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Faculty of Health Sciences

Department of Medical Biology

Triggering mechanisms in the molecular pathogenesis of FXTAS

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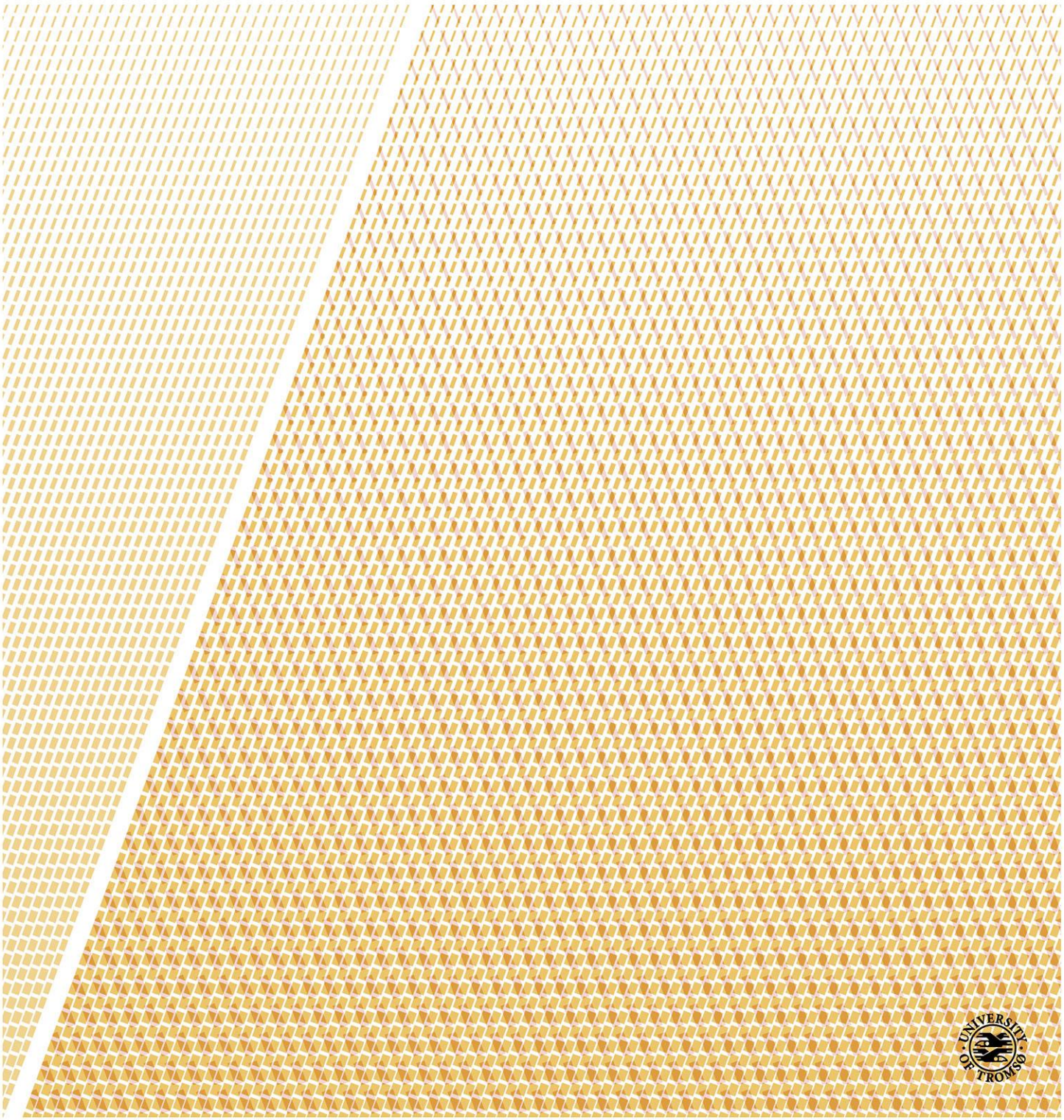
Gry Hoem

A dissertation for the degree of Philosophiae Doctor – July 2018



Triggering mechanisms in the molecular pathogenesis of FXTAS

By

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Table of contents

Abbreviations

ALS	Amyotrophic lateral sclerosis
ASFMR	Antisense fragile X mental retardation
CLEM	Correlative light and electron microscopy
DM1	Myotonic dystrophy type 1
DMPK	Myotonic dystrophy protein kinase
Dox	Doxycycline
FM	Full mutation
FMR1	Fragile X mental retardation 1
FMRP	Fragile X mental retardation 1 protein
FRD	Friedreich ataxia
FTD	Frontotemporal dementia
FXPOI	Fragile X-associated premature ovarian insufficiency
FXTAS	Fragile X-associated tremor/ataxia syndrome
HD	Huntington's disease
HDL2	Huntington disease-like 2
MBNL	Muscleblind-like
MCP	Middle cerebellar peduncle
mGluR	Metabotropic glutamate receptor
miRNA	microRNA
mRNA	messengerRNA
ORI	Origin of replication
PM	Premutation
polyG	polyglycine
polyQ	polyglutamine
PQC	Protein quality control
RAN	Repeat-associated non-AUG
ROS	Reactive oxygen species
SCA	Spinocerebellar ataxia
SMBA	Spinal and bulbar muscular atrophy
Tet	Tetracycline
TNR	Trinucleotide repeat
UTR	Untranslated region

Abstract

This study focused on the triggering mechanisms leading to development of the neurodegenerative disorder Fragile X-associated tremor/ataxia syndrome (FXTAS). FXTAS affects carriers of a so-called premutation in the Fragile X Mental Retardation 1 (*FMR1*) gene on the X chromosome. A premutation in this context, means the presence of 55-200 CGG repeats in the 5'untranslated region (UTR) of the gene. Unaffected individuals in the normal population generally have < 45 CGG repeats. The long CGG repeat tract results in expanded mRNA which forms stable hairpin structures. In addition, premutation carriers have 2-8 fold elevated levels of this expanded *FMR1* mRNA. The presence of this CGG-harboring mRNA in intranuclear inclusions found in brain tissue from FXTAS patients, has led to the hypothesis that the expanded mRNA itself mediates toxicity and is the cause of FXTAS.

Since the number of CGGs in each mRNA molecule, and the concentration of this mRNA are both increased in FXTAS patients, it was not known which factor led to downstream adverse effects. In the first part of our study, we set out to resolve this issue. We established a novel cellular model system for FXTAS, in which we could vary the mRNA levels for a range of CGG repeat sizes. Using reduced cellular viability as an outcome measurement, we found toxicity only upon expression of > 62 CGG repeats. The morphology of cells expressing mRNA with CGG repeat sizes in the normal (30 repeats) or premutation (95 CGGs) range, was evaluated using immunofluorescence microscopy. Only cells with 95 CGG repeats displayed features of cellular dysregulation, such as disrupted lamin architecture, lamin A/C positive inclusions and activation of the DNA damage repair response. We conclude that there is a threshold for CGG repeat number, between 62 and 95, below which no cellular dysregulation is observed.

Later publications in the field have focused on how the expanded CGG mRNA mediates toxicity and thus development of FXTAS. One of the hypotheses is that repeat-associated non-AUG (RAN) translation across the expanded CGG repeat tract causes formation of a toxic protein named FMRpolyGlycine (FMRpolyG). This novel protein was not well characterized. Specifically, it was not known whether its negative effects on cellular homeostasis depended on the presence of the CGG mRNA which forms a hairpin structure. In the second part of this study we therefore developed a cell-based model allowing expression of the FMRpolyG protein, without co-expressing the CGG mRNA hairpin. Using both stable cell lines and transient transfections, we found that FMRpolyG per se led to aggregate formation in several cell lines, reduced cell viability, and disrupted lamin ring structure. These

findings did not depend on the presence of a CGG mRNA hairpin. Using a flow-based assay, we determined that FMRpolyG is a stable protein which accumulates upon inhibition of the ubiquitin-proteasome system. Our results indicate that FMRpolyG itself may trigger development of FXTAS. Future studies looking at endogenous FMRpolyG expression, and its levels in FXTAS patients versus unaffected controls, are needed to determine the exact role of this protein in disease development.

List of papers

Paper I

Hoem G, Raske CR, Garcia-Arocena D, Tassone F, Sanchez E, Ludwig AL, Iwahashi CK, Kumar M, Yang JE and Hagerman PJ. **CGG-repeat length threshold for *FMR1* RNA pathogenesis in a cellular model for FXTAS**.

Human molecular genetics 2011; 20: 2161-70.

Paper II

Hoem G, Larsen KB, Øvervatn A, Lamark T, Sjøttem E and Johansen T. **The FMRpolyGlycine protein mediates aggregate formation and toxicity independent of the CGG mRNA hairpin in a cellular model for FXTAS**

Submitted Manuscript

1 Introduction

1.1 Genetic background

1.1.1 Trinucleotide repeat expansions causing human disorders

The human genome contains tens of thousands of areas where specific short DNA sequences (typically 3-6 bases) are repeated. These repeat tracts, often referred to as microsatellites, have a higher mutation rate than other parts of the genome. Arguably, this leads to increased genetic diversity. However, repeat tracts and their tendency to increase in size through generations, is also the cause of more than 20 human genetic disorders (Mirkin, 2007, Zhao and Usdin, 2015). Most of these conditions are due to expansions of a trinucleotide repeat (TNR) region. A classic example is the CAG-repeat region on chromosome 4 which causes Huntington's Disease (HD) when expanding to over 35 CAGs (Rubinsztein et al., 1996, Ho et al., 2001).

The first characterization of TNR expansion leading to human disease, appeared as late as the 1990s (Orr and Zoghbi, 2007). In 1991, both the expansion of CGGs in the 5'UTR of the fragile X mental retardation 1 (*FMR1*) gene causing Fragile X syndrome (FXS), and the CAG expansion in the coding sequence in X-linked spinal and bulbar muscular atrophy (SMBA), were described (Verkerk et al., 1991, La Spada et al., 1991). Soon after that, CTG-expansions in 3'UTR of myotonic dystrophy protein kinase (DMPK) in myotonic dystrophy type 1 (DM1) and CAG expansions in exon 1 in HD were also characterized (Mahadevan et al., 1992, MacDonald et al., 1993). These discoveries could explain the clinically observed "anticipation", i.e. that onset is increasingly earlier and the phenotype more severe as the disorder is passed through the generations.

Over the last decades, numerous TNR expansion disorders have been studied. The disease-causing mutations occur in both coding and non-coding regions of the genes involved, and despite the similarities, TNR expansions mediate toxicity through a wide range of mechanisms. TNR expansions occurring in the coding part of a gene, are translated and can cause alterations in the structure and function of the protein. Repeat expansions in the non-coding parts of the gene do not directly affect the protein structure, but both structure and function of the mRNA, as well as expression level of the protein, may change. It is beyond the scope of this introduction to go through all repeat expansion leading to human disorders. The focus will therefore mainly be on TNR expansions

1.1.2 Mechanisms by which microsatellite expansions contribute to pathogenesis

In this section the main mechanisms for pathogenesis caused by TNR expansions, with some examples of other repeat expansions, are described and exemplified. The position of the TNR expansion in a coding or non-coding region of the gene, can to some extent help elucidate what triggers disease development (Fig. 1).

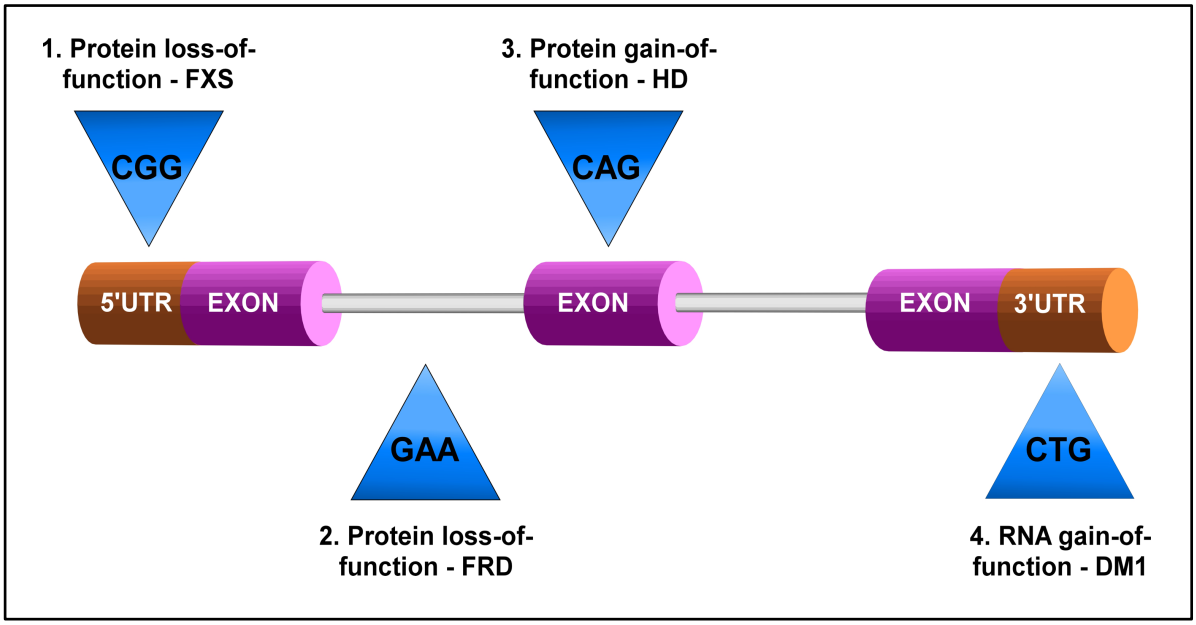

Figure 1. Different mechanisms for toxicity mediated by trinucleotide repeat expansions. The position of the TNR expansion in the gene (coding vs non-coding region) may predict the downstream mechanism for toxicity. While both the 5' and the 3' UTR are part of exons, they are non-coding regions. 1) CGG repeats in the 5'UTR lead to silencing of the gene, loss of the encoded protein and development of Fragile X syndrome (FXS). 2) GAA repeats in the first intron inhibit transcription, reduce levels of the protein Frataxin and cause Friedreich ataxia (FRDA). 3) CAG repeats in a coding part of the gene, an exon, lead to gain-of-function of the huntingtin protein and development of Huntington's disease (HD). 4) CTG repeats in the 3'UTR are the cause of a toxic effect mediated by the mRNA, and the resulting development of myotonic dystrophy 1 (DM1). Note that mutations in non-coding regions generally lead to protein-loss-of-function or RNA gain-of-function, while protein gain-of-function is seen upon mutation of the coding region. Descriptions of bidirectional transcription and repeat-associated non-AUG (RAN) translation complicate this picture.

Protein gain-of-function

A protein gain-of-function means that changes to the protein result in increased and/or new properties of this protein. The most frequent trinucleotide repeat leading to gain-of-function for proteins is the CAG-repeat. Upon translation, the $(CAG)_n$ gives rise to a polygutamine (PolyQ)-region in the protein. The known disorders caused by polyQ-harboring proteins, are all

neurodegenerative (Andrew et al., 1997, Fan et al., 2014). In addition, they usually involve formation of intranuclear aggregates in neurons, though in some cases there are also cytoplasmic aggregates (DiFiglia et al., 1997, Davies et al., 1997)(reviewed in (Michalik and Van Broeckhoven, 2003, Matilla-Duenas et al., 2014)). In vitro studies have shown that polyQ -tracts over a certain length form aggregates in solution, and this apparent threshold is conspicuously close to the number required for a polyQ-region to cause disease (~ 35-40 CAGs) (Scherzinger et al., 1999). However, whether the formation of aggregates in itself is a toxic or protective mechanism, is a topic still being debated (Arrasate et al., 2004, Ross and Tabrizi, 2011, Hoffner and Djian, 2015). The polyglutamine neurodegenerativ disorders include Huntington's disease, spinobulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA) and six spinocerebellar ataxias (SCAs). Examples of these are described in the following section. Finally, a short description of other TNRs involved in protein gain-of-function pathogenesis is included.

Huntington's disease

Huntingston's disease (HD) is a progressive neurodegenerative disorder with an autosomal dominant inheritance pattern. The clinical features include cognitive decline, chorea and dystonia as well as behavioral changes. George Huntington's description of the disorder's manifestations and inheritance pattern in 1872 gave it the name Huntingtons's disease (Huntington, 1872). However, the Norwegian physician Johan Christian Lund had also described a high prevalence of the same features in areas of Setesdalen, Norway, in 1860 (Stien, 1991). Here it was called "Setesdalsrykkja". The genetic basis for HD is an unstable CAG-expansion on the short arm of chromosome 4, coding for the Huntingtin protein (MacDonald et al., 1993). The unaffected population has 7-20 CAGs in this region (Kremer et al., 1994). Repeats in the range 36-40 give incomplete penetrance of HD, while > 40 CAGs result in full penetrance (Rubinsztein et al., 1996, McNeil et al., 1997, Ho et al., 2001). The wild-type Huntingtin (Htt) protein has been implicated in numerous cellular functions, including trafficking of vesicles and coordination of cell division (reviewed in (Saudou and Humbert, 2016)). However, since disruptions and deletions of the HD gene does not cause HD (Ambrose et al., 1994), and one mutant allele is sufficient to cause the disease (Ross and Tabrizi, 2011), it is not likely that reduction or loss of the wild-type protein is the main cause of HD. Evidence instead supports the notion that mutant Huntingtin protein has a toxic gain-of-function. An important finding here is the already mentioned in vitro studies showing aggregation of polyQ-tracts above 36 glutamines (Scherzinger et al., 1999). Expression of the mutant Htt appears to

cause aggregate formation and impairment of protein degradation pathways. In addition, the truncated Htt itself leads to formation of toxic N-terminal fragments and its nuclear translocation increases the toxic effects (reviewed in(Ross and Tabrizi, 2011)).

