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DEFINITION OF THE LANDSCAPE OF CHROMATIN STRUCTURE AT THE

FRATAXIN GENE IN FRIEDREICH'S ATAXIA

by

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DEFINITION OF THE LANDSCAPE OF CHROMATIN STRUCTURE AT THE

FRATAXIN GENE IN FRIEDREICH'S ATAXIA

A

DISSERTATION

Presented to the Faculty of

The University of Texas

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in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

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Houston, Texas

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Publication No._____

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Friedreich's ataxia (FRDA) is caused by the transcriptional silencing of the frataxin (*FXN*) gene. FRDA patients have expansion of GAA repeats in intron 1 of the *FXN* gene in both alleles. A number of studies demonstrated that specific histone deacetylase inhibitors (HDACi) affect either histone modifications at the *FXN* gene or *FXN* expression in FRDA cells, indicating that the hyperexpanded GAA repeat may facilitate heterochromatin formation. However, the correlation between chromatin structure and transcription at the *FXN* gene is currently limited due to a lack of more detailed analysis. Therefore, I analyzed the effects of the hyperexpanded GAA repeats on transcription status and chromatin structure using lymphoid cell lines derived from FRDA patients. Using chromatin immunoprecipitation and quantitative PCR, I observed significant changes in the landscape of histone modifications in the vicinity of the GAA tract in FRDA cells relative to control cells. Similar epigenetic changes were observed in GFP reporter construct containing 560 GAA repeats. Further, I detected similar levels of *FXN*

pre-mRNA at a region upstream of hyperexpanded GAA repeats in FRDA and control cells, indicating similar efficiency of transcription initiation in FRDA cells. I also showed that histone modifications associated with hyperexpanded GAA repeats are independent of transcription progression using the GFP reporter system. My data strongly support evidence that *FXN* deficiency in FRDA patients is consequence of defective transition from initiation to elongation of *FXN* transcription due to heterochromatin-like structures formed in the proximity of the hyperexpanded GAAs.

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Chapter 1. Introduction and Background

Some parts of this dissertation were modified from the following journal article.

Eunah Kim, Marek Napierala, Sharon Y.R. Dent. Hyperexpansion of GAA repeats affects post-initiation steps of *FXN* transcription in Friedreich's ataxia, Nucleic Acids Research, (2011) 39(19): 8366-8377.

1. FRDA

Clinical and pathological aspects

Friedreich's ataxia (FRDA) is an degenerative neuro-muscular disease that affects 1 in 50,000 individuals in the United States (Delatycki, Williamson and Forrest, 2000). The features of FRDA are recessively inherited with an incidence of one in 60 to 100 depending on the ethnic group (Cossee, Schmitt, Campuzano, Reutenauer, Moutou, Mandel and Koenig, 1997). FRDA is characterized by progressive ataxia, including uncoordinated gait and limb movements, weakened muscle strength, and diminished senses of position and vibration. FRDA involves degeneration of the posterior columns of the spinal cord that is extended to the brain stem. FRDA patients also exhibit atrophy in the spinocerebellar tract, a set of axonal fibers in the spinal cord terminating in the cerebellum. In the dorsal root ganglion within the spinal cord, large primary sensory neurons are also degenerated in FRDA patients. Quantitative analysis reveals that contact between Purkinje cells in the cerebellum and dendrites are decreased, while the cerebellar cortex is not affected in patients of FRDA. These phenomena indicate that FRDA patients are most likely defective in providing information from the spinal cord through the spinocerebellar tract, which lead loss of position and vibration sense. Additionally, hypertropic cardiomyocytes are observed in approximately 80% of FRDA patients (Campuzano, Montermini, Lutz, Cova, Hindelang, Jiralerspong, Trottier, Kish, Faucheux, Trouillas, Authier, Durr, Mandel, Vescovi, Pandolfo and Koenig, 1997). Such cardiomyocytes contain abnormal intracellular iron deposits, suggesting that this feature of FRDA is associated with defective function of iron metabolism.

The role of FXN in mitochondria

The FRDA disease gene is genetically mapped to chromosome 9q13 and encodes the

Frataxin protein (FXN), a nuclear encoded mitochondrial protein (Campuzano,

Montermini, Lutz, Cova, Hindelang, Jiralerspong, Trottier, Kish, Faucheux, Trouillas,

Authier, Durr, Mandel, Vescovi, Pandolfo and Koenig, 1997; Campuzano, Montermini,

Molto, Pianese, Cossee, Cavalcanti, Monros, Rodius, Duclos, Monticelli, Zara,

Canizares, Koutnikova, Bidichandani, Gellera, Brice, Trouillas, De Michele, Filla, De

Frutos, Palau, Patel, Di Donato, Mandel, Cocozza, Koenig and Pandolfo, 1996). FRDA is

characterized by insufficient levels of *FXN* mRNA and protein in all examined tissues

(Campuzano, Montermini, Lutz, Cova, Hindelang, Jiralerspong, Trottier, Kish, Faucheux,

Trouillas, Authier, Durr, Mandel, Vescovi, Pandolfo and Koenig, 1997). Most functions

of FXN have been demonstrated from yeast since yeast is viable without the yeast FXN

homolog, Yfh1p, while FXN is an essential protein in mouse (Adinolfi, Iannuzzi, Prischi,

Pastore, Iametti, Martin, Bonomi and Pastore, 2009; Bulteau, O'Neill, Kennedy, Ikeda-

Saito, Isaya and Szweda, 2004; Cossee, Puccio, Gansmuller, Koutnikova, Dierich,

LeMeur, Fischbeck, Dolle and Koenig, 2000). Yfh1p plays a role in the storage of iron

within mitochondria (Babcock, de Silva, Oaks, Davis-Kaplan, Jiralerspong, Montermini,

Pandolfo and Kaplan, 1997). During iron homeostasis, FXN acts as a chaperone (Bulteau,

O'Neill, Kennedy, Ikeda-Saito, Isaya and Szweda, 2004). Negatively charged FXN binds

ferrous iron to balanced levels of iron (Dhe-Paganon, Shigeta, Chi, Ristow and Shoelson,

