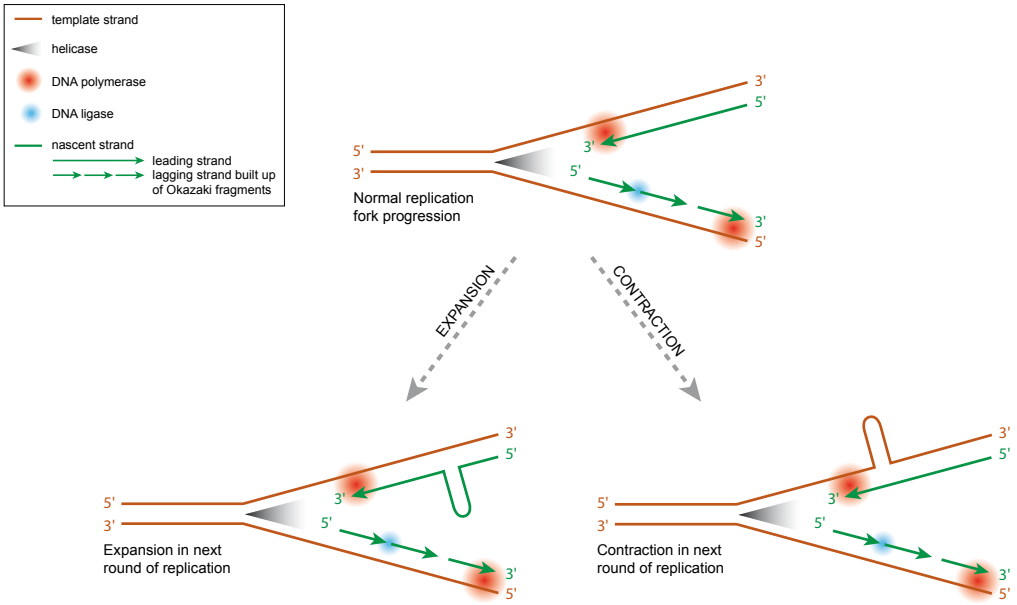


Figure 2.3 | Replication fork progression and repeat instability. During normal replication, helicases break the hydrogen bonds that keep the two DNA strands together, which yields the replication fork. DNA polymerase can only synthesise a new strand in a 5′ to 3′ direction. Hence, on one strand (leading strand) DNA polymerase reads the DNA and adds nucleotides to the nascent strand in a continuous matter. On the other strand (lagging strand) the complementary strand is synthesised in short segments (Okazaki fragments) at a time, that are later joined together by DNA ligase. Formation of secondary structures, such as hairpins can form in one of the strands. This can impair normal replication fork processing. The sequence of the strands, together with the position of the origin of replication with respect to the repeat sequence, determine which strand is more prone to form hairpins. Whether a hairpin is present in the template or nascent strand, in turn determines whether contraction or expansion results in the next round of replication.