Spinocerebellar ataxias

In addition to Huntington's disease, CAG-expansions also cause the spinocerebellar ataxias (SCA) 1, 2, 3, 6, 7 and 17. SCAs are a group of autosomal dominant disorders that usually progress at a slow rate. More than 35 SCAs have been described (Pestronk, 2018).The clinical features most commonly seen in SCAs are gait ataxia, dysarthria and oculomotor disorders (Manto, 2005). Loss of Purkinje cells and atrophy of the cerebellum can be found in all SCAs. The mean onset of the disorders is often around 30-50 years of age (Manto, 2005), and brain atrophy is usually already present before the ataxia is diagnosed (Dohlinger et al., 2008). The above mentioned SCAs with CAG-expansions, all have the polyQ-tract in the coding region of the protein. Except for this polyglutamine region, the proteins generally do not share common features. Ataxin 1-3 and -7 are the names of affected proteins for SCA 1-3 and -7, respectively. These proteins are involved in transcription regulation, RNA metabolism and de-ubiquitination. SCA 6 involves a subunit of the voltage dependent Ca^{2+} -channel (CACNA1A), while the polyQ-tract in SCA 17 is found in TATA-binding protein (TBP). The phenomenon of genetic anticipation, where symptoms appear at an earlier age as the disorder is passed through the generations, occurs in all these SCAs, even though the number of CAGs required to cause disease differs (summarized in (Orr and Zoghbi, 2007) and (Matilla-Duenas et al., 2014)).

While the polyQ SCAs differ in several aspects, most of the proteins affected in these disorders are somehow involved in regulation of transcription or posttranscriptional products. In addition, polyQ diseases usually form protein aggregates inside the nucleus (reviewed in (Matilla-Duenas et al., 2014)), one of the exceptions being SCA 6 where the vast majority of aggregates are cytoplasmic (Ishikawa et al., 1999, Giunti et al., 2015). This common feature of protein aggregation due to the expanded polyQ tract, is the basis for the proposed protein gain-of-function mechanism for development of SCAs (Shao and Diamond, 2007). However, consequences of this gain-of-function toxicity vary between the different SCA's. Other mechanisms, such as protein-loss of function, may also play important roles in some cases (Paulson et al., 2017).

Spinobulbar muscular atrophy

Spinobulbar muscular atrophy (SBMA) is also known as Kennedy's disease, after the physician Willam R. Kennedy who described SBMA in 1968 (Kennedy et al., 1968). It is a progressive neuromuscular disorder primarily affecting males with ≥ 38 CAG-repeats in the coding part of the Androgen receptor (AR)-gene on the X-chromosome. Clinically, it is characterized by increasing muscle weakness, eventually affecting bulbar muscles. In addition, signs of androgen insensitivity, such as gynecomastia and later reduced fertility, often start in adolescence. Histopathology reveals protein inclusions with the mutated AR-protein, myopathy in muscle and loss of anterior horn cells in the spinal cord (summarized in (Spada, 1999 (Updated 2017))). The mutated polyQ-containing protein in SBMA may be implicated in both loss- and gain-of-function mechanisms. It has been suggested that a loss-of-function gives rise to the endocrine dysfunction, while gain-of-function leads to the neurological symptoms (Adachi et al., 2007, Palazzolo et al., 2008).

For all the polyQ diseases, the formation of protein aggregates containing the mutated proteins support the idea that the polyQ does indeed lead to a protein gain-of-function mediated toxicity. In addition, studies of the polyQ-tract in isolation reveal that it may be more toxic than the full-length protein (reviewed in (Matilla-Duenas et al., 2014). Even though it is still being debated whether protein aggregates are favorable or toxic themselves, it seems evident that increasing the amount of free polyQ-proteins in cells has a negative effect (Chafekar et al., 2012, Arrasate et al., 2004).

Despite the evidence for toxic effects caused by the polyQ-proteins, we cannot exclude potential toxicity mediated by a loss-of-function for the many proteins containing polyQ-tracts (see table 1). This is especially important to keep in mind given that physiological functions of these proteins are not fully elucidated. In addition, the discovery of Repeat-Associated non-AUG (RAN) translation (described in detail later) opens up the possibility for yet another pathogenic function of the repeats.

Other repeats involving protein-gain-of function

Oculopharyngeal myotonic dystrophy (OPMD) is characterized by problems with swallowing and ptosis (drooping of upper eyelid). It is caused by a GCN repeat expansion in the coding part of the polyadenylate-binding protein 1 (PABN1) gene (previously PABP2)(Brais et al., 1998). The expansion is pathogenic when exceeding 11 repeats. The GCN codes for alanine.

Expression of PABPN1 with an expanded polyalanine tract leads to formation of insoluble protein aggregates in muscle tissue. This is thought to trigger the pathogenesis in OPMD (Fan and Rouleau, 2003). Interestingly, the GCN triplet is mitotically and meiotically stable, and is therefore not a dynamic mutation (Brais et al., 1998).

Huntington disease-like 2 (HDL2) involves cognitive, emotional and movement abnormalities that present in the middle of life and then progress (Margolis, 1993). It is caused by a CTG/CAG expansion over 41 repeats in the Junctophilin-3 (JPH3) gene. It has been suggested that proteins from both sense (CTG-direction) and antisense (CAG-direction) transcripts spanning the repeat region are involved in the pathogenesis. However, both the expanded CUG mRNA and the loss of the normal JPH3 may also contribute (reviewed in (Margolis and Rudnicki, 2016)).

The protein quality control

For all cases of protein gain-of-function, an important issue is how the cell handles these toxic proteins. In eukaryotic cells there are several protein quality control (PQC) mechanisms aimed at recognizing and handling misfolded or aggregated proteins (Enam et al., 2018, Ciechanover and Kwon, 2017). The two main system for degradation of proteins are the ubiquitin-proteasome system (UPS) and the autophagic system (Ciechanover and Kwon, 2017). In general, damaged or misfolded proteins are targeted for degradation by the UPS (Ciechanover and Kwon, 2017, Enam et al., 2018). In fact, the UPS degrade over 80% of intracellular proteins (Wang and Maldonado, 2006). However, the proteasome is a narrow, barrel-shaped structure which cannot degrade proteins unless they are unfolded. Aggregated proteins can block the opening of the proteasome, thereby avoid degradation and even reduce the activity of the UPS (Andre and Tabrizi, 2012, Ciechanover and Kwon, 2017). Autophagy, on the other hand, can direct aggregated proteins, and larger structures, for degradation in the lysosomes. The role of the autophagic machinery is degradation of cytosolic components (Johansen and Lamark, 2011, Klionsky and Schulman, 2014)

As previously mentioned, most polyQ diseases are characterized by formation of intranuclear protein aggregates. That is also the case for other microsatellite expansion disorders, like FXTAS (described later). Since no lysosomes are present in the nucleus, the PQC here depends on the proteasome, which cannot degrade aggregated proteins. The cell can handle this problem by gathering misfolded/aggregated proteins into inclusion bodies (Enam et al., 2018). However, the increased burden on the nuclear PQC in these disorders, can lead to failure of this system, and progression of disease (Enam et al., 2018).

Neurons are especially sensitive to the presence of toxic/misfolded protein species, as they cannot dilute the toxic components through cell division (Ciechanover and Kwon, 2017). In addition, neurons generally display reduced activity of the UPS and increasing levels of aggregated protein with age (Keller et al., 2000, Ciechanover and Kwon, 2017). In disorders leading to expression of toxic protein species, or inhibition of the cell's PQC, it is therefore not surprising that penetrance is often affected by age.

Protein loss-of-function

TNR expansions in non-coding parts of the gene can result in protein-loss-of-function. Examples of this are the Fragile X Syndrome, Fragile XE syndrome and Friedreich Ataxia. In Fragile X Syndrome there is a CGG repeat expansion in the 5' UTR of the Fragile X Mental Retardation 1 (*FMR1*) gene on the X-chromosome (Fu et al., 1991). Expansion of CGG repeat number to >200 results in hypermethylation of the promoter region, silencing of the gene and loss of the *FMR1* protein (FMRP) (Verkerk et al., 1991, Bagni et al., 2012). Lack of the important RNA-binding FMRP protein, is the direct cause of the neurodevelopmental disorder FXS. For more detailed information about the *FMR1* gene and Fragile X Syndrome, see chapter 1.1.3 and 1.1.5.

Fragile XE Syndrome (FRAXE) is caused by expansion of a CCG-repeat in the FMR2 gene, and as seen in FXS, an expansion to > 200 repeats shuts down the gene by hypermethylation, and results in loss of protein. The FMR2 protein is thought to act as a transcriptional activator, and its loss of function results in cognitive disability (Knight et al., 1994).

Friedreich ataxia (FRDA) is an inherited ataxia caused by a GAA repeat expansion in the first intron of the FRDA gene which encodes the protein frataxin (Campuzano et al., 1996). Here there is no hypermethylation of the promotor region, but reduced protein levels due to the GAA tract inhibiting transcriptional elongation. Loss of frataxin causes accumulation of iron in mitochondria, increased production of reactive oxygen species (ROS) and sensitivity to mitochondrial stress. The clinical manifestation of FRDA is mainly ataxia, but can include muscle weakness, vision-/ and or hearing impairment, diabetes and heart disorders (Pandolfo, 2009).

RNA gain-of-function

RNA gain-of-function is a proposed mechanism for several TNR expansion disorders with the repeat tract located in the non-coding part of the gene (see Fig. 1 and Table 1). Perhaps the best

described RNA gain-of-function pathogenesis is that found in myotonic dystrophy type 1 (DM1). DM1 is clinically characterized by muscle degeneration and muscle hyperexcitability (myotonia), in addition to cataracts, endocrine disorders and cardiac conduction defects (reviewed in (Meola and Cardani, 2015)). In DM1 there is a CTG/CAG expansion in the 3'UTR of the gene encoding the kinase DMPK (Brook et al., 1992). A milder phenotype can be found in individuals with a CCTG/CAGG-expansion in intron 1 of ZNF9/CNBP, and this disorder is called myotonic dystrophy type 2 (DM2)(Ranum et al., 1998). In both cases, studies have demonstrated that RNA containing the expansions is retained in the nucleus. Furthermore, this retained, expanded RNA, sequesters RNA-binding proteins and thus prevent these proteins from performing their normal function in the cell. Other RNA-binding proteins are upregulated in DM1 (reviewed in (Meola and Cardani, 2015)). Among the RNA-binding proteins affected are muscleblind-like proteins (MBNLs), heterogenous nuclear ribonucleoprotein (hnRNP) and CUG-binding protein (CUGBP) 1. Importantly, mouse models where the MBNL proteins are knocked-out demonstrate several of the same features found in DM (Kanadia et al., 2003), and overexpression of MBNL1 rescues the phenotype (Kanadia et al., 2006). It thus appears that the toxic-gain-of-function mediated through the expanded CTG/CAG or CCTG/GAGG tracts, is crucial to the pathogenesis of the myotonic dystrophies (Fig. 2).

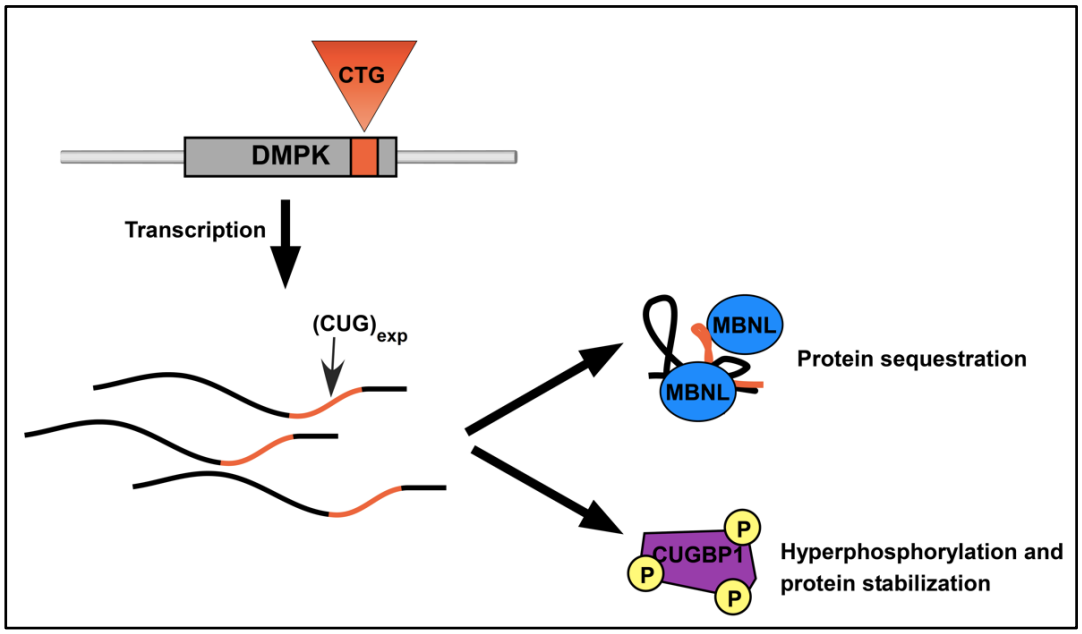

Figure 2. RNA gain-of-function in DM1. The CTG repeat tract is in the 3' untranslated region (UTR) of the DMPK gene. Expanded CUG repeat tracts in the DMPK mRNA are believed to cause the development of myotonic dystrophy type 1 (DM1) through several mechanisms. The CUG_{exp} mRNA sequesters the muscleblind-like (MBNL) proteins, leading to dysregulation of alternative splicing. The CUG binding protein 1 (CUGBP1) also interacts with the CUG_{exp} mRNA but does not co-localize with RNA foci in DM1. CUGBP1 is hyperphosphorylated and stabilized in tissues from DM1 patients. This may also contribute to the dysregulation of alternative splicing found in DM1.

Bidirectional transcription

Bidirectional transcription is not a pathogenic mechanism per se, but its occurrence is important because it increases the number of both transcripts and proteins potentially involved in the pathogenesis of TNR expansion disorders (Table 1). Both strands of the chromosome can serve as template for transcripts. Generally, RNA that encodes a protein is called the "sense" transcript, while RNA transcribed from the opposite DNA strand in the same area, is called the "antisense" transcript.

Bidirectional transcription from coding genes is wide-spread and appears to happen in most genes with a TNR expansion (Batra et al., 2010). It does not depend on the position of the TNR in the gene, and it is not known whether or how bidirectional transcription and TNR expansions influence each other (reviewed in (Budworth and McMurray, 2013)). An interesting example of bidirectional transcription across TNRs, is SCA8. In this case, the CTG/CAG-expansion gives rise to sense and antisense transcripts that both appear to be involved in the pathogenesis of the disorder. While the sense transcript leads to formation of a polyQ-protein, the antisense transcript is found in nuclear foci in patients (Moseley et al., 2006, Daughters et al., 2009). The *FMR1*-gene, with the CGG/CCG-expansion causing Fragile X syndrome and Fragile X-associated tremor/ataxia syndrome, is also subject to bidirectional transcription. Interestingly, the antisense transcript spanning this repeat tract, is up- and down-regulated in the same manner as the sense transcript (Ladd et al., 2007).

Repeat Associated non-AUG translation

Repeat Associated non-AUG (RAN) translation was first described by Laura Ranum and colleagues in 2011 (Zu et al., 2011). They studied the ATXN8-gene, involved in SCA8. In SCA8 there is a CTG/CAG expansion which is spanned by two genes, in opposite directions. In the CAG-orientation the gene is ATXN8, and the ATXN8-transcript contains the CAGs. In the CTG-orientation, i.e. opposite direction on opposite strand, the gene is called ATXN8 opposite strand (ATXN8OS). The transcript from ATXN8, but not from ATXN8OS, has a known ORF giving rise to a polyQ-protein found in intranuclear inclusions in SCA8-patients. However, the ATXN8OS-transcript is also found in nuclear foci in patients (Daughters et al., 2009).

Ranum and colleagues were studying the ATXN8-transcript when they found that deleting the only AUG-codon upstream of expanded CAG-repeats on this transcript, did not stop protein production (Zu et al., 2011). Instead, translation initiated in several reading frames, resulting in proteins with glutamine, alanine or serine tracts (Zu et al., 2011). Moreover, the mRNA-secondary structure attributed to the expanded CTG/CAG-repeats in this gene (ATXN8), was necessary for this non-AUG translation to take place. Reducing repeat number or GC-content in this region stopped the translation (Zu et al., 2011). The RAN-translation product SCA8-poly-Alanine, was found in cerebellar neurons in post-mortem patient material. In the same paper, they also show that RAN-translation from DMPK antisense mRNA with expanded CAG-repeats (involved in DM1), gives rise to a polyglutamine protein (Zu et al., 2011). RAN-translation has since been found to occur from both sense and antisense transcripts in FXTAS (CGG/CCG repeats), C9ORF72 Amyotrophic lateral sclerosis (ALS)/frontotemperal dementia (FTD) (G_4C_2 repeats) and HD (CAG/CTG repeats), in addition to SCA8 (CAG/CTG repeats) and DM1 (CTG/CAG repeats) (reviewed in (Cleary and Ranum, 2014)) (Table 1).