2000). FXN has a ferroxidase activity when the concentration of ferrous iron is low in

mitochondria leading to iron detoxification. Insufficient levels of FXN expression result

in abnormal biosynthetic processes through a defective utilization of iron such as

decreased activities of iron-sulfur cluster (ISC) enzymes, accumulation of iron in mitochondria, and increased susceptibility to oxidative stress (Wilson, 2003). Oxidative stress is caused by oxygen excess in mitochondria due to an impaired ability to maintain homeostasis by detoxifying oxidative molecules. Decreased activity of ISC is associated with a dysfunction of respiratory chain leading to exacerbation of oxidative stress conditions (Jensen, Sanchez, Srinivasan, Valentine and Culotta, 2004). Oxidative stress observed in FRDA patient possibly results from FXN deficiency through impaired ISC in mitochondria. However, the underlying mechanisms of FRDA have not yet been fully elucidated. FXN is evolutionarily conserved from yeast to humans, in particular in the C-terminal region. Interestingly, an exogeneous protein consisting of the C-terminus of human FXN fused to the N-terminus of Yfh1p complements the phenotype of Yfh1p deficient yeast (Wilson and Roof, 1997). Yfh1p precursor is processed to a 122-amino acid protein through cleavage by the mitochondrial processing protease (MPP) (Gordon, Shi, Dancis and Pain, 1999). Human FXN is similarly processed to a 128-amino acid protein from a 210-amino acid precursor. A N-terminal portion of FXN contains a mitochondrial signal sequence that is removed by the MPP. Unlike Yfh1p, FXN is processed through two-step cleavages (Schmucker, Argentini, Carelle-Calmels, Martelli and Puccio, 2008). The first cleavage occurs between Gly41 and Leu42 by MPP, generating a 169-amino acid intermediate protein in mitochondria. The second cleavage between Lys80 and Ser81 brings into being the 130-amino acid mature form of FXN. Only the final mature 130-amino acid form is functional in mitochondria. Ectopic expression of the mature form of FXN efficiently rescues the lethality of FXN null fibroblasts (Schmucker, Argentini, Carelle-Calmels, Martelli and Puccio, 2008).

Consistently, residues 90-210 of FXN have been demonstrated to be rigid by crystallography and NMR structure (Dhe-Paganon, Shigeta, Chi, Ristow and Shoelson, 2000; Musco, Stier, Kolmerer, Adinolfi, Martin, Frenkiel, Gibson and Pastore, 2000). This suggests that C-terminal region of FXN is functional as mentioned above while N-terminal region is responsible for localization of FXN into mitochondria. Indeed, residues 90-210 correspond to an α1-helix and β1-sheet of the core structure of FXN that has potential to either function to donate iron to its partner or to form multimers (Dhe-Paganon, Shigeta, Chi, Ristow and Shoelson, 2000). Although the processed forms of *FXN* are associated with FRDA pathology, it is noteworthy that defects in the process of FXN maturation are not. This emphasizes that enhancement of FXN transcription will be critical for curing FRDA.

Differential expression of the *FXN* gene

Since there is no evidence that FXN RNA is abnormally processed to make mature *FXN* RNA in FRDA, insufficient levels of FXN in FRDA likely result from the stage of *FXN* transcription. The *FXN* gene consists of 7 exons (Figure 1). The major transcripts contain exon 1 to 5a and are pronounced in all examined tissues of the patients (Kostrzewa, Klockgether, Damian and Muller, 1997). Other isoforms are occasionally detected in the heart. The *FXN* gene is differentially expressed in various tissues. The human *FXN* gene is mainly expressed in primary sites of degeneration in FRDA patients such as liver, heart and CNS, particularly in the spinal cord (Campuzano, Montermini, Molto, Pianese, Cossee, Cavalcanti, Monros, Rodius, Duclos, Monticelli, Zara, Canizares, Koutnikova, Bidichandani, Gellera, Brice, Trouillas, De Michele, Filla, De Frutos, Palau, Patel, Di Donato, Mandel, Cocozza, Koenig and Pandolfo, 1996).

Figure 1. Schematic diagram of the human *FXN* gene.

Black bars represent exons (exon1, 2, 3, 4, 5a, and exon 5b). Light gray bar represents non-coding exon that is involved in regulating the expression of the gene and does not lead to additions to the protein sequence. Exons 5a and 5b are alternatives and result in slightly different protein sequences. Exon1~5a produce major *FXN* transcripts. The *FXN* gene is displayed in correct proportion.

Figure 1

Human Frataxin (Chromosome 9: 71,650,175-71,715,094, forward strand)
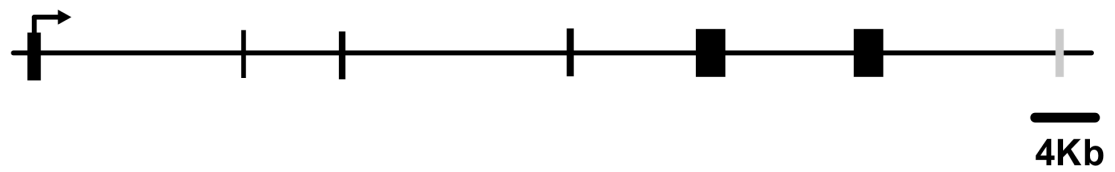
The mouse *FXN* gene is differentially expressed during development. Expression is gradually increased from embryonic day 10 (E10) to later stages, appearing the highest at E14. In the spinal cord, a characteristic pattern of *FXN* expression becomes evident at E12.5, particularly in the thoracolumbar region that is most likely composed of large neuronal cells in the DRG (Jiralerspong, Liu, Montermini, Stifani and Pandolfo, 1997). In the adult brain, expression is restricted to the ependymal layer, chorid plexus, and granular layer of the cerebellum. This pattern indicates that FXN expression in various tissues is correlated with FRDA pathology, suggesting that changes in FXN transcription is likely responsible for FRDA.

Mutation at the *FXN* gene in FRDA patients (Expansion of GAA repeats at the *FXN* gene in FRDA)

Approximately 98% of FRDA patients are characterized by abnormal GAA repeat expansions within intron 1 of the *FXN* gene in both alleles (Figure 2), while 2% of FRDA patients have either point mutations or missense mutations in the *FXN* gene that act as null mutations (Bidichandani, Ashizawa and Patel, 1998; Campuzano, Montermini, Molto, Pianese, Cossee, Cavalcanti, Monros, Rodius, Duclos, Monticelli, Zara, Canizares, Koutnikova, Bidichandani, Gellera, Brice, Trouillas, De Michele, Filla, De Frutos, Palau, Patel, Di Donato, Mandel, Cocozza, Koenig and Pandolfo, 1996; Ohshima, Sakamoto, Labuda, Poirier, Moseley, Montermini, Ranum, Wells and Pandolfo, 1999). The GAA repeat at the *FXN* gene is detected in the middle of an *Alu* repeat element. In addition, a polymorphic mononucleotide tract of adenines (poly A tract) was identified in

Figure 2. Mutation of the *FXN* gene in FRDA patients.

Expansion of GAA repeats is located within intron 1 of the *FXN* gene in FRDA patients. Black bars represent exon 1 and 2, while light gray bar represents non-coding exon 6. More than 1,700 GAA repeats are observed in FRDA patient, whereas less than 66 GAA repeats is detected in unaffected individuals. The region between exon 1 and exon 2 is displayed in correct proportion.