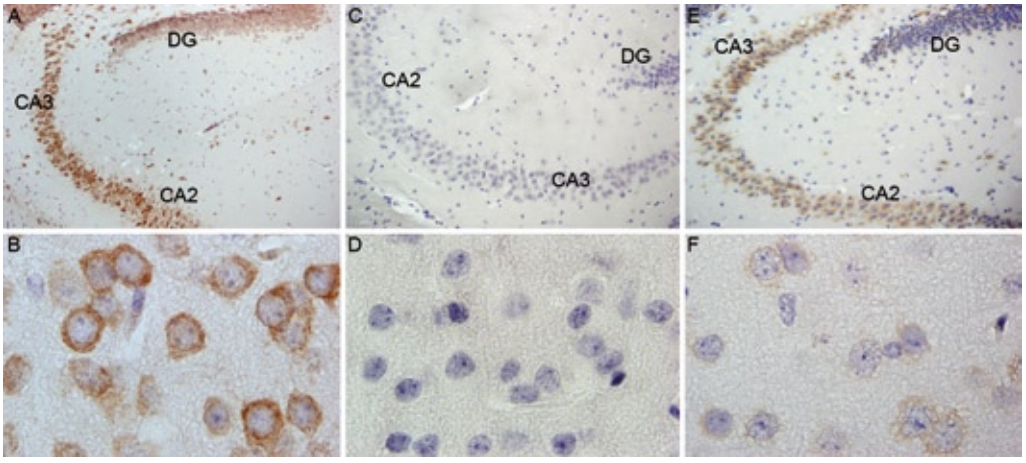


Figure 3.5 | Mouse brains from wt (A and B), Fmr1 KO (C and D) and the mouse with >230 CGGs (E and F) were stained with antibodies against Fmrp. Photos A,C and E show hippocampus and B,D, and F show cortex tissue. DG: dentate gyrus

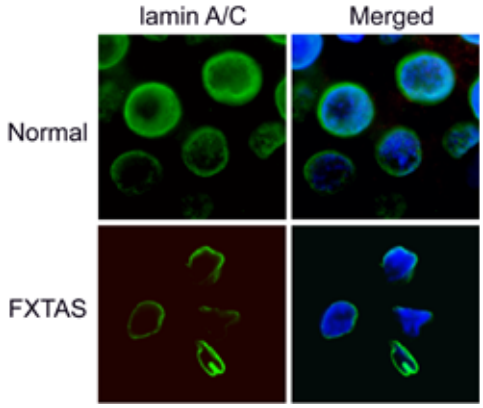


Figure 4.3 | *Post mortem* skin biopsy in a case (468-05) with **FXTAS displays frequently distorted nuclear ring-like distribution of lamin A/C** (lower panel) compared to an age-matched control (upper panel). Green, lamin A/C; blue, DAPI staining of nuclei.

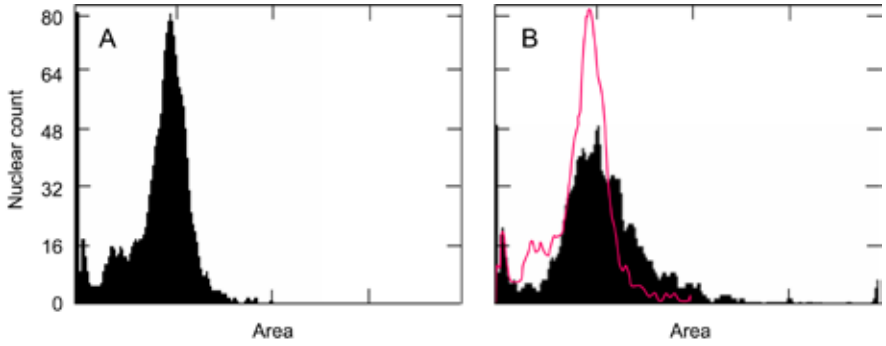


Figure 4.5 | Increased nuclear area in human fibroblasts from a patient with FXTAS. Morphometric values of individual human skin fibroblasts contoured on the basis of DAPI fluorescence. (**A**) Distribution of the nuclear areas of 2013 fibroblasts derived from an 87-year old normal control (C3). (**B**) The nuclear areas of 2009 fibroblasts derived from an 80-year old patient with severe FXTAS (P6) exhibit a broader distribution of nuclear areas, with an average 1.3-fold increase, compared to the age-matched control (overlay tracing of the distribution in part A).