***Table 1 Overview of different mechanisms involved in repeat expansion disorders** **

Disorder	Gene	Microsatellite expansion	Main proposed pathogenic mechanism(s)	Bidirectional transcription	RAN-translation
HD (Cleary and Ranum, 2017, Orr and Zoghbi, 2007)	HTT	CAG/ CTG	PGOF – Huntingtin w/polyQ	Yes(Batra et al., 2010)	Yes, HD-polyAla, -polySer, -polyLeu and -polyCys
HDL2 (Margolis and Rudnicki, 2016)	JPH3	CTG/ CAG	PLOF – JPH3 RNA GOF – $(CUG)_n$ PGOF - uncertain	Yes(Batra et al., 2010)	Possible, not described in patients
SCA1 (Paulson et al., 2017)	ATXN1	CAG/ CTG	PGOF- polyQ-proteins PLOF and RNA GOF proposed for some (Paulson et al., 2017)	Yes(Batra et al., 2010)	Not described
SCA2 (Paulson et al., 2017)	ATXN2	CAG/ CTG			Possible, not described in patients
SCA3 (Paulson et al., 2017)	ATXN3	CAG/ CTG			Possible, not described in patients
SCA6 (Paulson et al., 2017)	CACNA1A	CAG/ CTG			Not described
SCA7 (Paulson et al., 2017)	ATXN7	CAG/ CTG			Not described
SCA8 (Zu et al., 2011)	ATXN8OS ATXN8	CTG/ CAG	PGOF - polyQ-protein RNA GOF - ATXN8OS $(CUG)_n$	Yes(Batra et al., 2010)	Yes, SCA8-polyAla
SCA12 (Cohen and Margolis, 2016)	PPP2R2B	CAG/ CTG	PGOF – but not due to polyQ	Yes(Batra et al., 2010)	Not described

SCA17 (Toyoshima and Takahashi, 2018)	TBP	CAG/ CTG	PGOF and/or PLOF - TBP	Yes	Not described
SBMA(Adachi et al., 2007, Palazzolo et al., 2008)	AR	CAG/ CTG	PGOF and PLOF - polyQ-protein	Yes(Batra et al., 2010)	Not described
DRPLA(Veneziano and Frontali, 1999 (Updated 2016 Jun 9))	ATN1	CAG/ CTG	PGOF - polyQ-protein	Yes(Batra et al., 2010)	Not described
OPMD	PABPN1	GCN/ NGC	PGOF – PolyA protein	Yes(Batra et al., 2010)	Not described
FXS (Penagarikano et al., 2007)	FMR1	CGG/ CCG	PLOF - FMRP	Yes(Batra et al., 2010)	Not described
FXTAS (Boivin et al., 2017)	FMR1	CGG/ CCG	RNA GOF – $(CGG)_n$ PGOF - RAN-translated protein	Yes (Batra et al., 2010)	Yes, FMRpolyGly, FMRpolyAla, FMRpolyPro
FRAXE (Knight et al., 1994)	FMR2	CCG/ CGG	PLOF	Not described	Not described
FRDA (Pandolfo and Pastore, 2009)	FXN	GAA/ TTC	PLOF - Frataxin protein	Yes(Batra et al., 2010)	Not described
DM1 (Meola and Cardani, 2015)	DMPK	CTG/ CAG	RNA GOF – $(CUG)_n$ (PGOF – RAN-translated protein)	Yes(Batra et al., 2010)	Yes, DM1-polyGlu
DM2 (Meola and Cardani, 2015)	CNBP	CCTG/ CAGG	RNA GOF	Not described	Not described
ALS/FTD (Cleary and Ranum, 2017)	C9ORF72	G_4C_2/ G_2C_4	RNA GOF PGOF – RAN-translated proteins	Yes	Yes, C9-polyGlyPro, -polyGlyArg, -polyGlyAla, -polyAlaPro, -polyProArg, -polyGlyPro

* Examples of repeat expansion disorders caused by different pathogenic mechanisms. The discovery of both bidirectional transcription and repeat-associated non-AUG (RAN) translation complicates the picture. Several disorders previously thought to be caused by either pure RNA gain-of-function (RNA GOF), protein gain-of-function (PGOF) or protein loss-of-function (PLOF) are now hypothesized to be caused by several mechanisms. The table shows in which cases bidirectional transcription and RAN translation have been found to occur. Note that this does not indicate that these mechanisms have been proven to be part of the pathogenesis. The table includes the repeat expansion disorders mentioned in this chapter (1.1.2.)

1.1.3 The *FMR1* gene

The Fragile X Mental Retardation (FMR) 1 gene is situated at the end of the long arm on the X-chromosome (Xq27.3). In the 5'UTR of this gene there is a CGG repeat tract which normally

contains around 30 CGGs (Snow et al., 1993) (Fig. 3). However, the CGG repeat tract is unstable and can expand to become a "premutation" (55-200 CGGs) or a "full mutation" (> 200 CGGs) (Oostra and Willemsen, 2009). The full mutation is the cause of the neurodevelopmental disorder Fragile X syndrome (FXS).

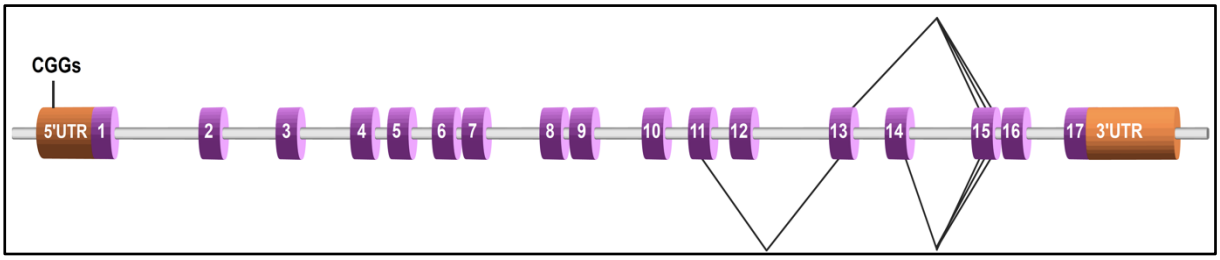

Figure 3. Schematic of *FMR1* gene structure and pattern for alternative splicing. The *FMR1* gene contains 17 exons. In the 5' untranslated region (UTR) is the CGG repeat tract. Due to alternative splicing of the exons near the 3'UTR, at least 12 mRNA isoforms are made. Exons are represented by purple structures, introns by the thick gray line, and alternative splicing by thinner angled lines.

The discovery of the *FMR1* gene started in the 1940s with Martin and Bells description of individuals with what appeared to be an X-linked disorder with intellectual disability (Martin and Bell, 1943). This disorder is now called the Fragile X Syndrome. More than two decades later, a "constriction" at the end of the long arm of the X chromosomes (later termed a "fragile" site) was found in several of the affected individuals (Lubs, 1969). Extensive studies of the fragile site lead to a breakthrough in 1991. A gene containing a CGG repeat region in the 5'UTR, was found to be transcribed from the chromosome region which is expanded in affected individuals (Verkerk et al., 1991). This was the *FMR1* gene. The same year it was also published that the number of CGG repeats in the FMR1 5'UTR was unstable, and this could explain the observed reduced penetrance and how seemingly unaffected individuals could pass on the genetic cause of the phenotype (Fu et al., 1991). Furthermore, it was demonstrated that affected individuals had a hypermethylation of the CpG island upstream of the CGG repeats, and that the size of this region was unstable (Oberle et al., 1991, Bell et al., 1991). Importantly, Pieretti et al. (Pieretti et al., 1991) showed that this hypermethylation resulted in loss of transcription from the *FMR1* gene. Together, findings in the early 90s revealed that: 1) There is an unstable CGG region in the 5'UTR of the *FMR1* gene on the X-chromosome, 2) large expansions of this CGG region results in hypermethylation of CCGs and a CpG island upstream, 3) this hypermethylation leads to silencing of the *FMR1* gene, and 4) silencing of the *FMR1* gene and lack of the FMR protein is the cause of the Fragile X syndrome (summarized in (Penagarikano et al., 2007)).

The Fragile X Mental Retardation 1 protein (FMRP)

The human *FMR1* gene was, as mentioned, described due to its's role in the neurodevelopmental disorder Fragile X Syndrome (discussed in more detail in chapter 1.1.5). The gene consists of 17 exons and covers 38 kb at the Xq27.3 site (Eichler et al., 1993). Homologs are found in all vertebrates. In most of these species, two genes with similar functions, namely FXR1 and FXR2, are co-expressed (Zhang et al., 1995).

The *FMR1* transcript is alternatively spliced, giving rise to several isoforms of both mRNA and *FMR1* protein (FMRP) (Huang et al., 1996, Sittler et al., 1996). While the full-length isoform 1 has been most extensively studied, it is isoform 7 which, at least at the mRNA level, is the most common one (Brackett et al., 2013). The human FMRP is a 71 kDa protein with several RNA-binding motifs including both an RGG-box (region with high content of arginine-glycine-glycine) and three K homology (KH) domains (Fig. 4). These are all characterized as RNA-binding domains, both in FMRP and other proteins. A point mutation in the KH2 domain leads to a severe form of FXS (De Boulle et al., 1993), indicating that this domain is vital to FMRP's normal function. FMRP also harbors two Agenet domains (also referred to as a double-tudor domain) involved in interactions with other proteins. They are also suggested to cause FMRP's interaction with methylated histones and a possible role in DNA damage response (Adams-Cioaba et al., 2010, Alpatov et al., 2014). Finally, FMRP has nuclear localization and nuclear export signals (NLS and NES) (Eberhart et al., 1996), but only about 4% of FMRP is found in the nucleus (Feng et al., 1997). Expression of FMRP is especially high in the brain and testes (Devys et al., 1993, Tamanini et al., 1997).

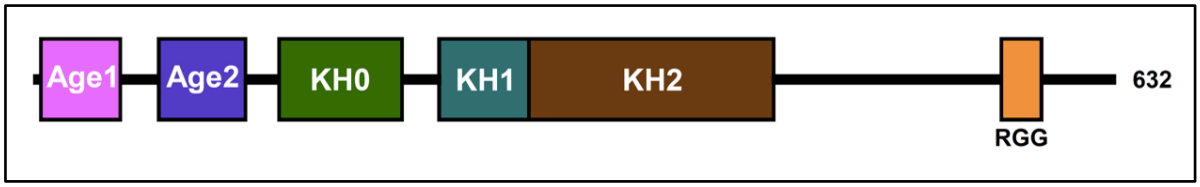

Figure 4: Schematic of the FMR1 protein. FMRP contains two Agenet (Age) domains, three K homology (KH) domains and a region with a high content of arginine-glycine-glycine (an RGG box). The FMRP also contains a Nuclear Localization Signal (NLS) and a Nuclear Export Signal (NES), that are not shown in the schematic above.

FMRP appears to have several functions, but most prominent is the effect on transport, stabilization and translation of mRNAs at the synapse (reviewed in (Bassell and Warren, 2008)).

Through its binding to polyribosomes, FMRP generally inhibits local translation of several mRNAs at the neuronal synapse. When the correct neuronal stimuli is received, FMRP releases the mRNAs and a wave of synaptic translation occurs (reviewed in (Willemsen et al., 2004)). The loss of FMRP results in abnormal translation at the synapse. This aberrant protein translation dysregulates synaptic plasticity (alterations in synaptic strength due to patterns of activity) which is proposed to be vital for memory and learning (Kiebler and DesGroseillers, 2000, Steward, 2002). The metabotropic glutamate receptor (mGluR) pathway plays a key part in this process. Signaling through mGluR both increases the amount of FMRP (Weiler et al., 1997) and is believed to mediate FMRPs release of mRNAs and subsequent protein translation. This led to the mGluR theory of FXS. The theory suggests that under normal conditions activation of mGluR causes synthesis of FMRP. FMRP, in turn, acts as a brake on protein synthesis mediated by mGluR activation. In FXS, where FMRP is absent, this brake is removed. The consequence is abnormally upregulated protein synthesis affecting several important functions of synaptic signaling (Bear et al., 2004, Huber et al., 2002), including enhanced long-term depression (LTD)(Dolen and Bear, 2008). Importantly, the mGluR theory suggests that down-regulation of mGluR signaling could ameliorate FXS. Numerous studies have therefore focused on targeting the mGluR to improve symptoms in FXS. However, while many compounds have shown promising results in animal models, only a few drugs have made it through to clinical trials, and no effective targeted treatment exists (Castagnola et al., 2017).

In the decades after the discovery of the *FMR1* gene, it became clear that mutations in this gene did not only cause a lack of FMRP and development of FXS but were also responsible for other phenotypes. The different phenotypes are all associated with expansions of the CGG repeat region.

1.1.4 Instability of the *FMR1* CGG repeat tract

The CGG repeat tract in the 5'UTR of the *FMR1* gene is meiotically unstable when the number of repeats is above the normal range. The expanded allele can go through both expansions and contractions, but contractions are less frequently seen for the middle and higher range of so-called "premutation" (PM) alleles (55-200 CGGs) and appear more often when the CGG repeat tract is intermediate (45-54 CGGs) or in the lower premutation range (Nolin et al., 2003). Expansions from a PM allele to the full mutation (FM) allele (> 200 CGGs) occur during

maternal transmission. In contrast, males do not have FM alleles in their sperm, even if they carry the FM themselves (Reyniers et al., 1993).

The likelihood of expansion correlates with the repeat number. However, several other factors are also important. One or two AGG interruptions in the CGG repeat tract are usually present in normal alleles, and these AGG interruptions reduce the risk for expansion (Yrigollen et al., 2012, Nolin et al., 2015). It is not necessarily only the number of AGGs that matters, but how long the uninterrupted CGG repeat tract is (Nolin et al., 2015). In addition, families with known cases of FXS have a higher risk for expansions during transmission, given the same number of both CGGs and AGG interruptions (Nolin et al., 2011). This latter finding indicates that other genetic elements affect the expansion risk.

The timing of the instability is still being debated. One model predicts that the expansions/contractions occur prezygotically, i.e. during meiosis when maternal/paternal gametes are formed. This would mean that both expansion to FM and contraction to PM can be found in gametes at the embryonic stage. In support of this theory, ovaries from female FM fetuses and testes from a 13-week old male FM fetus contain the FM. At 17-weeks, male FM fetuses have germ cells expressing FMRP, indicating the presence of some cells without a FM (Malter et al., 1997). The findings suggest that the FM may already be present in the maternal oocyte, and that contraction from FM to PM in male sperm occurs in the immature testes (Malter et al., 1997). However, oocytes from female fetuses carrying a PM have not been analyzed, and it is therefore not known if expansions of PM alleles have already occurred in the immature oocytes. A model for postzygotic expansion in the early stages of embryogenesis cannot be ruled out (Moutou et al., 1997). If expansions occur during embryonic development, they are likely to take place in early stages since FM expansions are present in isolated embryonic stem cells (Eiges et al., 2007, Urbach et al., 2010, Avitzour et al., 2014, Gerhardt et al., 2014).

Somatic instability occurs in several TNR expansion disorders. For the *FMR1* CGG expansion, somatic instability has been hypothesized due to the finding of different repeat sizes in samples from patients with the full mutation. This can be observed as a smear on southern blots of the CGG tract in the *FMR1* gene (Rousseau et al., 1991). In some cases, FM individuals also have PM alleles in some cells (mosaicism)(Nolin et al., 1994, de Graaff et al., 1995). Interestingly, a study of the repeat lengths in two monozygotic twins, carried out at two different time points

11 years apart, did not reveal any instability (Devys et al., 1992). In contrast to this, fibroblast from a female fragile X embryo displayed clear instability when cultured over several passages (Sun and Han, 2004) while adult male fragile X fibroblast lines appear to be stable (Sun and Han, 2004). Interestingly, a more recent study describes somatic instability of the *FMR1* premutation in both a mouse model and humans (Lokanga et al., 2013)

The molecular basis for the instability of the *FMR1* CGG repeats is still being investigated. Several hypotheses exist. While some are founded on the idea that expansion occurs during normal replication, others focus on the possibility that abnormal DNA repair, as a response to the secondary structures formed by repeats, can trigger instability (Mirkin, 2007, Usdin et al., 2014).