Figure 2
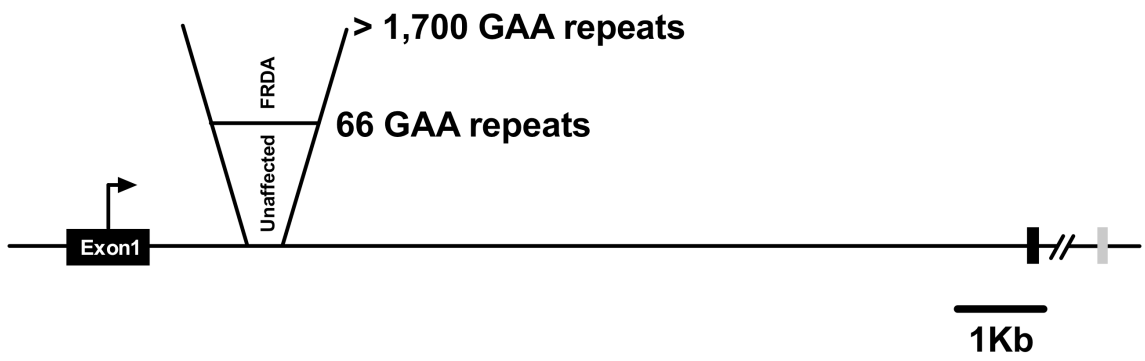
the vicinity of the GAA triplet repeat (Monticelli, Giacchetti, De Biase, Pianese, Turano, Pandolfo and Cocozza, 2004). Reduced levels of the *FXN*

gene expression in FRDA patients are correlated with the hyperexpanded tract of

repeated GAA triplets (Campuzano, Montermini, Molto, Pianese, Cossee, Cavalcanti, Monros, Rodius, Duclos, Monticelli, Zara, Canizares, Koutnikova, Bidichandani, Gellera, Brice, Trouillas, De Michele, Filla, De Frutos, Palau, Patel, Di Donato, Mandel, Cocozza, Koenig and Pandolfo, 1996; Grabczyk and Usdin, 2000a). In FRDA patients, the GAA tract frequently consists of more than 1500 triplets, whereas unaffected individuals have 66 or fewer repeats at the *FXN* gene (Campuzano, Montermini, Molto, Pianese, Cossee, Cavalcanti, Monros, Rodius, Duclos, Monticelli, Zara, Canizares, Koutnikova, Bidichandani, Gellera, Brice, Trouillas, De Michele, Filla, De Frutos, Palau, Patel, Di Donato, Mandel, Cocozza, Koenig and Pandolfo, 1996; Pandolfo, 1998; Pianese, Turano, Lo Casale, De Biase, Giacchetti, Monticelli, Criscuolo, Filla and Cocozza, 2004). About 85% of unaffected individuals have fewer than 12 GAA repeats whereas the remainder has 12 to 33 repeats.

Expansion of the GAA triplet

Expansion or contraction in size of the GAA repeat tracts is commonly observed during intergenerational transmission from parents to children. Although the underlying mechanism for how the size of the repeats is expanded has not yet been demonstrated, it has been suggested that DNA metabolism such as repair, replication, and recombination is involved in the flexibility of the GAA triplet size among generations. The mismatch repair (MMR) pathway is involved in inheritance and stability of the pathological repeats among generations (Krasilnikova, Kireeva, Petrovic, Knijnikova, Kashlev and Mirkin,

2007; Mirkin, 2007). In longer GAA repeats, single-stranded DNA is exposed to form hairpin DNA structures. MMR machinery is recruited to the hairpin structure, which leads to repeat expansion (Krasilnikova, Kireeva, Petrovic, Knijnikova, Kashlev and Mirkin, 2007). During DNA replication, exposed single stranded DNA containing GAA repeats enables folding into secondary DNA structures, which cause expansion of GAA repeats through misalignment of repetitive DNA (Ohshima and Wells, 1997). Recombination pathways also contribute to expansion of the GAA repeats. Longer GAA repeats are associated with a high frequency of recombination in E.coli, which results in expansion of the GAA repeats (Napierala, Dere, Vetcher and Wells, 2004). However, the detailed mechanisms are not clear.

The effect of the GAA repeats on gene expression

Previous studies demonstrate that the long GAA tracts cause transcriptional pausing in *in vitro* as well as in cell culture systems (Grabczyk and Usdin, 2000b; Krasilnikova, Kireeva, Petrovic, Knijnikova, Kashlev and Mirkin, 2007). Oligonucleotides containing longer GAA repeats show abnormal migration mobility on agarose gels when compared to linear DNA consisting of random sequences (Ohshima, Montermini, Wells and Pandolfo, 1998). Abnormal DNA structures of constructs containing longer GAA repeats have been observed by nuclear magnetic resonance (LeProust, Pearson, Sinden and Gao, 2000). Polypurine/polypyrimidine DNA sequences containing mirror repeat symmetry form distinctive non-canonical structures. Either a pyrimidine or a purine rich single-stranded DNA is intertwined into the major groove of a double helix consisting of the hyperexpanded GAA repeats forming intermolecular interactions between the strands in

in vitro systems (Sakamoto, Chastain, Parniewski, Ohshima, Pandolfo, Griffith and Wells, 1999). Integration of oligodeoxyribonucleotides into longer GAA repeats block formation of the non-canonical DNA structure leading to restoration of full-length FXN transcripts in a concentration-dependent manner (Grabczyk and Usdin, 2000a). Small molecules recognizing specific DNA sequences such as polyamides de-repress FXN expression in cellular systems (Dervan and Edelson, 2003; Gottesfeld, 2007; Gottesfeld, Turner and Dervan, 2000). T7 RNA polymerase is impeded in the vicinity of GAA repeats in *in vitro* systems(Grabczyk and Usdin, 2000b). These results explain the correlation between size of the GAA repeats and defective transcription in FRDA.

Treatments of FRDA

Although the mechanism of how hyperexpanded GAA repeats down-regulate FXN expression in FRDA patient remains unclear, a number of studies have been carried out to identify compounds that enhance the expression of FXN in FRDA. To date, several compounds have been reported to increase FXN expression in cellular models, including hemin, butyric acid (Sarsero, Li, Wardan, Sitte, Williamson and Ioannou, 2003), and erythropoietin (Sturm, Stupphann, Kaun, Boesch, Schranzhofer, Wojta, Goldenberg and Scheiber-Mojdehkar, 2005) by unknown mechanisms.

As FXN deficiency in FRDA results in increasing free radicals accompanied by abnormal accumulation of iron in mitochondria, both antioxidants and iron chelators have been applied to patients as a therapy to alleviate symptoms of FRDA. To date, only idebenone, a synthetic analogue of coenzyme Q10, has been demonstrated in a phase III trial as free

radical chelator (a treatment pipeline in the Friedreich's Ataxia Research Alliance (www.fara.org)). Unfortunately, a recent study has reported that idebenone does not improve neurological symptoms of FRDA in pediatric patients (Lynch, Perlman and Meier, 2010).