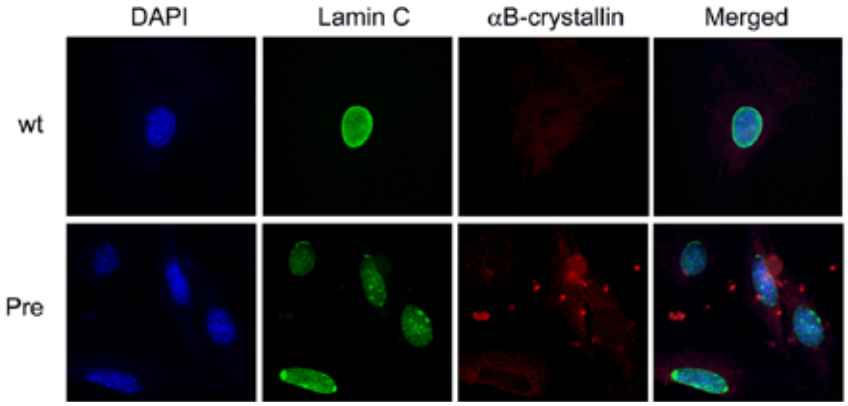


Figure 4.6 | Immunocytochemical localisation of lamin C and αB-crystallin in mouse embryonic fibroblasts. Lower panel: mouse fibroblasts harbouring the expanded-CGG-repeat display irregular nuclear morphology (nuclei counterstained with DAPI, blue), marked lamin C disruption and aggregation with loss of the normal ring-like architecture (green), and an increased number of perinuclear aggregates containing αB-crystallin (red).

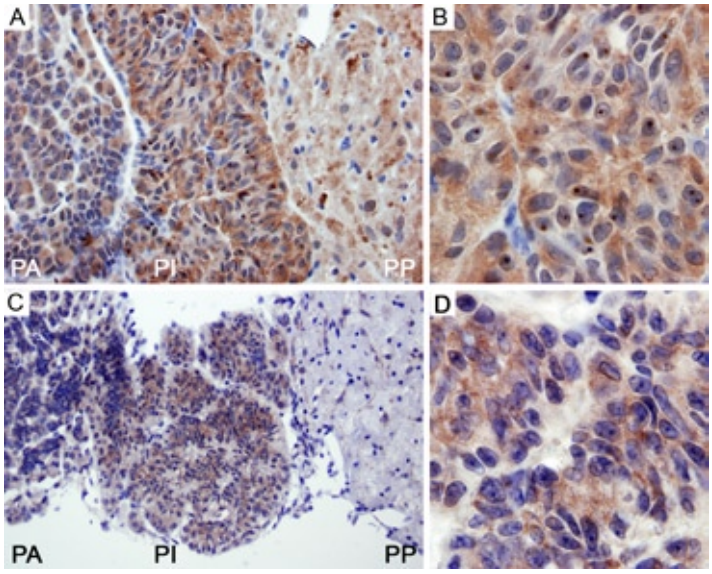


Figure 5.3 | Immunohistochemistry with antibodies against ubiquitin and Fmrp in pituitary gland of 100-week old CGG mice. **A**: Many intranuclear ubiquitin-positive inclusions (arrows) are seen in the pars intermedia, while hardly any were observed in the pars posterior. **B**: Ubiquitin-positive inclusions in the pars intermedia. **C**: Highest levels of Fmrp are observed in the pars intermedia. **D**: Fmrp expression in the pars intermedia. PA: pars anterior, PI: pars intermedia, PP: pars posterior.