In the first category, one of the first proposed mechanisms is slippage of the replication fork during replication. This alone cannot account for neither contractions nor the larger expansions from PM to FM (reviewed in (Brouwer et al., 2009)). A model proposing fork stalling and restarting of the replication fork, however, can explain both large expansions and contractions. This is an extension of the slippage theory, but takes into account the strong hairpin structure formed by the CGGs (Handa et al., 2003), and proposes that different origins of replication (ORI) are used (Mirkin and Smirnova, 2002). Briefly, this model (also called the ORI switch model) suggests that strong secondary structures (like the CGG repeat hairpin) slows or stops the DNA polymerase. If replication starts at an upstream ORI the CGG repeat hairpin will be on the lagging strand template. A stop and restart of the replication fork here can cause skipping of one Okazaki fragment and consequently a repeat contraction. If replication starts from a downstream ORI, the CGG repeat hairpin structure will be in the Okazaki fragment, and this could lead to extra repeats being added (see Fig. 5) (reviewed in (Brouwer et al., 2009, Mirkin, 2006, Usdin et al., 2014, Usdin et al., 2015)).

Other models suggest that the expansion event takes place because of repair of the secondary structures formed by expanded repeats during transcription, and that DNA damage outside the context of genomic replication can be the basis for the instability (reviewed in (Mirkin, 2007, Usdin et al., 2014, Pearson et al., 2005)) (Fig. 5). In support of this notion, somatic instability is observed in terminally differentiated neurons expressing both CAG repeats involved in HD (Gonitel et al., 2008), and the CGG repeats in the *FMR1* gene (Lokanga et al., 2013). Thus, some replication-independent mechanism(s) are also likely to be involved in repeat instability. Several DNA repair pathways have been suggested to play a part here. The MutS homologue 2

(MSH2) is involved in CGG repeat expansions in a mouse model (Lokanga et al., 2014). MSH2 is best known for its involvement in mismatch repair (MMR), but is also a player in other DNA repair pathways, like base excision repair (BER). In mice with an expanded CGG repeat tract, loss of MSH2 actually prevents expansions during transmission from parent to offspring (Lokanga et al., 2014). The Cokayne syndrome B (CSB) protein, necessary for transcription coupled repair (TCR), is implicated in somatic expansions in *FMR1* premutation mice (Zhao and Usdin, 2014). In addition to its role in somatic instability in non-dividing cells, DNA repair may play a role in replication-coupled instability in several TNR expansion disorders (reviewed in (Usdin et al., 2015).

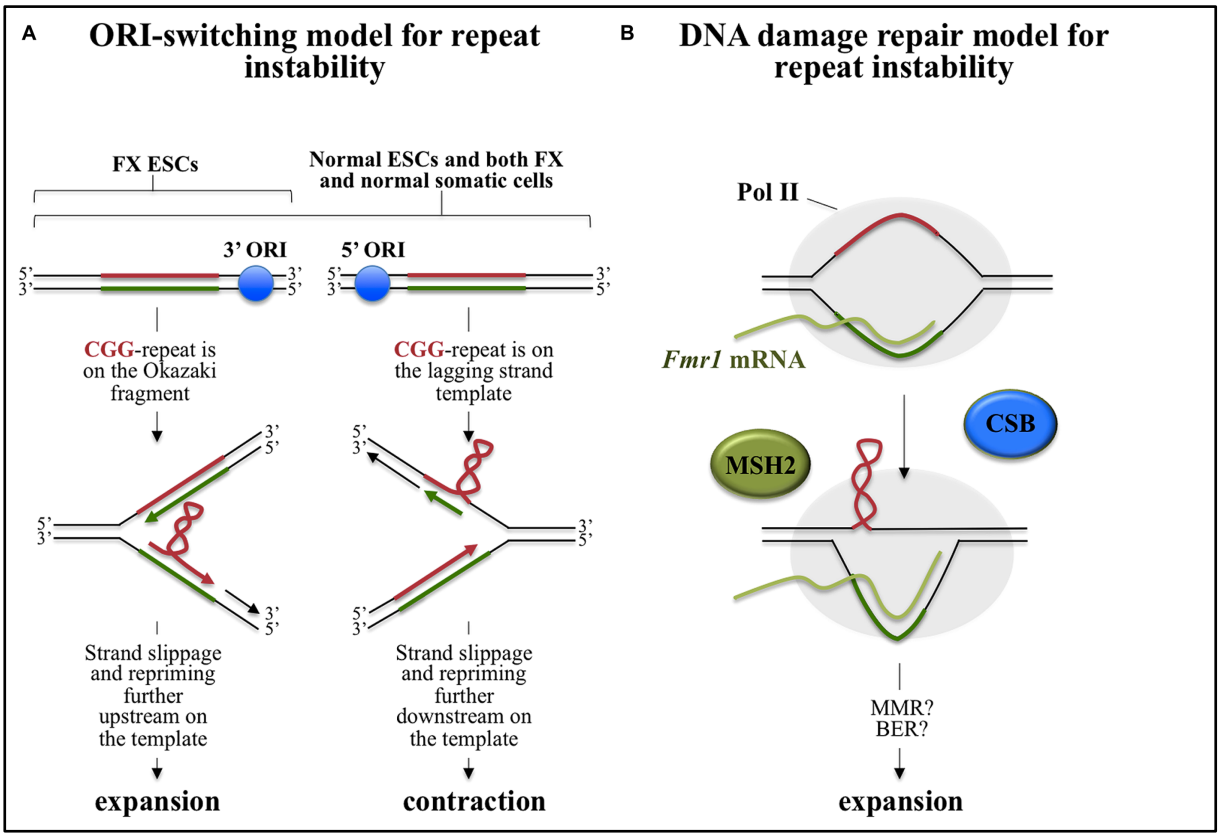

Figure 5. Models for instability of the CGG repeat region in *FMR1*. A) Replication from a downstream ORI can result in expansions while replication from an upstream ORI can lead to contraction of the CGG repeat region. B) Aberrant DNA damage repair may also contribute to expansion of the CGG repeat region. The non-template strand can form secondary structures processed through an MSH2-dependent pathway leading to extension. ESC = embryonic stem cell. CSB = Cockayne syndrome protein B. MSH2 = mutS protein homolog 2. MMR = Mismatch repair. BER = Base excision repair. Reprinted from (Usdin et al., 2014) with permission from Karen Usdin (karenu@niddk.nih.gov) [CC BY-SA 3.0 (https://creativecommons.org/licenses/by/3.0/)].

In conclusion, evidence exists for involvement of both replication fork mechanisms and DNA repair pathways in causing the instability of the CGG repeat tract (Fig 5). It is not unlikely that both a version of the "ORI switch" model, and aberrant DNA repair together leads to the complex picture of CGG repeat contractions and expansions. Several good reviews discuss this topic in more detail (Usdin et al., 2014, Mirkin, 2006, Zhao and Usdin, 2015, Brouwer et al., 2009)

1.1.5 Disorders of *FMR1* CGG repeat expansion

The CGG expansion tract in the *FMR1* gene on the X-chromosome is the cause of several phenotypes. It is now widely recognized that the *FMR1* CGG repeat can be dived into four allelic classes (reviewed in (Garber et al., 2008)). Unaffected individuals in the general population have 5-44 CGG repeats, usually 29 or 30 (Snow et al., 1993). Repeat numbers ranging from 45 to 54 are termed "grey zone" or intermediate alleles. These are generally not associated with a specific phenotype, but they can be meiotically unstable and can expand through the generations. Premutation (PM) alleles contain 55 – 200 CGG repeats. PM carriers are at risk of developing FXTAS and Fragile X-associated Primary Ovarian Insufficiency (FXPOI). In addition, PM alleles are meiotically unstable and can expand to larger PM alleles or to a full mutation (Snow et al., 1993, Fu et al., 1991). The fourth class of alleles is the full mutation (FM) with over 200 CGGs in the *FMR1* 5'UTR. Males with the FM usually display the FXS phenotype. Full mutation females are less likely to display the entire spectrum of FXS features and usually have a milder phenotype (de Vries et al., 1996, Sobesky et al., 1996). Interestingly, while FXS, FXTAS and FXPOI are all due to the same expansion on the same gene, FXS is caused by loss of transcription whereas FXTAS and FXPOI are caused by increased transcription of the *FMR1* gene (Fig. 6). The prevalence of FM alleles is around 1 per 7000 males and 1 per 11 000 females (Hunter et al., 2014), while PM alleles have a frequency of around 1 per 850 males and 1 per 290 females (Hunter et al., 2014).

Not all phenotypes that can be related to the *FMR1* CGG expansion will be described here. The focus will be on the three most commonly recognized disorders/medical conditions: FXS, FXPOI and FXTAS.

Fragile X syndrome

FXS is the most common hereditary cause of intellectual disability. The fragile X phenotype is almost always caused by inactivation of the *FMR1* gene due to a CGG expansion above 200 repeats. However, other mutations deleting FMRP or disrupting its function, can also result in a FXS phenotype (De Boulle et al., 1993, Wells, 2009, Gronskov et al., 2011, Myrick et al., 2014).

The FXS phenotype typically involves moderate intellectual disability with language delay and IQ scores < 55 (Raspa et al., 2017). There is, however, substantial variation in the cognitive phenotype with some FXS individuals in the borderline normal range, and others displaying severe intellectual disability. Although the reason for this variation is not fully understood, one contributing factor appears to be mosaicism of the *FMR1* mutation. Mosaicism can be divided into two forms: 1) Size mosaicism with regards to the CGG expansion where some cells have the PM instead of the FM and, 2) Methylation mosaicism where some cells with the full mutation don't have complete methylation and silencing of the *FMR1* gene (de Vries et al., 1998). In both cases, the presence of cells without a fully methylated *FMR1* promotor region, results in some FMRP production and thus a milder phenotype (de Vries et al., 1998, Hagerman et al., 1994). The IQ levels correlate with amount of FMRP expressed (Tassone et al., 1999). In addition, it is likely that both genetic background and adequate stimulus with early intervention in areas of language development, general learning and social interactions, affect the cognitive phenotype.

Individuals with FXS also share several common behavioral features. These include hyperactivity, gaze avoidance, hypersensitivity to stimuli, impulsivity, emotional lability, tantrums and repetitive movements such as hand flapping (Hagerman, 2002, Baumgardner et al., 1995, Cornish et al., 2008, Grefer et al., 2016). As many as 30% of individuals with FXS fulfill the criteria for an autism diagnosis (Harris et al., 2008, Rogers et al., 2001, Hatton et al., 2006, Hagerman et al., 2010). The behavioral phenotype is often what causes clinicians to suspect FXS and continue to diagnostic testing. Importantly, also the behavioral phenotype tends to be milder in female FM individuals.

The physical phenotype of FXTAS is mostly found in affected males. The classic appearance includes large protruding ears, a long and narrow face, tall forehead, strabismus and a prominent jaw (Hagerman et al., 1984). Examination typically reveals enlarged testes (marcroorchidism) in adult/adolescent males, hyperextensibility of finger and wrist joints, flat feet and valgus

malalignment of knees and elbows (Hagerman et al., 1984, Lachiewicz and Dawson, 1994). Several of these findings can be explained by connective tissue dysplasia and low muscle tone found in FXS individuals (Opitz et al., 1984).

Females who harbor the *FMR1* FM are protected by their unaffected X chromosome. It is not surprising that the degree of clinical involvement in these females depends on the activation ratio (Abrams et al., 1994, Riddle et al., 1998, de Vries et al., 1996), i.e. the ratio of cells where the unaffected X-chromosome is the active one vs the total number of cells.

Fragile X-associated Primary Ovarian Insufficiency (FXPOI)

Primary ovarian insufficiency (POI) is defined by the presence of cycle irregularities for at least four months, and two independent recordings of elevated follicle-stimulating hormone (FSH), in a woman under the age of 40 (Welt, 2008). POI is often used synonymously with menopause before the age of 40. FXPOI is the term used for POI in an individual with an *FMR1* PM. As many as 20% of female PM carriers develop FXPOI (Sullivan et al., 2011) and therefore display reduced fertility and early estrogen deficiency. This means that *FMR1* PM carriers have a 20-fold increased risk of POI compared with the general population. While far from all women with the PM develop FXPOI, some experience menopause as early as in their twenties (reviewed in (De Caro et al., 2008)).

FXPOI not only leads to early menopause. Diminished ovarian reserve, and therefore reduced fertility, is usually present more than ten years before POI (Sherman et al., 2016). In addition to reduced fertility, the early estrogen deficiency in FXPOI leads to increased risk of impaired endothelial function, coronary heart disease and cardiovascular mortality (Atsma et al., 2006, Mondul et al., 2005), low bone density and earlier osteoporosis and bone fractures (Kalantaridou et al., 2004, Gallagher, 2007).

The reason for the incomplete penetrance of FXPOI among PM carriers is not completely understood. In addition to the genetic background from other genes than *FMR1*, the CGG-repeat number within the PM range impacts the phenotype. Interestingly, it is not the women with the longest PMs who have the highest risk of FXPOI, but those who have around 80-100 CGG repeats (Ennis et al., 2006, Mailick et al., 2014, Sullivan et al., 2005). Skewed X activation does not appear to impact the risk (Sullivan et al., 2005, Bione et al., 2006, Tejada et al., 2008, Spath et al., 2010).

At the molecular level it is the expanded *FMR1* CGG mRNA that is thought to cause FXPOI. This is in line with the mRNA gain-of-function suggested for FXTAS (Hagerman et al., 2001), the other disorder caused by *FMR1* CGG expansions in the premutation range. The main reasons why the expanded CGG mRNA is believed to cause FXPOI, include the elevated *FMR1* mRNA levels in premutation carriers (Tassone et al., 2000), toxicity upon expression of PM length CGG-repeats (Jin et al., 2003, Handa et al., 2005, Hashem et al., 2009), and the observation that FXPOI is only seen in PM and not in FM females (Sullivan et al., 2011).

The expanded *FMR1* CGG mRNA, and its expanded antisense transcript, is hypothesized to cause toxicity through two distinct non-exclusive mechanisms: 1) The expanded CGG mRNA forms a hairpin structure which sequesters proteins and thereby prevents them from performing their tasks in the cell. 2) The expanded CGG mRNA acts as a template for RAN-translation, resulting in production of a polyglygine protein (FMRpolyG) that is toxic to human cells (reviewed in (Sherman et al., 2014) for the specific FXPOI context).

The two main mouse models for FXPOI can help gauge the contribution of each of the two mechanisms. One mouse model causes formation of both expanded *FMR1* mRNA and the FMRpolyG protein (Peier and Nelson, 2002) while the other only expresses the expanded *FMR1* mRNA (Hoffman et al., 2012). Both models demonstrate features of ovarian dysfunction, indicating that expressing the CGG mRNA, without producing FMRpolyG, can contribute to development of the FXPOI phenotype.

Fragile X-associated tremor/ataxia syndrome

FXTAS is the other disorder which can affect *FMR1* PM carriers specifically. FXTAS is a late-onset neurodegenerative disorder. It affects around 40% of men and 8-16% of women over the age of 50, who have a *FMR1* PM (Jacquemont et al., 2004, Rodriguez-Revenga et al., 2009, Coffey et al., 2008).

Clinical and epidemiological aspects of FXTAS, as well as the molecular basis for the pathogenesis, are described in the following section (chapter 1.2)

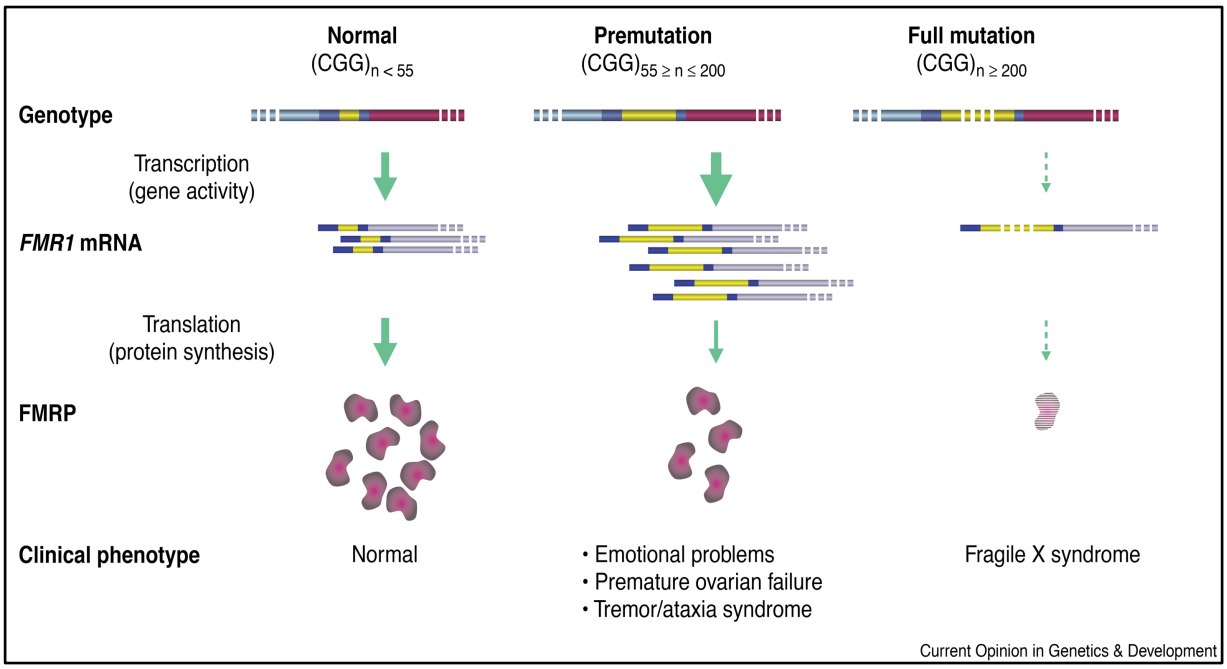

Figure 6. The consequences of different CGG repeat sizes. Overview of correlation between CGG repeat number and phenotype. The figure also demonstrates how protein levels are slightly reduced in premutation carriers and almost absent in the full mutation. mRNA levels, on the contrary, are increased only in premutation carriers. Reprinted from (Hagerman and Hagerman, 2002), with permission from Elsevier.