Although the treatments mentioned above have improved the progress of FRDA, there are no effective treatments for curing FRDA. Enhancement of FXN expression from FXN cDNA harboring viral vectors partially alleviates FXN deficiency and defective response to oxidative stress (Fleming, Spinoulas, Zheng, Cunningham, Ginn, McQuilty, Rowe and Alexander, 2005; Gomez-Sebastian, Gimenez-Cassina, Diaz-Nido, Lim and Wade-Martins, 2007; Lim, Palomo, Mauritz, Gimenez-Cassina, Illana, Wandosell and Diaz-Nido, 2007). These studies suggest that restoring FXN levels is likely to be more effective for FRDA treatment than targeting downstream consequences of FXN deficiency. These studies make the point that understanding the underlying mechanisms of how *FXN* expression is affected by hyperexpanded tracts of GAA repeats in patients with FRDA is critical to developing therapeutic targets for curing FRDA.

Models for study of FRDA

As previously mentioned, an effective treatment has not yet developed for curing FRDA. Nevertheless, various systems have been applied to investigate the molecular pathology of FRDA. Each system has different purpose for study.

- **Escherichia coli (*E.coli)*

E.Coli has been used to investigate the propensity of longer GAA repeats to form non B-DNA structures that are associated with abnormal replication and defective transcription in a length-dependent manner (Grabczyk and Usdin, 2000b). Moreover, persistent RNA_DNA hybrids in the presence of longer GAA repeats was first identified in *E.coli* (Grabczyk, Mancuso and Sammarco, 2007). RNA transcribed from long GAA repeats is hybridized with exposed single-stranded DNA to form persistent RNA_DNA hybrids, which inhibit transcription. Therefore, *E.coli* has been useful to define how non-B DNA structures consisting of longer GAA repeats act as *cis*-inhibitors of DNA metabolism.

- **Yeast**

Yeast is an appropriate model system to investigate the function of FXN since YFH1 deficient yeast survive (Wilson and Roof, 1997). Moreover, similar to human and mouse FXN, yeast FXN homologue contains a potential mitochondrial targeting sequence in the N-terminus of YFH1. Therefore, deletion of YFH1 in yeast strain provided evidence to demonstrate that FXN acts as an iron chaperone in mitochondria (Babcock, de Silva, Oaks, Davis-Kaplan, Jiralerspong, Montermini, Pandolfo and Kaplan, 1997).

- **Mouse models**

Mouse models for FRDA have been generated to study the mechanism of the pathology and to test pharmacological therapies.

  o FXN null/conditional KO/inducible KO

FXN null mice that have constitutive inactivation of FXN by homologous recombination are embryonic lethal, indicating that FXN is essential for development (Cossee, Puccio,

Gansmuller, Koutnikova, Dierich, LeMeur, Fischbeck, Dolle and Koenig, 2000).

However, these mice did not exhibit defective iron metabolism during development,

suggesting that lethality of the null embryos is independent of the iron metabolism

defects caused. To further understand the lethality caused by knockout (KO) of FXN,

conditional KO mice were generated using Cre-lox system specific to skeletal and cardiac

muscle (Puccio, Simon, Cossee, Criqui-Filipe, Tiziano, Melki, Hindelang, Matyas, Rustin

and Koenig, 2001). The conditional FXN KO mice were viable and resembled the

phenotype of FRDA patients such as large sensory neuron dysfunction and deficient

activity of mitochondria. Although this model did not exhibit susceptibility to oxidative

stress, this phenomenon is also controversial in FRDA patients. This result suggests that

the susceptibility to oxidative stress is not a major consequence of FXN deficiency.

Therefore, these mice models that have insufficient levels of FXN without GAA repeats

are appropriate to investigate the role of FXN and the pathological process of FRDA

independent of the GAA repeats.

    o GAA in mice

In an effort to define the effects of the GAA repeats in the pathology of FRDA, a

transgenic mouse model (knock in-knock in (KIKI) mice) that contains a tract of GAA

repeats was generated by insertion of 230 GAA into the first intron of the mouse Fxn

gene and then mated with Fxn -/- mice (Miranda, Santos, Ohshima, Smith, Li, Bunting,

Cossee, Koenig, Sequeiros, Kaplan and Pandolfo, 2002). The offspring (Fxn $^{230/-}$) have

25~36% reduction of FXN expression relative to wild type. Unfortunately, these mice did

not phenocopy a FRDA in despite of reduced levels of Fxn caused by the insertion of the

GAA repeats. This finding suggests that 230 GAA repeats are insufficient to stimulate

pathology of FRDA. Moreover, this study also suggests that the appropriate location of the GAA repeat at a given gene might be a major factor in exhibition of FRDA pathology, as the location of the repeat in the mouse Fxn gene is not comparable to the location of the GAA repeats in human *FXN* gene. Nevertheless, this is a first FRDA mouse model to exhibit FXN deficiency caused by the presence of the GAA repeat.

        o   YAC-GAA in mice

To overcome embryonic lethality of FXN KO model, transgenic mice were generated by introducing 190 or 190/90 GAA repeats in intron 1 of the human *FXN* gene within a human YAC clone in the background of *FXN* null mice (Al-Mahdawi, Pinto, Ruddle, Carroll, Webster and Pook, 2004; Pook, Al-Mahdawi, Carroll, Cossee, Puccio, Lawrence, Clark, Lowrie, Bradley, Cooper, Koenig and Chamberlain, 2001). These transgenic mice exhibited typical FRDA phenotypes such as coordination deficits and a progressive decrease in motor activity in younger mice (3 month old), indicating that location of the GAA repeat is considerable when compared to KIKI mice. Histological studies in this model showed similarities to FRDA patients such as large vacuoles in dorsal root ganglia cells and iron deposits in cardiomyocytes, whereas electrophysiological studies showed a mild, progressive peripheral neuropathy (Al-Mahdawi, Pinto, Varshney, Lawrence, Lowrie, Hughes, Webster, Blake, Cooper, King and Pook, 2006). Moreover, this model showed tissue-specific and age-dependent somatic instability specifically in the cerebellum and dorsal root ganglia (Clark, De Biase, Malykhina, Al-Mahdawi, Pook and Bidichandani, 2007). Especially, a HDACi enhances *FXN* expression accompanying with increased levels of acetylation on histones in the KIKI mice. This outcome is meaningful that removal of marks associated heterochromatin-like structures has potential to alleviate

FXN deficiency in FRDA. However, it is unclear that the observed phenomenon resulted from the GAA repeats rather than from the construct *per se*.

- **Lymphoid cell lines**

Patient-derived lymphoid cell lines immortalized by Epstein-Barr virus available in the Coriell Institute (http://www.coriell.org). Moreover, cells from unaffected control individuals that contain a range of the GAA repeats are available for comparison studies. Therefore, the lymphoid cell lines are perhaps the best system to address my hypothesis since the presence of expanded GAA repeats at the *FXN* locus does not result in degeneration of these lymphoid cell lines.