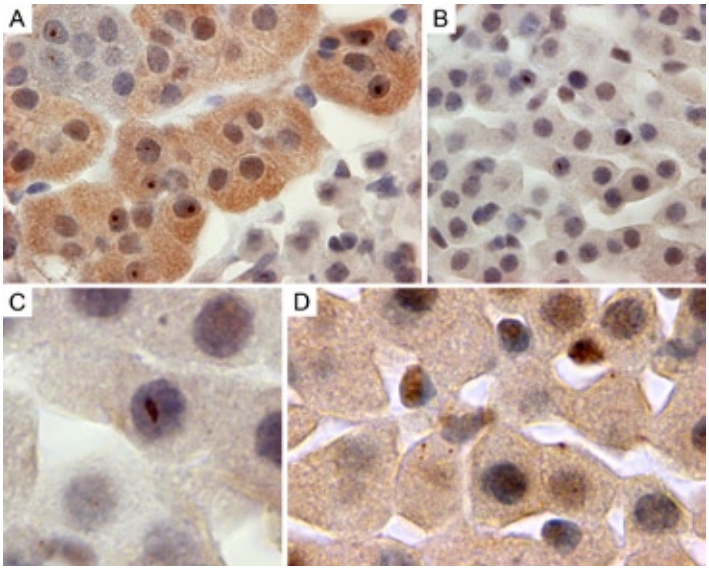


Figure 5.4 | Immunohistochemistry with an antibody against ubiquitin show ubiquitin-positive inclusions in adrenal gland of 100-week old CGG mice. **A**: Ubiquitin-positive intranuclear inclusions (arrows) in chromaffin cells of the adrenal gland. **B+C:** Elongated, irregularly shaped ubiquitin-positive intranuclear inclusions in the *zona fasciculata* of the adrenal gland. **D:** Cytoplasmic inclusions (arrow heads) in the *zona fasciculata* of the adrenal gland.

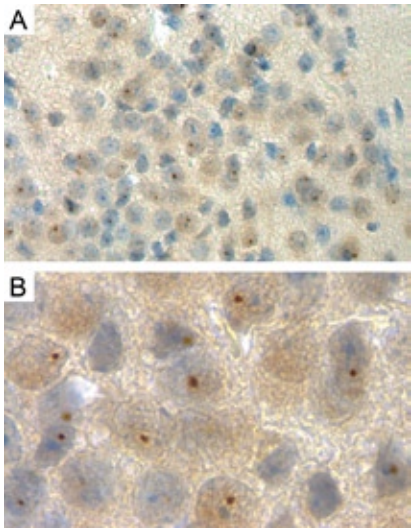


Figure 5.5 | Ubiquitin-positive intranuclear inclusions in the amygdala of a 100-week old CGG mouse. **A** en **B** show the posteromedial amygdalohippocampal nucleus, where B is a higher magnification of what is seen in A.

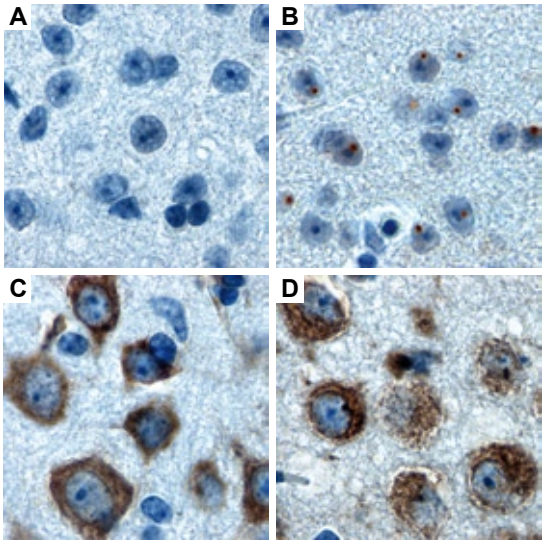


Figure 6.1 | No ubiquitin-positive inclusions are seen in the colliculus inferior of 72-week old mice with $(CGG)_{70}$ (**A**), while mice with $(CGG)_{106}$ do show ubiquitin-positive inclusions (**B**) brown, round intranuclear staining). Immunohistochemistry for Fmrp in cortex of $(CGG)_{70}$ (**C**) and wt (**D**) animals gives a similar staining pattern.

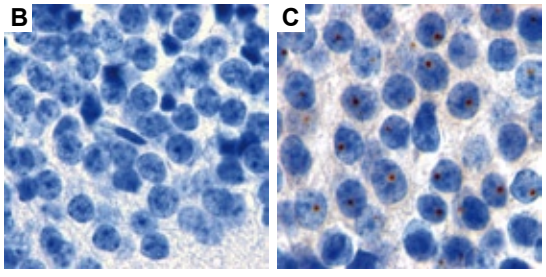


Figure 6.3 | B: Dentate gyrus of an animal with $(CGG)_{106}$ that does not show intranuclear inclusions, while a mouse with $(CGG)_{130}$ (**C**) does.