1.2 Fragile X-associated Tremor/Ataxia syndrome (FXTAS)

1.2.1 The discovery of FXTAS and its phenotype

The discovery of FXTAS

Fragile X-associated tremor/ataxia syndrome was first described in 2001 (Hagerman et al., 2001). The discovery of FXTAS started with Dr. Randi Hagerman's conversations with mothers of children with FXS. Dr. Hagerman worked as a behavioral and developmental pediatrician, treating children with FXS. She took note of the information given to her by the mothers of her patients. These women talked about neurological symptoms in their fathers, i.e. the grandfathers of children with FXS. Dr. Hagerman decided to examine these men, and contacted neurologist Dr. Maureen Leehey. This cooperation resulted in the description of intention tremor, parkinsonism and brain atrophy in male carriers of the *FMR1* premutation (Hagerman et al., 2001). The phenotype was named Fragile X-associated tremor/ataxia syndrome (FXTAS).

The clinical features of FXTAS have since been more extensively studied, both in men and women.

The FXTAS phenotype

The main clinical features of FXTAS include action tremor and cerebellar gait ataxia (Hagerman et al., 2001, Leehey et al., 2008). These motor symptoms are often accompanied by cognitive dysfunction, particularly executive dysfunction, parkinsonism and peripheral neuropathy (Grigsby et al., 2008, Juncos et al., 2011, Apartis et al., 2012, Niu et al., 2014). Autonomic dysfunction has also been reported in several studies (Juncos et al., 2011, Jacquemont et al., 2003, Hamlin et al., 2012). The onset of symptoms is often in the early sixties.

The fist manifestation of FXTAS is usually the presence of action tremor (Juncos et al., 2011, Leehey et al., 2007, Apartis et al., 2012). The typical tremor in FXTAS is quite symmetrical, is present when maintaining posture and during non-goal directed movements and increases during targeted movements. Women with FXTAS are not as often or severely affected by tremor as men are (Apartis et al., 2012). As the disease progresses, the amplitude of the action tremor increases and creates difficulties in performing daily tasks such as writing and eating. Cerebellar gait ataxia has a mean age of onset of 64 years (Tassone et al., 2007a). It involves increasing problems with tandem gait, general gait instability and a high risk of falling. Notably, the patient is not always aware of his/her tremor and/or ataxia (Juncos et al., 2011). Parkinsonism in FXTAS usually involves bradykinesia while rest tremor is less common (Juncos et al., 2011, Apartis et al., 2012, Niu et al., 2014).

Cognitive and neuropsychiatric symptoms of FXTAS can have a big impact on quality of life, not only of affected individuals, but also family members. Impaired executive function is the primary cognitive deficiency seen in FXTAS (Brega et al., 2008, Grigsby et al., 2007). In addition, as many as 50% of male FXTAS patients over 55 years of age, fulfill the criteria for dementia (Robertson et al., 2016). The lifetime prevalence of severe depression and anxiety disorders are 44% and 55%, respectively (Bourgeois et al., 2011).

1.2.2 Diagnostic criteria for FXTAS

In addition to the clinical features of FXTAS, both histopathological and neuroimaging findings play key parts in determining the diagnosis.

The histopathological hallmark of FXTAS is the presence of intranuclear inclusions in both neurons and astrocytes throughout the brain (Greco et al., 2002, Greco et al., 2006). These inclusions contain the expanded *FMR1* CGG repeat mRNA, but not the *FMR1* protein (Tassone et al., 2004, Iwahashi et al., 2006). Furthermore, even though several RNA-binding proteins, Lamin A/C and ubiquitin are found in the inclusions, no single protein species make up more than 7% of the protein mass (Iwahashi et al., 2006). Interestingly, the FXTAS-inclusions are not only found in brain, but also in the autonomic nervous system and in somatic tissues (Gokden et al., 2009, Greco et al., 2007, Hunsaker et al., 2011). For diagnostic purposes, it is so far only the inclusions in the CNS that are included in the diagnostic criteria for FXTAS (see Table 2). Histopathological analysis of CNS tissue is of course only performed post-mortem and can thus not aid in determining the diagnosis in a clinical setting. However, given the difficulties with diagnostics of FXTAS, the potential implications for other family members, and the scientific need for more information about these inclusions, histopathological analysis of CNS tissue can provide valuable information.

Neuroimaging findings in FXTAS patients was studied by Brunberg et al. in 2002 (Brunberg et al., 2002). On MRI, they found decreased T1 and increased T2 signaling intensity symmetrically in the two middle cerebellar peduncles (MCPs), indicating loss of fat and increased amount of water in these areas of the brain. The bilateral increased T2 signal intensity in the MCPs is now known as the "MCP-sign" and is considered to be the perhaps most distinct radiological sign of FXTAS. However, in the diagnostic setting it is important to know that the MCP sign is not pathognomonic for FXTAS (Storey and Billimoria, 2005). It is also seen in other disorders such as multiple system atrophy (Massey et al., 2012), and only around 60% of men and 13% of females with FXTAS have a positive MCP sign on MRI (Adams et al., 2007). Increased T2 weighted signal intensity is also often found in the splenium at the posterior end of corpus callosum (Renaud et al., 2015), and is now included in the diagnostic criteria for FXTAS (Hall et al., 2014). In addition, FXTAS patients have higher frequencies of abnormal cortical white matter signal (Brunberg et al., 2002, Cohen et al., 2006) and generalized cerebral atrophy (Brunberg et al., 2002, Greco et al., 2006).

The current diagnostic criteria are updated from the original ones presented in 2003 (Jacquemont et al., 2003), and are the result of the 1st international meeting on *FMR1* premutation carrier issues (Hall et al., 2014). The criteria are displayed in Table 2.

Table 2: Diagnostic criteria for FXTAS*.

Definite fragile X-associated tremor/ataxia syndrome		
One clinical and one radiological major criterion, or one clinical major criterion and intranuclear inclusions + premutation		
Probable fragile X-associated tremor/ataxia syndrome		
Two clinical major criteria, or one clinical minor criterion and one radiological major criterion + premutation		
Possible fragile X-associated tremor/ataxia syndrome		
One clinical major criterion and one radiological minor criterion + premutation		
	Major criteria	**Minor criteria**
Clinical signs	Intention tremor and gait ataxia	Parkinsonism, moderately to severely impaired short-term memory, loss of executive functions, or neuropathy
Radiology (brain MRI)	White matter lesions in the middle cerebellar peduncles or posterior regions of the corpus callosum	White matter lesions in the cerebrum. Moderate to severe generalized atrophy
Neuropathology	Intranuclear inclusions in neurons and astrocytes in the central nervous system	

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1.2.3 Epidemiology of FXTAS

The prevalence of FXTAS has not been examined directly in the total population. Instead, it is estimated based on the prevalence of the PM in the total population and the penetrance of FXTAS in PM carriers.

Several studies have examined the prevalence of the *FMR1* PM in a wide range of countries. In 2014 Hunter et al. (Hunter et al., 2014) published a systematic review and meta-analysis of the prevalence of both the FM and the PM in the general population. They included 22 observations of the PM allele in the total population (i.e. a random screening population with no selection bias) and additional 10 of PM allele frequency in the normal (i.e. excluding those with intellectual disability) female population (Hunter et al., 2014). The analysis include testing for the PM in 45 000 men and 88 000 women in the total population, and additional 42 000 females in the normal population. The frequency of the PM allele was found to be 1 in 855 men and 1 in 291 women (Hunter et al., 2014).

Since FXTAS patients may initially be diagnosed with ataxia, tremor or parkinsonism (Hall et al., 2005), a range of movement disorder populations have been screened for the *FMR1* premutation. Among these, the ataxia populations demonstrate the highest prevalence of the PM (Macpherson et al., 2003, Milunsky and Maher, 2004, Brussino et al., 2005, Van Esch et al., 2005, Zuhlke et al., 2004, Rodriguez-Revenga et al., 2007, Rajkiewicz et al., 2008). However, the populations tested mostly included patients where the diagnosis of SCA had already been excluded and are therefore likely to have a higher prevalence of the *FMR1* PM. Screening of patients with Parkinson's disease (PD) have not provided sufficient evidence for increased prevalence of the PM in these populations (Annesi et al., 2004, Toft et al., 2005, Tan et al., 2005, Reis et al., 2008). Populations with essential tremor have been screened without revealing virtually any PM carriers (Garcia Arocena et al., 2004, Clark et al., 2015, Tan et al., 2004, Deng et al., 2004). For multiple system atrophy (MSA), the picture is similar to that found in PD populations, with less than 1% carrying the PM (Kamm et al., 2005, Garland et al., 2004, Yabe et al., 2004, Seixas et al., 2005). The low frequency of PM in these populations is a bit puzzling, given that many FXTAS patients are initially diagnosed with tremor, parkinsonism or ataxia (Hall et al., 2005). However, it is worth noting that inclusion criteria for several of the above mentioned studies, could exclude FXTAS patients as they seldom present with symptoms typical for pure essential tremor or Parkinson's disease (reviewed in (Hall et al., 2006)).

The penetrance of FXTAS in a PM population, has been examined by Jacquemont and colleagues (Jacquemont et al., 2004) who found that around one third of PM carrier men over the age of 50 had tremor and ataxia. In addition, the penetrance increases with age, and is around 50% after the age of 70. The different allele sizes within the PM range were not controlled for in this study. This could have a big impact since the penetrance of FXTAS is lower for smaller

PM alleles (Tassone et al., 2007a, Leehey et al., 2008) and half of PM alleles are in the lower range (55-60 CGGs) (Jacquemont et al., 2006). Pooling together the penetrance found in several studies, around 40% of male PM carriers and 8-16% of female premutation carriers over the age of fifty, develop FXTAS (Jacquemont et al., 2004, Tassone et al., 2007a, Coffey et al., 2008, Rodriguez-Revenga et al., 2009).

Estimating the prevalence of FXTAS based on the prevalence of the PM in the total population and the penetrance of FXTAS in PM carriers, results in the prediction that 1 in 2000 males over the age of 50 is affected by FXTAS (Hunter et al., 2014, Grigsby, 2016). However, taking into account the correlation between presence of neurological signs in patients and their CGG repeat number within the PM range (Jacquemont et al., 2006), the estimated prevalence drops to about 1 in 4000 males over the age of 50 (Hall and Mailick, 2016).

1.2.4 Treatment of FXTAS

No targeted treatment for FXTAS exists, and current management is based on alleviating symptoms. While several drugs have ameliorated FXTAS symptoms in case reports, none have displayed significant effects in clinical trials (Hall et al., 2016). This underscores the need for targeted treatment. The development of treatment specific for the pathogenic mechanisms in FXTAS, requires a good understanding of the molecular basis of the disorder. Promising strategies for therapeutic interventions include development of small molecules or antisense oligonucleotides (AOS) that target the CGG mRNA hairpin. Studies performed in vitro, in cultured cells, and in a Drosophila model for FXTAS, indicate that such compounds can inhibit several possible triggering mechanisms in the FXTAS pathogenesis (reviewed in (Kong et al., 2017)). However, the safety and efficacy of these potential drugs have yet to be evaluated in animal models, and they are far from reaching clinical trials.

1.2.5 Molecular understanding of FXTAS

The events leading to the development of FXTAS have been studied intensively. Patient material, animal models, various cell-based systems and in-vitro assays have all been used to shed light on the processes behind this neurodegenerative disorder. This chapter will focus on the triggering events in the FXTAS pathogenesis, at a molecular level.

Co-transcriptional mechanisms: R-loop formation and aberrant DNA damage response

What separates all FXTAS patients from both non-affected individuals with a normal *FMR1* allele, and full mutation carriers with FXS, is the occurrence of transcription from an *FMR1* gene with the CGG repeat expansion (Hagerman and Hagerman, 2016). During transcription from this gene, mRNA with the expanded CGG repeat is produced. Transcription through GC rich gene regions (often CpG islands) can result in the mRNA binding stronger to the template DNA than the corresponding DNA strand does, and thereby prevent the reformation of the double stranded DNA. This is more likely to take place when there is a GC skew, i.e. that one of the DNA strands has a higher G-content than the other. The result is a three-stranded structure called an R-loop (Fig. 8). Formation of R-loops occur because it is thermodynamically more favorable for the G-rich RNA to bind to the C-rich DNA strand, than for the two DNA strands to bind one another (Ginno et al., 2012). This occurs for several CpG islands in the genome and can actually protect them from methylation and thus prevent silencing of the gene (Ginno et al., 2012). However, excess formation of R-loops has been hypothesized to activate the DNA damage repair response and result in an accumulation of γH2AX (Fig. 8) (Paulsen et al., 2009, Sordet et al., 2010).

In the context of the *FMR1* gene, it has been demonstrated that premutation alleles cause an increase in the R-loop formation (Loomis et al., 2014). This led to the idea that R-loop formation might be involved in the development of FXTAS through activation of the DNA damage repair response machinery (Fig. 8). In concordance with this, the histone variant γH2AX, an important signaling molecule in early steps of the DNA repair response, is found in the FXTAS inclusions (Iwahashi et al., 2006).

RNA gain-of-function

The elevated mRNA levels in premutation carriers, the presence of this mRNA in patient inclusions, and the fact that individuals with a FM do not get FXTAS, led to an RNA gain-of-function hypothesis for the FXTAS pathogenesis (Hagerman and Hagerman, 2004). A similar model had already been proposed for DM1. The CTG repeat expansion in the 3'UTR of the DMPK leads to formation of expanded mRNA that sequesters MBNL proteins, thereby preventing them from regulating alternative splicing, localization and stability of mRNAs (Goodwin et al., 2015) (reviewed in (Meola and Cardani, 2015)). Importantly, for DM1, overexpression of one of the sequestered proteins, MBNL1, rescues the phenotype in mice expressing expanded CUG repeats (Kanadia et al., 2006). Several studies have therefore focused on which proteins could be sequestered by the expanded CGG mRNA found in FXTAS.

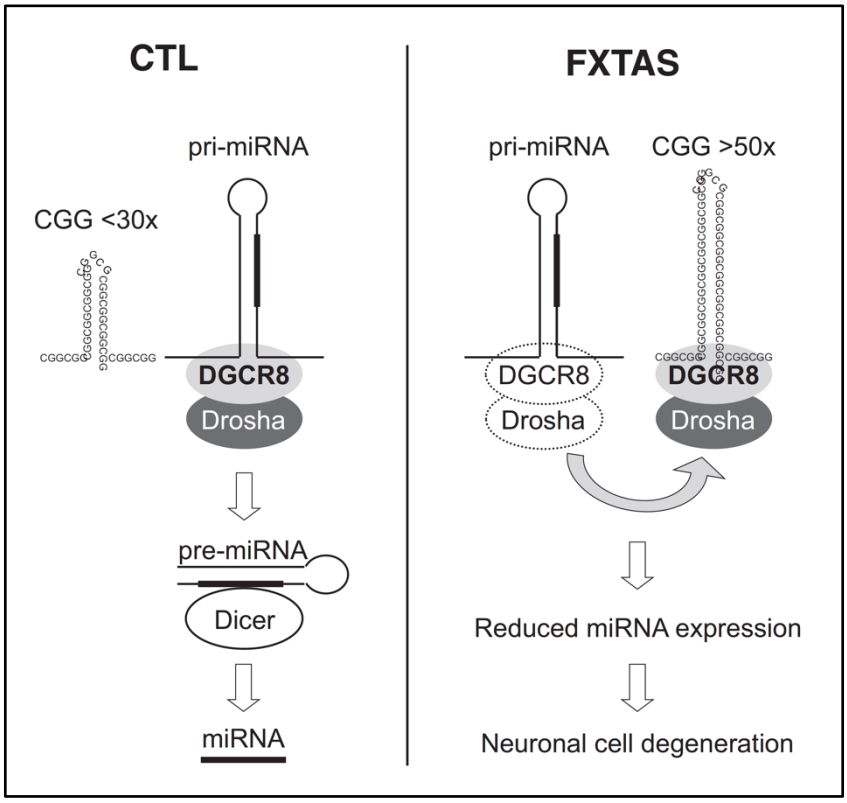

Figure 7: The CGG hairpin sequesters DGCR8. mRNA containing the CGG repeats form a hairpin structure. When the hairpin is formed by expanded CGG repeat tracts, it sequesters the RNA-binding protein Di George Syndrome Critical Region Gene 8 (DGCR8). DGCR8 usually binds to pri-microRNA in the nucleus and helps attach the RNase Drosha to these molecules. Drosha processes the pri-microRNA into pre-microRNA. The presence of an expanded CGG repeat hairpin which sequester these proteins, reduces the level DGCR8 and Drosha available to perform the processing of pri-microRNA into pre-microRNA. The result is reduced levels of mature micro-RNA. Reprinted from (Sellier et al., 2013) with permission from Elsevier. The material is under a Creative Commons license: [CC BY-NC-ND 3.0 (https://creativecommons.org/licenses/by-nc-nd/3.0/)].