- **The GFP_GAA system**

To avoid effects from inherent characteristics of the *FXN* gene so that changes induced by the hyperexpanded GAA repeats *per se* can be determined, the GAA repeat derived from a FRDA patient (the GM16210 cell line from Coriell Institute) was engineered into an intronic region originating from the human *Pem1* gene that is known to be spliced efficiently during RNA processing (Figure 3). The *Pem* intronic region containing the GAA repeat is embedded into the middle of the GFP coding region to divide the GFP coding region. The CMV promoter controls expression of this construct to avoid inherent

Figure 3. The GFP_GAA system.

Schematic diagram of the *GFP* gene containing GAA repeats in the GFP_(GAA•TTC) reporter system. The *GFP* gene was divided into two exons (gray boxes) by the intron containing 560 GAA repeats. This reporter system is expressed under control of the CMV promoter (black box) and the tetracycline operator/repressor $(TetO)_2$ (white boxes) that allows regulating transcription initiation by tetracycline. BGH pA represent bovine growth hormone and polyadenylation signal that is used during mRNA processing.

Figure 3
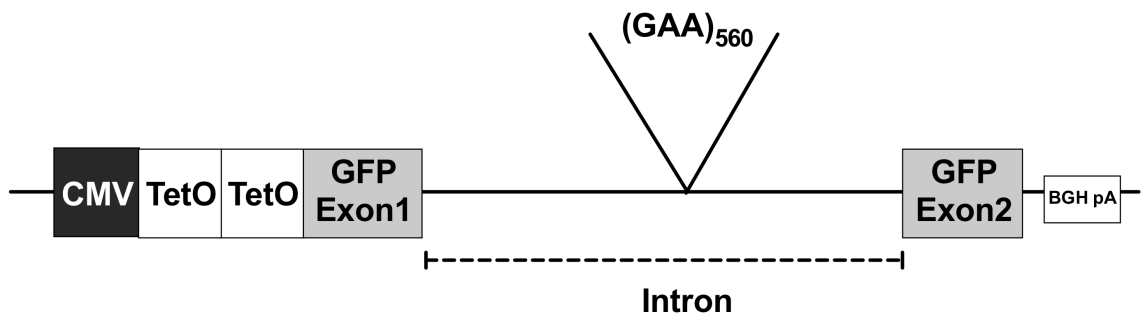
characteristics of the FXN promoter. A tetracycline operator element was also inserted in

the promoter region so that GFP expression is regulated by tetracycline treatment,

reducing the possibility of genomic instability associated with transcription of repetitive

DNA sequences. This reporter construct genetically recapitulates the location of the GAA

repeats as characteristics of the endogenous *FXN* gene. Moreover, the GFP_GAA system

is validated as a model system to investigate molecular pathway associated with FXN

expression changes (Soragni, Herman, Dent, Gottesfeld, Wells and Napierala, 2008). The

insertion of the GAA repeats results in reduction of *GFP* mRNA. The intronic region in

the proximity of the GAA repeat has increased histone modifications marking

heterochromatin and decreased modifications marking euchromatin. Furthermore, small

drugs that affect modifications on chromatin structure restore GFP expression in the

GFP_(GAA•TTC) $_{560}$ cell line.

Section 2 of this chapter will describe general information about epigenetics, which

might play a role in down-regulating *FXN* transcription through the hyperexpanded GAA

repeats in FRDA.

2. Epigenetics regulation of FXN expression

In eukaryotes, the genome is organized into arrays of nucleosomes composed of histones

and DNA. The histones are classified as H1, H2A, H2B, H3, and H4. Two copies of

H2A, H2B, H3, and H4 compose a core octamer around which is wrapped around 146

base pair (bp) DNA to form a nucleosome (Luger, Mader, Richmond, Sargent and

Richmond, 1997). Chromatin is categorized into either euchromatin or heterochromatin,

depending on the conformation of structure, status of transcription, and post-translation modifications of histones. Configuration of chromatin structure is less packed in euchromatin. Moreover, transcription occurs more in euchromatin. In contrast, heterochromatin is characterized by condensed chromatin structure, rich in repetitive DNA, which is rarely transcribed in eukaryotes and plants (Grewal and Jia, 2007). Heterochromatin comprises most portions of both centromeres and telomeres in mammals. Genes localized in heterochromatin are most likely silenced (Grewal and Jia, 2007). This phenomenon suggests that gene expression is regulated by not only *trans* through transcription machinery but also *cis* through organization of chromatin structures.

Regulation of Chromatin structures

- **Post-translational modifications of Histones**

Regulation of chromatin structure is involved in various cellular functions such as DNA repair, replication as well as transcription by specific covalent marks in the N-terminal tails of histones such as acetylation (ac) and methylation (me) (Li, Carey and Workman, 2007). Although a number of lysine residues have been identified as targets for modifications, this thesis will focus on well characterized sites of acetylation and methylation of histone H3 and H4 (Figure 4).

Lysine residues in the amino-terminal regions of the histones are acetylated dependent on the status of transcription and on temporal and spatial conditions such as tissue specificity and development. Most acetylated histones are readily detected at transcriptionally active

Figure 4. Diagram of histone modifications at the amino-terminal tails of histone H3 and H4 in each chromatin structure (euchromatin and heterochromatin). Each chromatin structure has distinct combination of histone modifications as shown in diagram. DNA wrapped around histone octamer consisting of pairs of H2A, H2B, H3 and H4. Each histone exhibits a flexible histone tail that can be dynamically modified. Shown here are histone modifications examined in this study. Blue oval represent a methyl group on lysine residue. Green oval represent acetyl group on lysine residue.