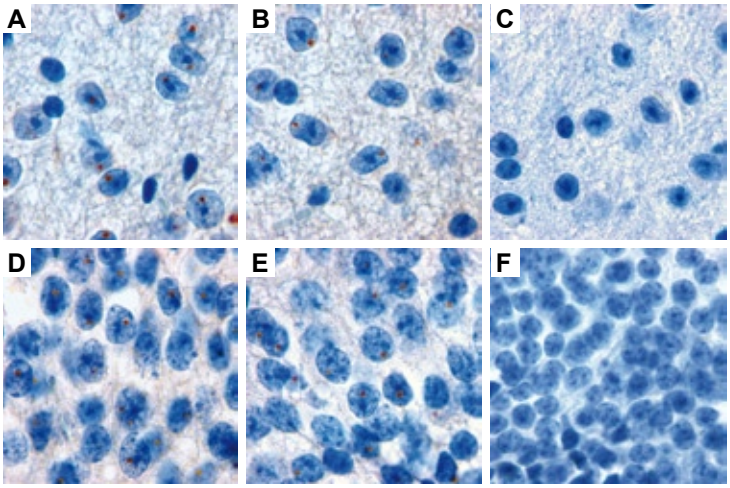


Figure 6.4 | Ubiquitin-positive inclusions are present in colliculus inferior (A-C) and dentate gyrus (D-F) of mice with $(CGG)_{130}$ and $(CGG)_{180}$, but not in mice with (CGG)>200.

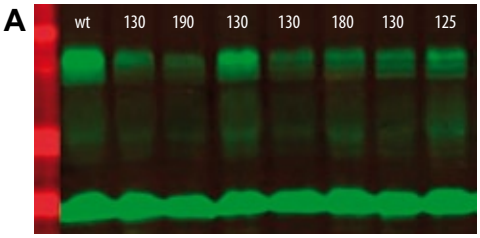


Figure 6.5 | Fmrp levels in mouse brain of the different $(CGG)_n$ length categories. A shows an example of a Western Blot, visualised with the 2F5 antibody against Fmrp, Gapdh was used as a loading control. Approximate $(CGG)_n$ lengths are indicated.

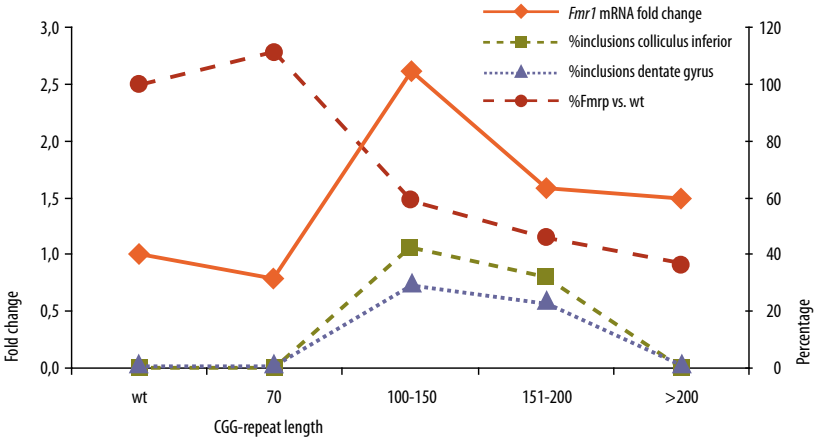


Figure 6.6 | Summary of the molecular correlates. Fold change Fmr1 mRNA (left y-axis), % Fmrp and % of inclusion-bearing neurons in colliculus inferior and dentate gyrus (right y-axis) are given. Please refer to the individual graphs for an indication of the variability of the observed data.

Curriculum Vitae
List of publications
Dankwoorden / Words of thanks

Curriculum Vitae

Judith Rixt Brouwer was born in Leeuwarden on August 31, 1979. She attended secondary school at the Christelijk Gymnasium in Leeuwarden, followed by the Esdal College in Emmen. After her graduation from secondary education in 1997, she travelled through North America and Europe for a year, in a group of 170 young people of 24 nationalities, with the cultural educational organisation Up With People.