Numerous RNA-binding proteins have been found to co-localize with the CGG repeat mRNA in model systems, and FXTAS patient material. Drosophila models have shown that both heterogenous nuclear ribonucleoprotein (hnRNP) A1/B1 and purine-rich binding protein α (purα) can be associated with the expanded CGG repeat mRNA, and that their overexpression rescues the phenotype (Sofola et al., 2007, Jin et al., 2007). While hnRNP A2/B1 has been found together with the inclusions in patient material, this is not the case for purα (Iwahashi et al., 2006, Sellier et al., 2010). In 2010 Sellier et al. (Sellier et al., 2010) reported that expanded CGG mRNA recruited SAM68, an RNA-binding protein involved in regulation of alternative splicing. Furthermore, several transcripts that are targets for SAM68 showed changes in alternative splicing when the expanded CGG mRNA was expressed (Sellier et al., 2010). Interestingly, the same group later found that Di George Syndrome Critical Region Gene 8 (DGCR8) binds to CGG repeat mRNA directly (Sellier et al., 2013). DGCR8 is part of a protein complex involved in processing of microRNA. Together with its partner,

Drosha, DGCR8 binds pri-microRNA (miRNA) and process them into pre-miRNAs (Lee et al., 2003, Denli et al., 2004, Gregory et al., 2004, Han et al., 2004). This is a necessary step to get mature miRNA. However, DGCR8 can also bind other double stranded RNAs, like RNA hairpins formed by the CGG repeats (Sellier et al., 2013) (Fig. 7). The expanded CGG repeats form a strong double stranded hairpin structure (Handa et al., 2003, Zumwalt et al., 2007, Kumar et al., 2011). Interestingly, DGCR8 binds stronger to CGG mRNA over 60 CGGs than mRNA with 40 or fewer repeats (Sellier et al., 2013). Furthermore, Drosha and DGCR8 are stuck at the CGG mRNA hairpin and have reduced activity when expanded CGG mRNA is present (Sellier et al., 2013). Importantly, brain samples from FXTAS patients demonstrated decreased levels of mature miRNAs, in line with the hypothesis that the expanded CGG mRNA sequesters DGCR8 and Drosha and thereby prevents maturation of miRNAs (Sellier et al., 2013). Overexpression of DGCR8 rescued the phenotype in cultured mouse neurons (Sellier et al., 2013).

Another important issue regarding the hypothesized RNA gain-of-function mechanism, has been the localization of the expanded CGG mRNA. Even though the *FMR1* mRNA is present in the intranuclear inclusions, the majority of the mRNA seems to be exported to the cytoplasm (Tassone et al., 2007b). This is in contrast to DM1 where the expanded mRNA is retained in the nucleus (Davis et al., 1997). Interestingly, expression of the CGGs without the surrounding native *FMR1*-sequence causes the RNA to be held back in the nucleus where they form RNA foci (Sellier et al., 2013, Sellier et al., 2010). CGG repeat tracts surrounded by the native *FMR1* 5'UTR form very few of these nuclear foci (Sellier et al., 2017). Since CGG repeat mRNA expressed from the native *FMR1* gene is mainly exported from the nucleus, it is available as a template for protein translation.

RNA gain-of-function toxicity has also been suggested to arise from the *FMR1* antisense transcript (ASFMR), containing a CCG repeat region. Just like *FMR1* mRNA, ASFMR is upregulated in premutation carriers and not present in individuals with a FM (Ladd et al., 2007). This antisense transcript has an open reading frame including this repeat and could lead to formation of a polyproline containing protein (Ladd et al., 2007, Khalil et al., 2008).

Repeat associated non-AUG translation

In 2013 Todd and colleagues discovered that the *FMR1* CGG-repeat mRNA causes repeat-associated non-AUG (RAN) translation, and formation of a polyglycine containing protein

named FMRpolyG (Todd et al., 2013). In short, *FMR1* mRNA with a CGG repeat expansion leads to translation initiation at a near-cognate start codon upstream of the CGG repeats. Translation initiation here also entails a frameshift which causes the CGGs to be read as GGCs. The result is formation of a protein where the CGGs (read as GGC) code for a polyglycine stretch flanked by a short N-teminus and a longer C-terminus (Todd et al., 2013). Furthermore, Todd and colleagues show that FMRpolyG induces toxicity when expressed in human cells and Drosophila. It causes formation of intranuclear inclusions and can be detected in brains of FXTAS patients (Todd et al., 2013). RAN translation across the CGG repeats may also result in production of two other proteins, FMRpoly-Alanine and FMRpoly-Proline (Todd et al., 2013). Whether they are endogenously expressed in patients, is not clear.

The FMRpolyG protein has since been detected, using immunohistochemistry (IHC) staining, in several animal models for FXTAS (Sellier et al., 2017, Hukema et al., 2015), together with FXTAS inclusions in patient material (Buijsen et al., 2014, Sellier et al., 2017), and in ovarian cells from a woman with FXPOI (Buijsen et al., 2016). Even though staining for the FMRpolyG protein in human brain samples seems to be specific for FXTAS patients, it is not known whether levels of FMRpolyG are elevated in premutation carriers compared to unaffected individuals with normal alleles.

Little is known about how the different parts of the FMRpolyG protein contribute to toxicity. Polyglycine itself forms insoluble aggregates in solution when consisting of more than nine consequetive glycines (Ohnishi et al., 2006), and the polyglycine stretch in FMRpolyG is needed for aggregate formation in cells (Sellier et al., 2017). However, it is the C-terminal part of FMRpolyG which appears to be most important for its toxic effects (Sellier et al., 2017).

The notion that the RAN translated FMRpolyG protein can be toxic and contribute to the pathogenesis of FXTAS, has prompted the investigation of how this unconventional translation is regulated. Increasing number of CGG repeats results in a more efficient RAN translation (Kearse et al., 2016). However, Sellier et al. (Sellier et al., 2017) see the GFP-tagged RAN translation product even when all CGGs are removed (Sellier et al., 2017). The RAN translation initiation upstream of the CGGs, appears to be dependent on the canonical translation initiation machinery, i.e. where a 5'G-cap recruits initiation factors and the preinitiation complex (Kearse et al., 2016). Another study demonstrates that the integrated stress response causes a relative up-regulation of RAN translation compared to conventional translation (Green et al., 2017). It is not clear which specific factors cause this increase in RAN-translation.

The FMR1 antisense transcripts, ASFMR, can also serve as a template for RAN translation (Krans et al., 2016). It remains to be investigated whether the resulting proteins can play a part in the development of FXTAS.

The molecular pathogenesis of FXTAS is not completely understood. Several non-exclusive mechanisms may contribute (Fig. 8). Further insight into the triggers of FXTAS development can help us identify the right targets for therapeutic interventions.

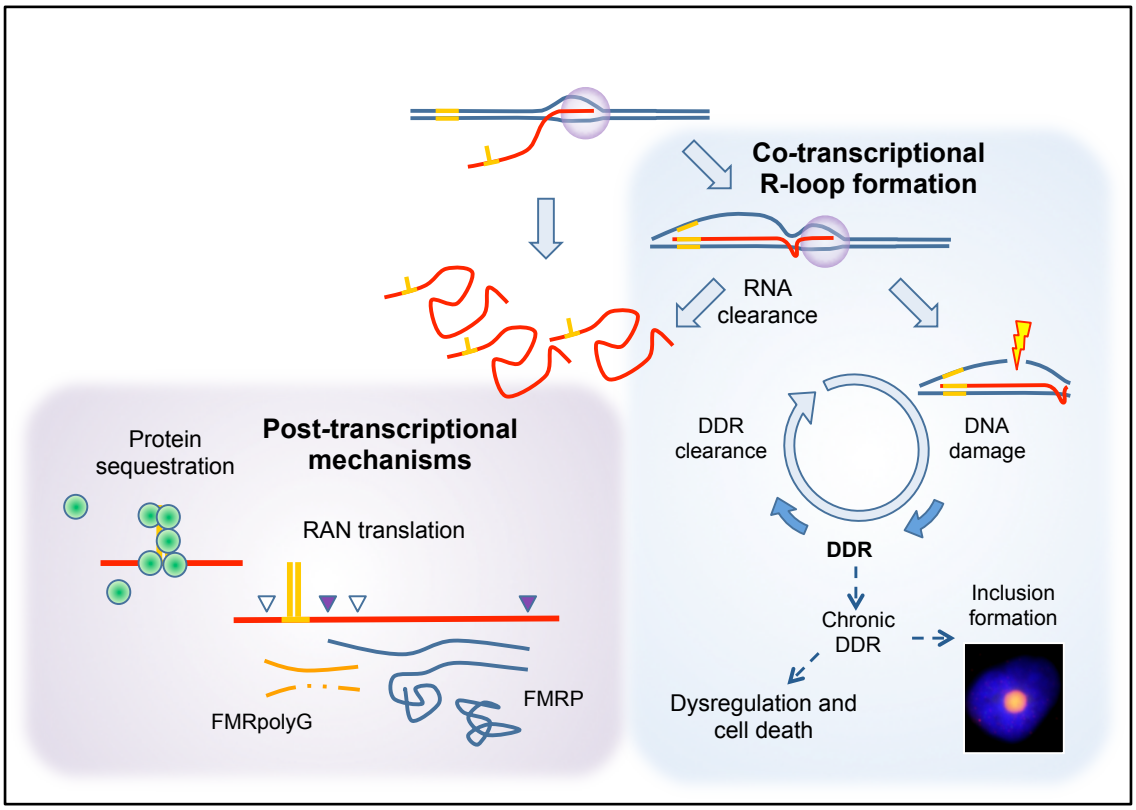

Figure 8. Models for the pathogenesis of FXTAS. The current models all depend on a transcriptionally active *FMR1* gene with a premutation (i.e. CGG repeat nr. 55-200). At the transcriptional level this can lead to formation of DNA:RNA hybrids (R-loops) that activates the DNA damage repair (DDR). If this is not resolved, it can lead to cellular dysregulation. The post-transcriptional mechanisms include sequestration of proteins, mediated by the expanded CGG repeat mRNA, and RAN translation. The RAN translation product FMRpolyG is translated from the 5'UTR, and this region spans the first part of the FMR protein (FMRP) coding region. All of the above mentioned mechanisms are non-exclusive and can contribute to the development of FXTAS. Reprinted with permission from (Hagerman and Hagerman, 2015) ©2015 New York Academy of Sciences.

2 Aims of study

This study focuses on the early events in the pathogenesis of FXTAS. The overall aim was to shed light on the cellular mechanisms triggering the development of this disorder.

Paper I

*Hypothesis***:** CGG-repeat-induced cellular toxicity will depend primarily on the length of the CGG repeat, with a secondary dependence on RNA concentration.

Specific Aim: Using a dox-inducible, stably-transfected episomal system, define the CGG-repeat- and RNA-concentration-dependencies of cellular dysregulation, using both morphological changes and reduced cell viability as readouts of such dysfunction.

Paper II

Hypothesis I: FMRpolyG expression will not lead to cellular toxicity in the complete absence of a CGG repeat mRNA hairpin.

Specific Aim I: Using transient and stable expression in human cell lines, study the relative contribution of the repeat-associated non-AUG-initiated (RAN) translated protein FMRpolyG, in the manifestation of pathogenic cellular phenotypes in FXTAS.

Hypothesis II: The FMRpolyG protein is a stable protein which is neither degraded by the autophagic machinery nor the ubiquitin-proteasome system

Specific Aim II: Using flow cytometry, study the rate of degradation of FMRpolyG in the presence and absence of inhibitors of autophagic/lysosomal and proteasomal degradation.

3 Summary of papers

Paper I

Hoem G, Raske CR, Garcia-Arocena D, Tassone F, Sanchez E, Ludwig AL, Iwahashi CK, Kumar M, Yang JE and Hagerman PJ. **CGG-repeat length threshold for *FMR1* RNA pathogenesis in a cellular model for FXTAS**.

Human molecular genetics 2011; 20: 2161-70.

In this study, we established a novel cellular model system for the neurodegenerative disorder FXTAS. With this system, we were able to separate the respective contributions of CGG repeat number and CGG mRNA levels on cellular dysregulation. We used a flow-based viability assay to show that cell death was dependent on a CGG repeat number above 62. Immunofluorescence staining revealed dysregulated lamin architecture and rare inclusions only in cells expressing 95 CGGs for 72 hrs. Expression of 30 CGGs did not result in cell death, inclusion formation or disruption of lamin rings, even when the amount of mRNA was 100-fold higher than endogenous levels. Furthermore, expression of 95 CGGs resulted in increased amount of the protein γH2AX, an important component of the DNA damage repair response. Isolated nuclei from FXTAS patients were stained for γH2AX, revealing its co-localization with FXTAS inclusions and a potential role in development of the disorder. Together, our findings show that both cell death and more subtle signs of cellular dysregulation depend primarily on CGG repeat number.

Paper II

Hoem G, Larsen KB, Øvervatn A, Lamark T, Sjøtten E and Johansen T. **The FMRpolyGlycine protein mediates aggregate formation and toxicity independent of the CGG mRNA hairpin in a cellular model for FXTAS**

Submitted Manuscript

In this study, we made a construct encoding the FMRpolyG protein without the CGG repeats. We used this to express FMRpolyG without the CGG mRNA hairpin, both in transient transfection experiments and in a stable cell line. A flow-based viability assay demonstrated that cell death was increased upon FMRpolyG expression, while co-expression of CGG mRNA did not have any additional effect. Immunofluorescence staining revealed that disruption of lamin architecture required FMRpolyG expression, but not the CGG mRNA hairpin. Both confocal microscopy of fixed cells and long-term imaging of live cells, demonstrated aggregate formation upon expression of FMRpolyG-GFP without the presence of a CGG mRNA hairpin.

FMRpolyG-GFP aggregates were further analyzed using both immunofluorescence staining and correlative light and electron microscopy (CLEM). Key proteins in the UPS co-localized with the aggregates. In addition, degradation of FMRpolyG-GFP was reduced upon inhibition of the UPS. Our findings demonstrate the FMRpolyG per se can cause aggregate formation and toxicity in human cells. Furthermore, it is a stable protein which is degraded primarily by the UPS.

4 Discussion

In both **paper I** and **paper II** we use overexpression of RNA and protein to study potential toxic effects on human cells. While FXTAS develops over decades, these systems show signs of dysregulation already after 24 hrs. This allows us to analyze several aspects of the CGG-repeat-mediated toxicity in cells over a relative short time period. Another important advantage to using cellular overexpression systems, is that it allows us to separate complex and intertwined processes occurring in FXTAS patients. We can then gauge how each of them may contribute to the pathogenesis. Whether it was the number of CGG repeats within the premutation range, or the amount of *FMR1* mRNA molecules, that was important for triggering FXTAS pathogenesis, could not be studied in patient material. In FXTAS patients, the elevated levels of the *FMR1* mRNA correlate with increasing CGG repeat number. The episomal system in paper I allowed for separate evaluations of effects mediated by increasing CGG repeat number and by increasing mRNA concentration. This enabled us to conclude that a CGG repeat expansion was the primary requirement for downstream adverse effects. In paper II we analyzed the effect of the FMRpolyG protein per se, versus that of FMRpolyG together with the CGG mRNA hairpin. Similar to the case in paper I, we here wanted to differentiate between the effects mediated by two factors, the FMRpolyG protein and the CGG mRNA hairpin, that are inseparable in patient material. The use of an FMRpolyG encoding sequence without CGGs, allowed us to investigate the specific contribution of FMRpolyG to cellular dysregulation.

However, overexpression studies have their limitations. Further studies using patient material are needed before we can evaluate if the observations in paper I or II reflect processes in individuals with FXTAS. Both the cell's general mechanisms and the properties of the overexpressed gene product itself may be affected by a several-fold increased expression from one single gene. A more specific issue in our studies is that the use of tags, such as GFP, increases the size of gene products and can change their characteristics. Importantly, it has been suggested that adding a GFP-tag, but not a smaller FLAG-tag, to FMRpolyG may prevent formation of intranuclear aggregates (Sellier et al., 2017). In our study, however, substituting the GFP-tag with a FLAG-tag, did not have any mayor effect on localization of FMRpolyG aggregates 24 and 48 hrs after transfection (Fig. 9). To control for possible adverse effects of overexpression per se, we compare cells expressing the gene of interest to cells expressing a reporter or control gene.