Figure 4

Euchromatin

N-ART**K**QTAR**K**STGG**K**APRKQLATKAAR**K**SAP..V**K**KP..**K**...
     4   9   14   27   36 79

H3

H4

N-SGRGKGGKGLGKGGAKRHR**K**VLRDNIQGIT........
                    20

Heterochromatin

N-ART**K**QTAR**K**STGG**K**APRKQLATKAAR**K**SAP..V**K**KP..**K**...
     4   9   14   27   36 79

H3

H4

N-SGRGKGGKGLGKGGAKRHR**K**VLRDNIQGIT........
                    20
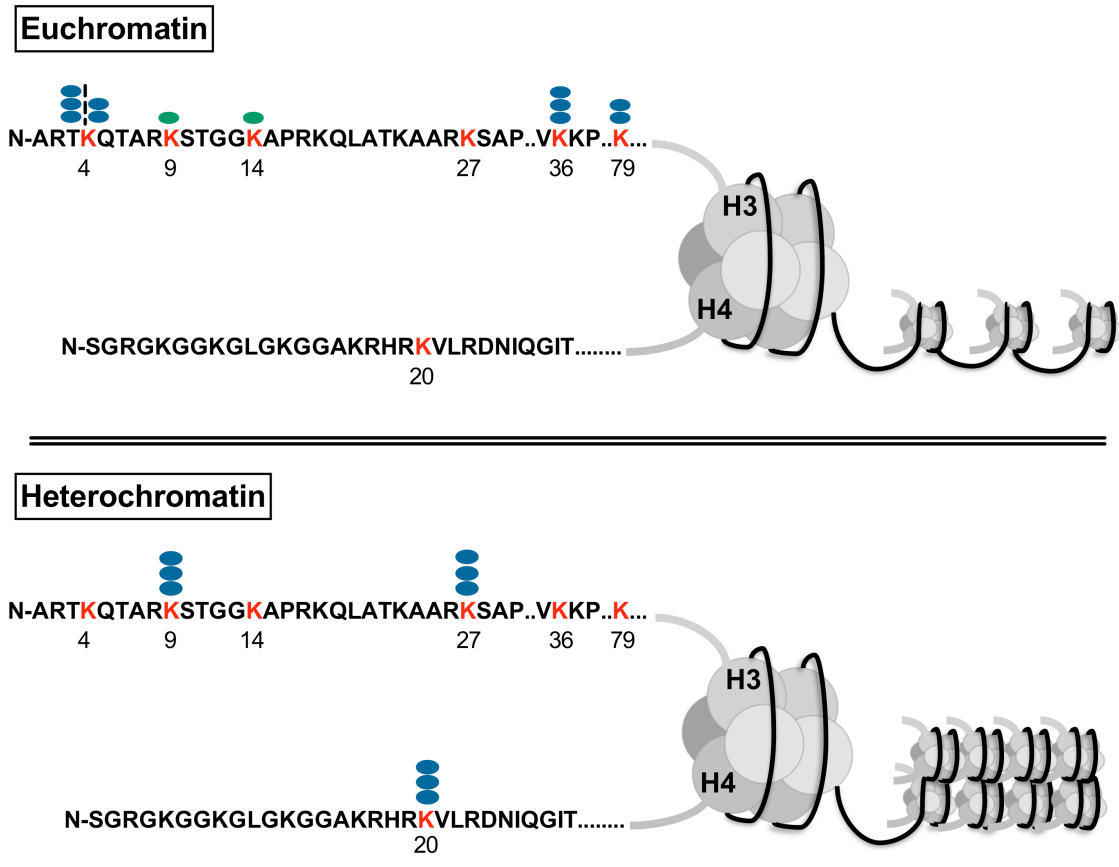
genes, particularly, in regulatory regions. Acetylation of lysines in histones neutralize the

positive charge on histones and thereby decrease interactions between histones and

DNAs. Therefore, transcription machinery is better able to access chromatin structures

that are hyper acetylated. In contrast, lack of acetyl group on histone results in

compaction of chromatin structure leading to gene silencing. Inhibition of histone

deacetylation using HDACi is promising to enhance expression of a given gene. Indeed,

many diseases such as neurodegenerative diseases and cancers have been treated with

HDACi to alleviate symptoms through regulation of histone acetylation. In

heterochromatin, acetylation is rarely detected as compared to euchromatin. Moreover,

higher eukaryotic heterochromatin is associated with heterochromatin protein (HP1), first

identified at pericentric heterochromatin in *Drosophila*. HP1 contributes to formation

and maintenance of heterochromatin by multimerization of HP1 through 'chromo

domain' at the N-terminal and 'chromo shadow domain' at the C-terminal domain of the

HP1. Gene silencing associated with HP1 is relived by inhibition of histone deacetylase,

TSA, suggesting that hypoacetylation plays a role in HP1 mediated

heterochromatinization. However, HP1 is also observed in euchromatin where genes are

transcribed (Ayyanathan, Lechner, Bell, Maul, Schultz, Yamada, Tanaka, Torigoe and

Rauscher, 2003), suggesting that additional proteins or histone modifications may be

involved to HP1-mediated gene silencing. Acetylation of lysine of proteins can serve as

binding sites for effector molecules that contain 'bromodomains'. GCN5 is an

acetyltransferase that is found in SAGA (Spt-Ada-Gcn5-acetyltransferase) complex. The

GCN5 bromodomain-mediated interaction with acetylated histones promotes stability of

SAGA complex at target genes, which maintain chromatin structure as euchromatin along

with increased levels of acetylation on histones. These results suggest a role of bromodomain as a modulator of chromatin modifying/remodeling complexes (Li and Shogren-Knaak, 2009).

Methylation of histones is involved in either active or repressive chromatin structures, depending on either the number of methyl groups present on the targeted residue and which residue is targeted in the histones (Li, Carey and Workman, 2007). While acetylation occurs only at lysine residues, either lysine or arginine residues can be methylated. Methylation at lysine 9 of histone H3 is enriched in heterochromatin, while acetylation at lysine 9 of histone H3 is enriched in euchromatin, where genes are actively transcribed. Methylation at lysine 9 of histone H3 prevents acetylation, promoting gene silencing. Methylation at lysine 9 of histone H3 at heterochromatin provides a binding site for HP1 protein to stimulate condensing of chromatin structures through the chromo domain and chromo shadow domain (Jenuwein and Allis, 2001). HP1 at the region where H3K9me3 are enriched act as anchor to recruit chromatin-remodeling complex to further modify histones. Although the majority of H3K9me3 is associated with repressed and silenced regions throughout the genome, this histone modification is also found in the coding regions of transcriptionally active genes (Vakoc, Mandat, Olenchock and Blobel, 2005). In centromeric heterochromatin, where gene expression is repressed, enrichment of H4K20me3 is accompanied by enrichment of H3K9me3 (Kourmouli, Jeppesen, Mahadevhaiah, Burgoyne, Wu, Gilbert, Bongiorni, Prantera, Fanti, Pimpinelli, Shi, Fundele and Singh, 2004; Schotta, Lachner, Sarma, Ebert, Sengupta, Reuter, Reinberg and Jenuwein, 2004).

During development, genes are appropriately either turning on or off at a given stage. Methylation at lysine 27 of histone H3 is well-characterized and is important in the regulation of gene expression during development. H3K27me3 is mainly detected in repressive chromatin structures such as the inactive X chromosome as well as in inducible genes that are required for a given stage of development. In embryonic stem cells, H3K4me3 is co-localized with H3K27me3 at the promoter of some genes, called bivalent genes. The silenced gene expression at the bivalent gene is reversed by the removal of H3K27me3, and then H3K4me3 becomes pronounced for these genes as cells differentiate. To switch off gene expression, methylation of H3K4 is removed by a demethylase such as a lysine-specific demethylase (LSD) 1 leading to over representation of H3K27me3 at the bivalent gene during differentiation or development (Seenundun, Rampalli, Liu, Aziz, Palii, Hong, Blais, Brand, Ge and Dilworth, 2010). This phenomenon is also observed in non-developmental inducible genes such as a heat-shock gene, *Hsp70,* in *Drosophila* (Gilmour and Lis, 1986). RNA polymerase II (RNA pol II) are accompanying with H3K4me3 at the promoter. Once the repressive mark is removed, paused RNA pol II is released from the promoter to produce full-length transcripts. During on going transcription, H3K4me3 is mainly observed at the 5' end of genes, representing initiation of transcription (Barski, Cuddapah, Cui, Roh, Schones, Wang, Wei, Chepelev and Zhao, 2007). The presence of H3K4me3 accompanying with H3S10p is preventing recruitment of a enzyme that methylases a lysine 9 of histone H3 as a repressive mark (Dormann, Tseng, Allis, Funabiki and Fischle, 2006). During elongating transcript, the lysine 36 of histone H3 are methylated by a Set domain containing methyltransferase that is recruited to an active form of RNA pol II phosphorylated at

Figure 5. Distribution of histone modifications across genes to define chromatin structures.