Upon returning to The Netherlands, she studied Biomedical Sciences at the Radboud University in Nijmegen. Next to her major in Toxicology, she took Neurobiology and Biology of Reproduction as minors. As a student she took part in research into the effects of elevated maternal stress hormone levels on the development of schizophrenia in newborn rats (Department of Psychoneuropharmacology, Radboud University Nijmegen).

During a second internship in Southampton, England, she investigated the immunological composition of breast milk in relation to atopy status (Department of Child Health, Southampton University). In a fifth, extra study year, Judith extended her knowledge in Neuroscience and explored cognitive neurosciences at the FC Donders Centre for cognitive neuroimaging in Nijmegen.

After her graduation in 2003 she gained experience in molecular biology at the department of Molecular Animal Physiology (Radboud University Nijmegen). Since May 2004 Judith has been working as a PhD student at the department of Clinical Genetics of the ErasmusMC in Rotterdam, under supervision of Prof.dr. BA Oostra and dr. R Willemsen. As part of this research period she was a visiting researcher in the lab of Prof.dr. PJ Hagerman (Department of Biochemistry and Molecular Medicine, UCDavis School of Medicine, Davis, CA, USA). The results obtained from this PhD research project were presented at several national and international conferences, have been published in international, peer-reviewed scientific journals and have resulted in this dissertation.

Until October 2008, Judith is working as a scientific researcher at the department of Clinical Genetics. From November 2008 onwards, she will continue her scientific career in the group guided by dr. G Gourdon, at INSERM U781 Department of Genetics and Epigenetics of Neurometabolic Diseases and Birth Defects, Université Paris 5 René Descartes, in Paris. She will work on the further elucidation of epigenetic mechanisms involved in myotonic dystrophy and trinucleotide repeat instability.

List of publications |

Brouwer JR, Mientjes EJ, Bakker CE, Nieuwenhuizen IM, Severijnen LA, Van der Linde HC, Nelson DL, Oostra BA, Willemsen R. 2007. Elevated *Fmr1* mRNA levels and reduced protein expression in a mouse model with an unmethylated Fragile X full mutation. Exp Cell Res 313:244-253.

Musumeci SA, Calabrese G, Bonaccorso CM, D'Antoni S, **Brouwer JR,** Bakker CE, Elia M, Ferri R, Nelson DL, Oostra BA, Catania MV. 2007. Audiogenic seizure susceptibility is reduced in fragile X knockout mice after introduction of *FMR1* transgenes. Exp Neurol 203:233-240.

Brouwer JR, Severijnen E, de Jong FH, Hessl D, Hagerman RJ Oostra BA, Willemsen R. 2008. Altered hypothalamus-pituitary-adrenal gland axis regulation in the expanded CGG-repeat mouse model for fragile X-associated tremor/ataxia syndrome. Psychoneuroendocrinology 33:863-873.

Brouwer JR, Willemsen R, Oostra BA. 2008. Microsatellite repeat instability and neurological disease. BioEssays: In press.

Brouwer JR, Willemsen R, Oostra BA. The *FMR1* gene and its involvement in mental retardation and FXTAS. Am J Med Genet B Neuropsychiatr Genet: Submitted.

Garcia-Arocena D, Iwahashi C, **Brouwer JR**, Tassone F, Berry-Kravis EM, Goetz CG, Sumis AM, Zhou L, Ludwig A, Raske C, Leehey M, Greco C, Willemsen R, Hagerman RJ, Hagerman PJ. Lamin A/C dysregulation and cellular stress response in fragile X-associated tremor/ataxia syndrome Brain: Submitted.

Brouwer JR, Huizer K, Severijnen E, Hukema RK, Berman RF, Oostra BA, Willemsen R. Correlation between CGG-repeat length and phenotype in FXTAS. Submitted.

Willemsen R, Li Y, Berman RF, **Brouwer JR**, Oostra BA, Yin P. 2008. Animal models for FXTAS. In: Tassone F, Berry-Kravis EM. ed. Fragile X-associated tremor/ataxia syndrome: Springer. In press.

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En nu: Paris, j'arrive!
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