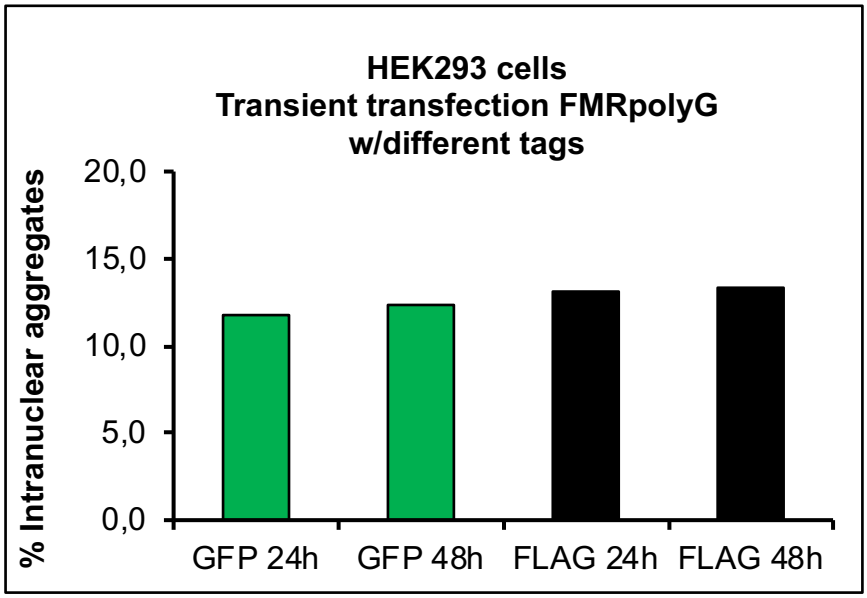

Figure 9: Localization of aggregates does not depend on the tag. Percentage of aggregates that were intranuclear 24 and 48 hrs after transfecting HEK293 cells with wtHP-99G-GFP or wtHP-99G-FLAG. A minimum of 50 aggregates were counted for each tag at each time point.

A CGG repeat threshold exists for aggregate formation and toxicity

Our main finding in **paper I** is a threshold for CGG repeat numbers below which no CGG-mediated cellular dysregulation is observed. It was already established that expression of high levels of expanded CGG mRNA was toxic to cells. However, we did not know whether it was the number of CGG-repeat-containing mRNA molecules (molarity) or the size of the CGG repeat region that determined this effect. For the TNR expansion disorder DM1, it is well-established that there is an mRNA gain-of-function mechanism where the CUG-repeat sequesters proteins. In DM1, the CUG mRNA elicits toxicity even when it is not expanded beyond the normal range, given high overexpression/ concentration of this mRNA (Mahadevan et al., 2006). Our observation that unexpanded CGG mRNA does not lead to toxic effects in human neural cells, even at concentrations around 100-fold higher than endogenous levels, demonstrates that CGG mRNA toxicity is dependent on a repeat expansion. Thus, our finding suggests that development of FXTAS does not have to occur through the same pathways as in DM1. The observed threshold, where over 62 CGG repeats are required to cause cellular dysregulation, implies that mechanisms other than pure protein sequestration may be triggering the pathogenesis. However, CGG mRNA with an expansion above the threshold could still contribute to the pathogenesis by binding and thereby remove proteins from other pathways and interaction partners. Indeed, a CGG-repeat-size-dependent sequestration of RNA-binding proteins was demonstrated by Sellier et al. (Sellier et al., 2010, Sellier et al., 2013) Nuclear CGG mRNA foci were not observed in cells expressing less than 40 CGGs, rarely in those

expressing 40 CGGs, but consistently when repeat number exceeded 60 (Sellier et al., 2010). These RNA foci sequestered RNA binding proteins, including Sam68 (Sellier et al., 2010). In addition, for the RNA binding protein DGCR8, the interaction with the CGG mRNA occurred primarily when CGG repeat numbers exceeded 60. This CGG-repeat-size-dependent sequestration resulted in dysregulated alternative splicing and microRNA processing (Sellier et al., 2010, Sellier et al., 2013). A threshold around or above 60 CGG repeats is consistent with these findings.

Establishing that there is a threshold for the number of CGG repeats required for cellular dysregulation and cell death, helps focusing the FXTAS-research on the expanded repeats. Future studies can therefore look primarily at proteins and pathways affected by the expanded CGG repeats instead of all possible interactions mediated by expression of CGG repeat tracts in the normal range.

Extended CGG repeats may induce the DNA damage repair response

In paper I, cells expressing 95 CGG repeats display an increase in γH2AX levels. This histone variant is an early responder to DNA damage, and aids in recruitment of DNA damage proteins to the site of damage (Mah et al., 2010). It indicates activation of the DNA damage repair response. γH2AX is also present in the inclusions found in FXTAS patients. In addition, previous studies demonstrated that both ataxia telangiectasia mutated (ATM) and – Rad 3-related (ATR), two other proteins involved in the DNA damage repair response, are activated in the presence of the *FMR1* premutation (Entezam and Usdin, 2009). This upregulated activity of the DNA damage repair response, could be due to increased oxidative stress and mitochondrial dysfunction associated with the premutation (Ross-Inta et al., 2010). However, the premutation alleles more recently described propensity to form R-loops during transcription (Loomis et al., 2014), can also explain the activation of the DNA damage repair response. R-loops are RNA:DNA hybrids that can be formed during transcription through GC rich regions, like the *FMR1* CGG repeat. Aberrant R-loop formation can activate the DNA damage repair response (Paulsen et al., 2009). Since expanded alleles increase R-loop formation, the R-loop theory is in line with the observed CGG repeat threshold.

Does FMRpolyG expression trigger FXTAS development?

After the publication of paper I, several studies provided new information regarding the molecular aspects of FXTAS. Perhaps the most influential finding was that of repeat-associated

non-AUG (RAN) translation across the CGG-repeats (Todd et al., 2013, Zu et al., 2011). RAN translation, as the name implies, is not dependent on a canonical start codon. Through this mechanism, a single mRNA can potentially give rise to peptides in all reading frames. For the *FMR1* mRNA, it has been proposed that the CGG mRNA hairpin stalls the ribosome (Todd et al., 2013, Kearse et al., 2016). Translation is then initiated from a near-cognate start codon (ACG) upstream of the repeats (Sellier et al., 2017). This results in a polyglycine-containing protein named FMRpolyG, present in FXTAS inclusions and found to be toxic when overexpressed in cell lines, and in a Drosophila model. (Todd et al., 2013).

The focus of paper II was to determine whether the FMRpolyG protein per se was toxic when expressed in human cells. Expression of the FMRpolyG protein together with the CGG mRNA hairpin had already been compared to expression of the CGG mRNA hairpin alone (Sellier et al., 2017). However, the complete FMRpolyG protein had not been expressed without the CGG mRNA. We designed a construct without any CGGs, but coding for the same amino acid sequence found in FMRpolyG. This made it possible for us to study the effect of FMRpolyG expression without the presence of a CGG mRNA hairpin structure.

In paper II, our main finding is that FMRpolyG per se, causes both aggregate formation and reduction of cell viability. Hence, the CGG mRNA hairpin is not required for either of these effects in cultured cells. Interestingly, similar observations have been made using synthetic polypeptides expressed to study their involvement in C9ORF72 ALS-FTD (Mizielinska et al., 2014, May et al., 2014, Yamakawa et al., 2015). Here they also use alternative codons to create a polypeptide without expressing the natively corresponding mRNA structure. Like FMRpolyG, these polypeptides are expressed through the unconventional RAN translation. It thus appears that expressing RAN translated proteins, without the corresponding repeat-containing mRNA forming secondary or tertiary structures, is toxic in several repeat expansion disorders. This further complicates a traditional picture where repeat mediated toxicity is caused either by RNA gain-of-function, protein gain-of-function or protein loss-of-function. First, the discovery of RAN translation implies that repeat expansions in non-coding parts of genes not only result in RNA gain-of-function or protein loss-of-function, but also the formation of novel protein species. Second, observations that these RAN translated proteins do not depend on corresponding repeat mRNA to exert toxic effects, underscore their potential roles as triggers for development of disease. Valuable information about complex disorders, including FXTAS,

may therefore be obtained by investigating the consequences of expressing these RAN translated proteins in isolation.

In paper I we also observe reduced cell viability and aggregated protein structures. In cells expressing 95 CGG repeats for 72 hours, we find rare intranuclear inclusions that are lamin A/C positive. Our findings are in line with previous description of intranuclear inclusions in neural cell lines transiently transfected with expanded CGG repeats (Arocena et al., 2005), and the presence of lamin A/C in the inclusions found in patient material (Iwahashi et al., 2006). Since RAN translation had not yet been described when the study was carried out, we did not stain for FMRpolyG. We therefore cannot exclude that cytoplasmic and/or intranuclear aggregates containing this protein are present in the cellular model system used in paper I.

The loss of CGG repeats has a negative effect on FMRpolyG expression

Interestingly, expressing the FMRpolyG from the native CGG-harboring sequence (wtHP-99Gly-GFP) results in higher levels of FMRpolyG-GFP, and more aggregates, than expressing FMRpolyG from the sequence with alternative glycine codons (mutHP-90Gly-GFP). In fact, the levels of FMRpolyG-GFP expressed from the mutated construct was so reduced compared to the wild type construct that it made it problematic for us to directly compare the effects of these two constructs on aggregate formation and toxicity. Our solution was to focus all comparisons on the GFP positive cells, but this was not optimal. Numerous studies support the idea that the CGGs themselves have a profound impact on transcription rates (Tassone et al., 2000, Chen et al., 2003, Entezam et al., 2007, Brouwer et al., 2008, Tassone et al., 2007b). Thus, our findings are in concordance with previous ones, but our data show an augmented impact of replacing the CGGs with alternative codons, compared to reducing or removing the CGG-repeat tract. This could potentially also be due to a negative impact on transcription mediated by the alternative glycine codons themselves. Removing the positive effect of the CGGs on transcription and adding another G-rich repetitive region, could be considered two separate changes that together cause the observed gap in levels of transcript. The purpose of this study, however, was not to investigate the levels of mRNA or protein, but to evaluate the possible toxic effect of the FMRpolyG-protein with and without the presence of the CGG-repeat hairpin. Nevertheless, our data in paper II supports the idea that the CGG repeats may regulate the efficiency of transcription, and thereby also production of FMRpolyG.

Is FMRpolyG responsible for the disruption of Lamin rings?

Disruption of lamin architecture is an important outcome measurement for cellular dysregulation in our studies. As previously mentioned, Lamin A/C is present in FXTAS inclusions from patients (Iwahashi et al., 2006), and intranuclear inclusions formed in neuronal cells transiently transfected with expanded CGG repeats (Arocena et al., 2005). The nuclear lamins are important to maintain both the nuclear envelope and the structure of the nucleus itself, and their disruption can activate DNA damage response and eventually lead to cell death (Lees-Miller, 2006). We observe abnormal lamin ring structure in cells expressing CGG repeats above the threshold (paper I), and in cells expressing FMRpolyG alone (paper II).

In FMRpolyG-GFP expressing cells, disrupted lamin rings are more frequently seen when aggregates are present. Co-expression of CGG mRNA did not increase the portion of abnormal lamin rings in our experiments. Interestingly, it has been proposed that FMRpolyG's C-terminus interacts with the nuclear lamina-associated polypeptide (LAP) 2β to mediate lamin ring disorganization (Sellier et al., 2017). However, we did not see consistent co-localization between FMRpolyG-GFP aggregates and LAP2β in our study (data not shown). Furthermore, both LAP2β and the lamins are abundant proteins that maintain nuclear integrity in human cells (Furukawa et al., 1995, Foisner and Gerace, 1993). This raises the question of how much FMRpolyG would have to be present in FXTAS patients for it to sequester the abundant LAP2β and affect the organization of lamin proteins. So far, the amount of FMRpolyG in FXTAS patients, other premuation carriers, and individual with unexpanded alleles, has not been determined.

Properties of the FMRpolyG protein

Sellier et al. (Sellier et al., 2017) showed that the C-terminal part of FMRpolyG was most important for the protein's toxic effect, while the polyglycine stretch encoded by the CGG repeats mediates its aggregation (Sellier et al., 2017). Interestingly, the C-terminus of FMRpolyG is identical whether it is expressed from a normal or a premutation allele. We demonstrate that FMRpolyG containing more than 90 glycines is a very stable protein. Only 30% of the protein was degraded 48hrs after its expression was turned off. Furthermore, Sellier et al. report that FMRpolyG, without a large tag, is only detectable when the number of glycines is around 60 or higher (Sellier et al., 2017). This indicates that not only the expression, but also the stability of the protein, may depend on a CGG repeat expansion in the premutation range. It is possible that the expanded polyglycine stretch in FMRpolyG, when expressed from

premuation alleles, causes its aggregation and thus increased stability of the protein. This scenario, where FMRpolyG aggregation depends on the premutation and leads to increased stability of the toxic protein, may explain how a toxic effect mediated by the FMRpolyG's C-terminus could occur exclusively in premutation carriers. However, it is not certain that aggregation of FMRpolyG increases cellular dysregulation. In polyQ disorders, it is still being debated whether aggregate formation is a toxic or protective mechanism (reviewed in (Shao and Diamond, 2007, Hoffner and Djian, 2015)). Free polyQ proteins are suggested to have a negative effect on cells (Chafekar et al., 2012, Arrasate et al., 2004). In light of this, it would be very interesting to study the FMRpolyG concentration inside FXTAS inclusion and compare this to the amount of "free" FMRpolyG in tissues from both patients and control individuals.

Except for the above mentioned publication looking at how the C-terminus and polyglycine stretch of FMRpolyG behave in isolation (Sellier et al., 2017), the properties of this protein were largely unexplored. In our study, we show that FMRpolyG-GFP is found both in the cytosol and nucleus of human cells. While the protein moves freely in these compartments, it becomes immobilized in the aggregates. Interestingly, a previous publication has shown that inhibition of the UPS increased neurodegeneration in a Drosophila model for FXTAS, and that this depended on expression of FMRpolyG (Oh et al., 2015). Furthermore, they observed an improvement of the phenotype by overexpressing HSP70, known to increase UPS clearance of proteins. We demonstrate that amount of FMRpolyG-GFP increases upon inhibition of the UPS, while inhibition of autophagic degradation does not significantly affect FMRpolyG-GFP levels. In addition, FMRpolyG-aggregates stained positive for both the 20S proteasome and ubiquitin. Together these findings indicate that FMRpolyG is degraded primarily through the UPS. At the same time, it appears to inhibit this protein degradation pathway, possibly through sequestration of the UPS' main components.

A parallel can be drawn to expression of polyGlycine-Alanine (poly-GA), a RAN translated protein involved in C9ORF72 ALS/FTD. Poly-GA forms aggregates that sequester proteasomes and inhibit their activity (Guo et al., 2018). Inhibition of the UPS can have deleterious effects. If these glycine-containing proteins (i.e. poly-GA and FMRpolyG) can be targeted for degradation by the UPS before they form insoluble aggregates, toxicity might be avoided, or at least reduced. Indeed, new technologies facilitating the binding of selected proteins to a ubiquitin-ligase, and thus subsequent degradation through the UPS, are currently being developed (reviewed in (Enam et al., 2018). However, such strategies will only work if the selected protein can be efficiently degraded by the proteasome. In order to ameliorate toxic

effects triggered by FMRpolyG expression, elucidating the exact mechanism for its degradation may be a crucial step.

The CGGs may impact the amount of FMRpolyG by affecting transcription, translation and degradation

Even though the native FMRpolyG was first found in cells expressing expanded CGG repeat tracts, it is not clear exactly which role the number of CGG repeats play in regulating levels of this protein. The amount is of course dependent on both production and degradation. If the polyG stretch is important for aggregation, a longer polyG stretch, and thus a higher number of CGGs, is likely to inhibit degradation. Whether the polyG stretch also increases the stability of the protein through other mechanisms than aggregation, remains to be investigated.

The production of FMRpolyG depends on both transcription and translation. Since premutation carriers have a 2-8 fold higher levels of *FMR1* transcript (Tassone et al., 2000), the CGGs also increase the amount of mRNA available for RAN translation. In paper II we observed that replacing all the CGGs with alternative codons led to a drastic reduction of mRNA levels. This strongly supports an important role for the CGGs in regulating transcription from the *FMR1* gene. Increased transcription rather than increased stability of the mRNA seems to be the explanation for elevated levels of transcript (Tassone et al., 2007b).

At the translational level, it has been hypothesized that the large mRNA hairpin formed by expanded CGG repeats, stalls the ribosome and thereby facilitates translation initiation at non-canonical start codons 5' to the repeats (Todd et al., 2013, Kearse et al., 2016). In the first study of RAN translation across the CGG repeats, Todd et al. observed that inserting a CGG repeat tract upstream of a GFP reporter allowed its expression without an ATG start codon (Todd et al., 2013). GFP constructs lacking both a canonical start codon and the CGG repeat tract did not produce any significant amount of the reporter (Todd et al., 2013). Further support for a role of the CGG repeat tract in facilitating RAN translation, comes from experiments with mRNA transfection in HeLa cells. Here, RAN translation is increased with increasing CGG repeat number (Kearse et al., 2016). Conversely, in vitro RAN translation in the same reading frame did not depend on CGG repeat length (Kearse et al., 2016). The impact of CGG repeat expansions may thus be dependent on the translation machinery in living cells. Another interesting observation is that the number of CGG repeats required for detection of FMRpolyG expressed in HeLa cells, depend on the size of the entire protein (Sellier et al., 2017). With a

large GFP tag attached, FMRpolyG can be detected with only 30 CGGs. Using a smaller FLAG tag, a minimum of 60 repeats are required (Sellier et al., 2017). Thus, increasing the protein size by adding other sequences than CGG repeats, may also upregulate FMRpolyG levels. However, it is the number of CGG repeats that separates FXTAS patients from non-affected individuals with normal allele sizes.