This pattern is generated by genome scale screening by several groups of histone modifications in yeast and mammalian cells by ChIP-seq. The middle represents euchromatin that is enriched in H3K4me2/3 and H3K9ac at TSS of genes during transcription initiation and H3K79me2 and H3K36me3 in the body of genes during transcription elongation. Both ends represent heterochromatin that is compacted more than euchromatin as shown here. As I mentioned before, H3K9me3 and H4K20me3 are mainly enriched in heterochromatin.

Figure5
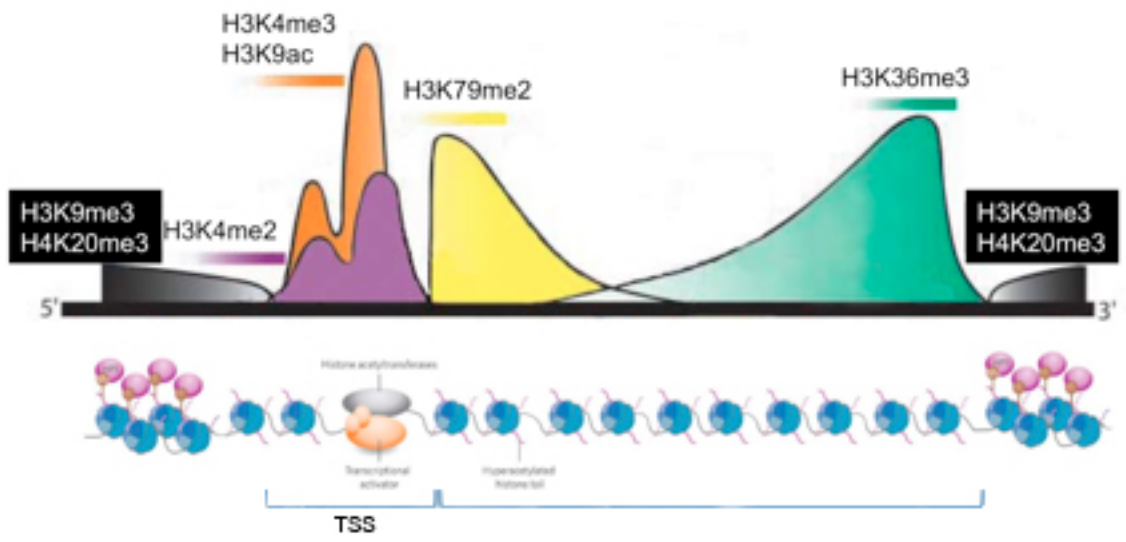
distributed across genes and are associated with transcriptional status (Figure 5). This discrete enrichment of each histone modifications at a given gene suggests that coordination of histone modifications is essential for regulation of gene expression.

- **DNA methylation**

DNA methylation is an epigenetic event that is frequently observed in silenced gene expression. The C-5 position of cytosine is predominantly methylated in CG dinucleotide rich regions that are mainly located within regulatory regions such as gene promoters. These CG rich regions are generally unmethylated, but become methylated upon gene silencing. There are two possibilities to explain how DNA methylation is associated with gene silencing. First, during transcription, the accessibility of regulatory elements embedded into DNA sequences to either transcription machinery or transcription factors is inhibited by DNA methylation. Alternatively, there are proteins that specifically recognize methylated DNA such as the methyl-CpG-binding domain (MBD) containing protein, MeCP2. MBD proteins exist in mammals and bind to methylated CG residues in *in vitro* (Ng, Zhang, Hendrich, Johnson, Turner, Erdjument-Bromage, Tempst, Reinberg and Bird, 1999). Secondly, the methylated DNA may influence the configuration of chromatin structures. Indeed, high levels of DNA methylation affect the preference of histones to incorporate into certain DNA sequence in *in vitro* (Davey, Pennings and Allan, 1997).

Interaction between histone methyltransferase and DNA methyltransferase supports that DNA methylation is associated with configuration of chromatin structure (Cedar and Bergman, 2009). In embryonic stem cells, SUV39H1 and SUV39H2 responsible for

H3K9me3 recruit DNA methyltransferase 3A and 3B to add methyl groups on CpG residues in satellite repetitive DNA (Lehnertz, Ueda, Derijck, Braunschweig, Perez-Burgos, Kubicek, Chen, Li, Jenuwein and Peters, 2003). The methylated CpG is possibly recognized by MBD proteins, which recruit chromatin modifying/remodeling complexes leading to further heterochromatinization (Jones, Veenstra, Wade, Vermaak, Kass, Landsberger, Strouboulis and Wolffe, 1998). However, the relation between chromatin structures and DNA methylation in gene expression is not yet completely clear.

Suggested effect of the GAA repeats at the *FXN* gene leading to FXN deficiency in FRDA patients.

Among various factors contributing to heterochromatin formation, high density of repetitive DNA, such as found in transposons, is a main stimulator across the genome (Grewal and Jia, 2007). Hyperexpanded GAA repeats have been reported to adopt a heterochromatin-like structure that is characterized by increased levels of di- and trimethylated lysine 9 of histone H3 (H3K9me2/3) and decreased acetylation on H3 and H4 at the *FXN* gene (Al-Mahdawi, Pinto, Ismail, Varshney, Lymperi, Sandi, Trabzuni and Pook, 2008; Rai, Soragni, Jenssen, Burnett, Herman, Coppola, Geschwind, Gottesfeld and Pandolfo, 2008; Saveliev, Everett, Sharpe, Webster and Festenstein, 2003; Soragni, Herman, Dent, Gottesfeld, Wells and Napierala, 2008). Inhibition of histone deacetylases restores levels of *FXN* expression in FRDA primary lymphocytes and in a murine model (Herman, Jenssen, Burnett, Soragni, Perlman and Gottesfeld, 2006; Rai, Soragni, Jenssen, Burnett, Herman, Coppola, Geschwind, Gottesfeld and Pandolfo, 2008), and manipulating histone modifications, particularly acetylation, can partially enhance expression of the *FXN* gene. Therefore, these studies suggest that changes in chromatin structure associated with the hyperexpanded GAA repeats induce *FXN* repression. However, it is an open question whether the heterochromatin-like structure induced by the hyperexpanded tract of GAA repeats impacts initiation and/or elongation of *FXN* transcription. Some studies show that the heterochromatin-like structure induced by the hyperexpanded GAA repeats impact promoter activity, which affect initiation of *FXN* transcription (De Biase, Chutake, Rindler and Bidichandani, 2009; Kumari, Biacsi and Usdin). Marks enriched in heterochromatin such as H3K27me3, H3K9me3 and