In conclusion, several studies support the notion that FMRpolyG levels are CGG-repeat-length-dependent in vivo, in accordance with the threshold theory. Furthermore, these studies support the hypothesis that expanded CGG repeat tracts increase the FMRpolyG amount by impacting its transcription, translation and degradation. To what extent FMRpolyG levels are elevated in premutation carriers, warrants further investigation.

Non-exclusive pathogenic mechanisms

The two papers in this thesis both focus on triggering mechanisms in the pathogenesis of FXTAS. The first paper was written before the discovery of RAN translation and therefore focuses on the potential toxic effects mediated by the CGG mRNA. In the second paper we take a closer look at the RAN translation product FMRpolyG. It could be argued that the two papers present evidence for opposing views on the mechanisms for FXTAS development. However, an RNA gain-of-function mechanism does not exclude a toxic effect mediated by RAN translation products, and vice versa. Moreover, the discovery of a CGG repeat threshold for toxicity, supports the idea that a CGG repeat expansion is required for production of FMRpolyG with a conformation, or at the concentration, required for toxic effects. Interestingly, the episomes contain most of the FMRpolyG-encoding sequence, including the non-canonical start-codon (ACG) upstream of the repeats. However, the last 14 amino acids are not part of the sequence due to a stop-codon in this reading frame. Consequently, if FMRpolyG mediates the cell death, induction of DNA damage repair response and lamin ring disruption observed in paper I, it does so without the last part of its C-terminus.

Whether the expanded CGG repeat tract causes toxicity through co-transcriptional mechanisms, protein sequestration by CGG mRNA, or a RAN translated protein, is still up for debate. They could all contribute to some extent. Our studies demonstrate that an expanded allele is required for cellular dysregulation, and that FMRpolyG expression from these alleles is toxic in human cells. Future studies on endogenous expression of FMRpolyG in patient and control material are needed to determine the exact contribution of this protein to disease development.

Perspectives for targeted treatment of FXTAS

Currently, no treatment reversing, stopping or slowing down the disease progression can be offered to FXTAS patients. However, recent discoveries give reason to believe that this might change. Antisense oligonucleotides (AOS) have been shown to reduce RAN-translated protein and RNA foci in a mouse model for ALS/FTD caused by a G_4C_2 repeat expansion in the C9ORF72 gene (Jiang et al., 2016). For the CGG repeats specifically, small molecules targeting the mRNA hairpin have been applied to inhibit both RAN translation (Su et al., 2014) and sequestration of RNA binding proteins in cells (Disney et al., 2012, Tran et al., 2014). In addition, a drug screen in a Drosophila model for FXTAS identified small molecules that improved the phenotype (Qurashi et al., 2012). However, the presence of a CGG repeat threshold underscores that any treatment targeting the CGG mRNA should be specific for the expanded repeats, to reduce the risk of adverse effects. One concern regarding direct targeting of the CGG mRNA is that it might affect translation from the downstream open reading frame, and thus reduce levels of FMRP. Since lack of FMRP is the cause of FXS, it is important to maintain normal translation from this open reading frame. While this already appears to be possible using small molecules (Su et al., 2014, Tran et al., 2014), it remains to determine whether these compounds have any other off-target effects. In addition, the observed beneficial effects have yet to be verified in mouse models of FXTAS.

The observation that FMRpolyG is toxic per se, suggests that it is possible to ameliorate FXTAS by targeting this protein instead of the *FMR1* mRNA. However, the amount of FMRpolyG seems to depend on the CGG repeat number and the presence of a CGG mRNA hairpin. If we can develop safe drugs from small molecules or AOS that bind the CGG mRNA hairpin to inhibit both RAN translation and repeat-size-dependent protein sequestration, there might not be a need for therapies targeting the FMRpolyG. However, as long as no disease-modifying treatment for FXTAS exists, all options are worth exploring. In this context, FMRpolyG certainly is an interesting target for therapeutic interventions.

5 Main Conclusions

Paper I

- The CGG repeat-induced cellular toxicity depend primarily on the length of the CGG repeat, with a secondary dependence on mRNA concentration
- There is a CGG repeat threshold between 62 and 95 CGGs, below which no sign of cellular dysregulation is observed

Paper II

- Expression of the FMRpolyG protein leads to aggregate formation and cellular toxicity, even in the absence of a CGG repeat hairpin
- The FMRpolyG protein is a stable protein which is degraded primarily by the ubiquitin-proteasome system

6 Methodological considerations

FXTAS is a late-onset neurodegenerative disorder. Due to both ethical and practical reasons, many FXTAS-studies cannot be performed in patients. In order to elucidate the triggering events in the pathogenesis of FXTAS within a reasonable time-frame, various model systems are used. All of the work in this thesis is based on experiments performed in human cell lines. This chapter will therefore focus on the cell-based models we have developed to answer our specific research questions. Finally, a brief consideration of the main outcome measurements in these systems is included.

Cloning of CGG repeat sequences

The CGG repeat sequence in the *FMR1* gene in humans, becomes more unstable as the number of CGGs increases (Fu et al., 1991). Instability is also seen when this repeat tract is inserted into plasmids that are propagated in *E. coli*. The copy number of the plasmid containing the CGGs, bacterial strain used for propagation, and orientation of the repeat sequence relative to the replication origin, all appear to affect the stability of the CGG repeat tract (Chen et al., 2003, Hirst and White, 1998). The instability means that all batches of purified plasmid must be checked using restriction digestion and/or sequencing. When the number of CGG repeats are in the premutation or full mutation range, sequencing can usually not be performed using conventional Sanger-sequencing, or next generation sequencing systems that don't make use of singe-molecule techniques (Loomis et al., 2013, Buermans and den Dunnen, 2014).

In the lab where the experiments included in paper II were performed, a common cloning strategy is to use the gateway system (Hartley et al., 2000). However, the gateway vectors for expression in mammalian cells that were available in the lab, were all high copy number plasmids. Placing the CGG repeat sequence in these vectors led to deletion of the repeats. The presence of GC-rich regions, like the CGG repeat tract, also causes difficulties with PCR based methods like site-directed mutagenesis. To avoid these problems, we kept the CGG repeat sequence in low/medium copy number plasmids and used cloning strategies solely based on the use of restriction enzymes, DNA polylinkers and DNA ligase. Neither the gateway system nor site-directed mutagenesis was used for cloning of plasmids with the CGGs. To further reduce the frequency of deletions, the plasmids were propagated in Stabl3 or SURE *E. coli* bacteria (strains with reduced ability to perform recombination), and growth of bacteria took place at room temperature. All batches of purified plasmids were analyzed using restriction enzyme

digestion and agarose gel electrophoresis. Despite the above mentioned measures to avoid deletions, they frequently occurred (Fig. 10). We were not able to sequence through the repeat region containing 99 CGGs. Therefore, only the flanking sequences could be checked by sequencing, and determining the repeat size was performed using restriction enzyme digestion as described. The expression vectors that retained the entire CGG repeat tract were used for both transient transfection experiments and establishment of stable cell lines.

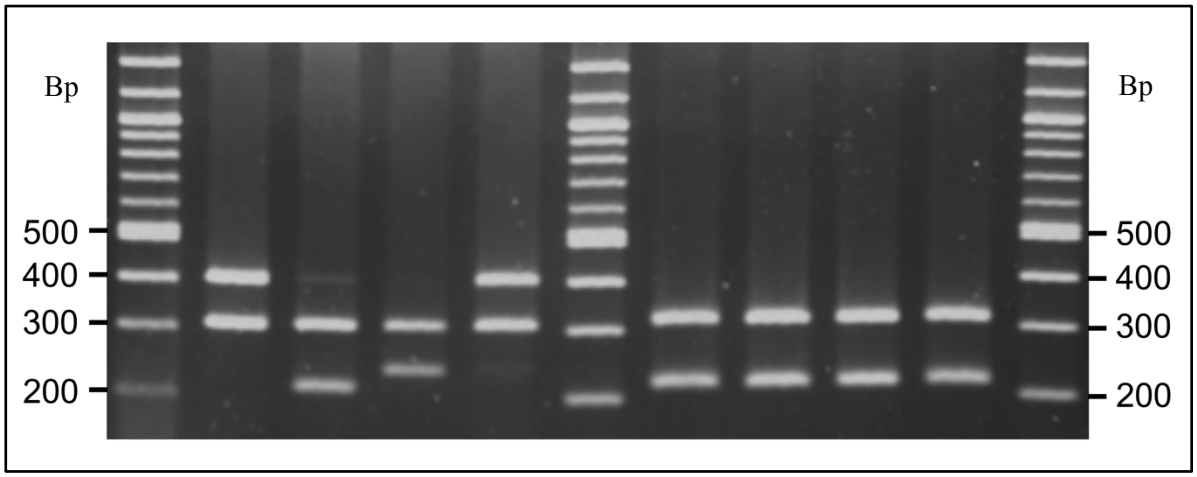

Figure 10: Agarose gel showing the results of restriction enzyme digestion of plasmids with CGG repeats. The four samples on the left contain 99 CGG repeats while those to the right contain 30 CGG repeats, and are thus in the normal, stable range. Plasmids were cut with BlpI and XhoI. The expected fragment sizes are 424 and 311 base pairs (bp) for the samples with 99 CGG repeats, and 217 and 311 base pairs for the samples with 30 repeats. The bands at 424 and 217 contain the repeat sequence. Despite growing both plated bacteria and liquid cultures at room temperature in SURE cells, both sample number 2 and 3 from the left clearly display deletions/contraction of the CGG repeat region.

Stable cell lines

A number of different approaches can be used to establish stable cell lines. They can be divided into those involving integration of the construct into the host cell's genome (examples are retroviral and lentiviral vectors), and those where transfected expression vectors are maintained without this integration (for example episomes (Van Craenenbroeck et al., 2000)). Which system will work better for a specific project, depend on factors such as which cell line(s) are used, desired levels of expression from the gene of interest (GOI), how large the GOI is, etc. In general, establishment of cell lines requires some sort of selection of the cells that are successfully transfected. The insertion of the GOI, and, if relevant, it's location in the genome, should be confirmed before conducting experiments with a new cell line. To avoid gradual loss of the GOI in subsequent experiments, cells can be maintained under mild selection (i.e. lower concentration of the antibiotic(s) used for initial selection), and/or be kept at a relatively low

passage number for all experiments. For our episome-based system, expression of GOI was confirmed by measuring amount of GFP reporter expressed. In addition, we only used cells at low passage numbers (5-13) and verified the presence and stability of the GOI using a PCR based method (see paper I). The stable cell line system used in paper II (Flp-In-T-Rex 293, Invitrogen) is well-established, and leads to integration of the transfected construct at one specific site in the genome. Here we also verified that expression was maintained by evaluating levels of the GFP-reporter, and used cells at passage number below 15 for all experiments.

Tetracycline inducible expression

Inducible promoters allow expression to be turned on and off at desired time-points, and, in some cases, to regulate the level of expression. Inducible promoters include both chemical-, alcohol-, and steroid-regulated promoters. In cell-based systems, the tetracycline-controlled promoters are the most commonly used chemical-regulated promoters. Tetracycline-controlled expression systems can be divided into two main categories: 1)Tetracycline-off systems (Tet-Off) where presence of tetracycline (or analogues like doxycycline) inhibits expression from a tet-inducible promoter, and 2) tetracycline-on (Tet-On) systems where addition of tetracycline or doxycycline induces expression from a promoter. The system with tetracycline-responsive promoters in mammalian cells was first described by Gossen and Bujard in 1992 (Gossen and Bujard, 1992).

In paper I, one of our questions was how the level of expression from a gene containing the CGGs, measured as concentration of mRNA, affected the phenotype. In order to answer this, we needed a system with inducible expression that could be controlled over a wide range of expression levels. The tetracycline (tet)/doxycycline (dox)-inducible episomal system met our needs. This system is a version of the Tet-On Advanced Inducible Gene Expression System (Urlinger et al., 2000). In brief, addition of dox induces transcription of the CGG-repeat with a GFP-reporter. The dox modulates the expression level of the CGG-GFP in a dose-dependent manner. A more detailed description of how our specific episomal-based system was developed is found in both the result- and method sections of paper I. The advantage to this system is that it gives us a fairly good control over the expression level of the GOI. This allowed us to answer the research questions in paper I. However, the system was leaky, i.e. low-level expression occurred even in the absence of any doxycycline or tetracycline. The system would therefore not be ideal to study any reversal of the phenotype after turning off expression from the GOI. In addition, the levels of expression at a given dox-concentration were not the same for the

different episomes. Comparisons therefore had to be based on the measured mRNA concentration, and not the amount of dox added.

In paper II we used a different cell-based system with tetracycline inducible expression, namely The Flp-In-T-Rex 293 (HEK-FlpIn) cell line from Invitrogen. This is also a Tet-On system where expression from the GOI is induced by adding tetracycline or doxycycline. In our system, the cell line was used to establish stable, inducible expression from either wtHP-99Gly-GFP or mutHP-90Gly-GFP. While experiments using transient transfections also answered many of research questions in paper II, it was important to assess whether the findings could be reproduced using stable cell lines with a lower level of overexpression. A significant reason for us to choose the HEK-FlipIn cell line, was that we successfully inserted the complete expanded CGG repeat region into the pcDNA5/FRT plasmid, which is compatible with the FlpIn system. Due to the instability of the repeats, this was not accomplished with other vectors used in our lab to establish stable cell lines. The tet-inducible system also had some obvious advantages for our studies of the FMRpolyG protein. By culturing cells in media with serum devoid of tetracycline, we avoided leakage from the system. This allowed us to measure the rate of degradation by following the change in GFP fluorescence, and hence the level of FMRpolyG-GFP, after first adding and then removing tetracycline from the media.

Outcome measurements

An important part of designing any study is to choose the outcome measurements, i.e. what to measure, and how. For FXTAS, which has been studied across model system, several findings in cell-based systems have been related to the pathogenesis in patients. We mainly focused on these established outcome measurements in our studies. It is beyond the scope of this chapter section to go into technical details regarding all measurements performed in paper I and II. These are described in the methods sections in each respective paper. The following section will instead briefly go through some of the specific considerations regarding the main outcome measurements.

Cell viability was assed using a flow based assay where we quantified the number of propidium iodide (PI) positive cells. PI is a fluorescent intercalating agent that does not cross the intact cell membrane of living cells. That means that it will stain dead or dying cells specifically, by binding to their DNA. PI emits strong red fluorescence when bound to DNA. Reasons for choosing the PI-assay are that the red fluorescence signal means it can be analyzed together

with GFP-tagged proteins (contrary to many live/dead kits), and that it is compatible with flow cytometry which allows high throughput. In addition, a previous study had successfully used the PI-assay to study cell death in neuronal cells expressing the expanded CGG repeat (Arocena et al., 2005). In the same paper they also used the TUNEL assay and found that cell death was not due to apoptosis (Arocena et al., 2005). Other cell viability assays based on measuring apoptosis, did thus not appear to be better options than the PI-assay.

Lamin architecture has been described as disrupted or abnormal in model systems for FXTAS (Arocena et al., 2005, Sellier et al., 2017). Lamin A is present in intranuclear inclusions in FXTAS patients, and recent findings indicate this is also the case for Lamin B1 (Iwahashi et al., 2006, Sellier et al., 2017). This establishes a direct link from findings in model systems to the pathogenic mechanisms taking place in patients. Since part of our aim was to detect changes in morphology, studying lamin architecture became an important part of our studies. In addition, good commercial antibodies for both Lamin A/C and Lamin B1 are available. We did not use any automated quantification of disrupted versus normal lamin rings. We therefore defined the criteria for disrupted versus normal lamin rings before counting was performed. Nevertheless, the quantitation is subjective to some extent. To further try to reduce any bias, the person performing the counting was blinded to the identity of the samples.

Aggregate formation is an important outcome measurement, especially in paper II. One of our aims was to study the contribution of FMRpolyG in causing a cellular manifestations of the phenotype. A hallmark of FXTAS is the presence of intranuclear inclusions, and the FMRpolyG protein is present in aggregates formed in model systems (Todd et al., 2013, Sellier et al., 2017). The FMRpolyG aggregates were thus considered a manifestation of the phenotype. With a GFP-tag attached to the FMRpolyG protein, these aggregates were easy to identify using both fluorescence and confocal microscopy. We also confirmed that aggregates formed when the GFP-tag was substituted with a FLAG-tag, by staining cells with a FLAG-specific antibody. Since patient material was not available to us when performing the study in paper II, it was not possible to directly compare the aggregates that are formed with the inclusions found in patients. In order to get as much information as possible regarding the FMRpolyG positive aggregates, we performed correlative light and electron microscopy (CLEM). This technique allowed us to identify specific aggregates found by confocal microscopy, in the electron microscopy images. While the CLEM technique is laborious, the results provided interesting information regarding the ultrastructure of these aggregates.

7 References

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PAPER I

PAPER II