heterochromatin protein (HP1) are detected at the transcription start site (TSS) of the *FXN* gene in FRDA fibroblast lines, leading to the failure of *FXN* transcription initiation. These repressive marks may also influence expression of *FXN* antisense transcripts in the proximity of the TSS, thereby interfering with initiation of *FXN* sense transcripts in FRDA patients (De Biase, Chutake, Rindler and Bidichandani, 2009). Other studies suggest that both defective initiation and transcript elongation is affected in FRDA (Kumari, Biacsi and Usdin, 2011b). Levels of both H3K4me3, indicator of active transcription initiation at the TSS of the *FXN* gene and H3K36me3, an indicator of transcription elongation, are decreased at the *FXN* gene in FRDA cell lines.

Besides altered histone modifications in the proximity of the hyperexpanded GAA repeats at the *FXN* gene in FRDA, high levels of DNA methylation have been reported in the region upstream of the GAA repeats in FRDA cell lines (Castaldo, Pinelli, Monticelli, Acquaviva, Giacchetti, Filla, Sacchetti, Keller, Avvedimento, Chiariotti and Cocozza, 2008; Greene, Mahishi, Entezam, Kumari and Usdin, 2007). Moreover, the levels of DNA methylation correlate with the number of the GAA repeats (Castaldo, Pinelli, Monticelli, Acquaviva, Giacchetti, Filla, Sacchetti, Keller, Avvedimento, Chiariotti and Cocozza, 2008). Taken together, these observations suggest that epigenetic alterations induced by the hyperexpanded GAA repeats are associated with regulation of FXN expression in FRDA.

Current knowledge gap in molecular mechanisms to understand *FXN* deficiency via the hyperexpanded GAA repeats in FRDA patients.

Epigenetic alterations caused by the hyperexpanded GAA repeats are one of the potential therapeutic targets for curing FRDA. Mouse models containing a longer GAA repeats tract exhibit sensitivity to epigenetic modifiers, suggesting that epigenetic regulation by the GAA repeats is likely a good target for potential drugs in restoring FXN expression (Festenstein, Sharghi-Namini, Fox, Roderick, Tolaini, Norton, Saveliev, Kioussis and Singh, 1999; Saveliev, Everett, Sharpe, Webster and Festenstein, 2003). Moreover, a number of studies showed that specific HDACi are capable of *FXN* expression and increasing histone acetylation in FRDA cells (Herman, Jenssen, Burnett, Soragni, Perlman and Gottesfeld, 2006; Rai, Soragni, Jenssen, Burnett, Herman, Coppola, Geschwind, Gottesfeld and Pandolfo, 2008; Soragni, Herman, Dent, Gottesfeld, Wells and Napierala, 2008). In contrast, levels of H3K9me3, a repressive mark, observed in the proximity of the long GAA repeats are maintained upon HDACi treatment (Herman, Jenssen, Burnett, Soragni, Perlman and Gottesfeld, 2006; Rai, Soragni, Jenssen, Burnett, Herman, Coppola, Geschwind, Gottesfeld and Pandolfo, 2008). Moreover, *FXN* expression has no association with inhibition of H3K9 methylation using a small molecule in FRDA cells (Grabczyk and Usdin, 2000a). These results suggest that two or more epigenetic silencing pathways may be simultaneous involved in regulation of *FXN* expression. Additionally, although DNA methylation is one of major factors associated with gene silencing, investigations of how DNA methylation might be involved in *FXN* deficiency have been limited to date. Therefore, this thesis provides a detailed definition of the spectrum of chromatin structure changes associated with hyperexpanded GAA

repeats in order to further understand the molecular mechanisms underlying *FXN*

deficiency in FRDA cells.

The purpose of this study is to determine whether the hyperexpanded GAA repeats foster epigenetic changes associated with the formation of heterochromatin-like structures leading to inhibition of *FXN* transcription in FRDA.

Chapter 2. Materials and Methods

Some parts of this dissertation were modified from the following journal article:

Eunah Kim, Marek Napierala, Sharon Y.R. Dent. Hyperexpansion of GAA repeats affects post-initiation steps of *FXN* transcription in Friedreich's ataxia, Nucleic Acids Research, (2011) 39(19): 8366-8377.

Cells

Lymphoid cell lines were purchased from NIGMS Human Genetic Cell Repository at

The Coriell Institute for Medical Research, Camden, NJ, USA. I selected three cell lines

derived from FRDA patients: GM15850 harboring two expanded alleles of approximately

650 and 1030 GAA repeats; GM16798 harboring two expanded alleles of approximately

750 and 1000 GAA repeats and GM16209 harboring approximately 800 GAA repeats on

both alleles of the FXN gene. As controls, I also used three lymphoid cell lines

(GM15851, GM03928 and GM05152) derived from healthy, unaffected individuals

containing short GAA repeat tracts within a normal range. Lymphoid cell lines were

grown in RPMI 1640 medium supplemented with 15% FBS, 100U/ml penicillin and 100

µg/ml streptomycin at 37°C in 5% CO_2. The GFP_GAA systems [GFP_(GAA•TTC)$_0$

and GFP_(GAA•TTC)$_{560}$] were generated by integration of a tract of GAA repeats

derived from the GM16210 affected cells as described previously (Soragni, Herman,

Dent, Gottesfeld, Wells and Napierala, 2008). A DNA fragment harboring approximately

560 GAA repeats was PCR amplified from genomic DNA isolated from the GM16210

cell line (NIGMS Human Genetic Cell Repository at The Coriell Institute for Medical

Research, Camden, NJ, USA) as described in (Soragni, Herman, Dent, Gottesfeld, Wells

and Napierala, 2008). PCR product was cleaved by Bsu36I and BssHII endonucleases

and the GAA repeat containing fragment was cloned into intron 1 (1.2Kbp from the

exon1/intron1 junction) of the *GFP* gene of the pGFP_Int plasmid (Seluanov, Mittelman,

Pereira-Smith, Wilson and Gorbunova, 2004; Wilson and Roof, 1997). Plasmids

containing 0 and 560 GAA repeats were integrated by site-specific recombination into the

genome of the HEK293Flp-InT-Rex cell line (Invitrogen). The use of identical sites of

integration for both GFP_(GAA•TTC)$_0$ and GFP_(GAA•TTC)$_{560}$ constructs allows

direct comparison between cell lines and eliminates any potential bias resulting from

random integration events in different chromosomal contexts. Integrants were selected

using hygromycin (200 μg/ml); individual hygromycin-resistant colonies were isolated

and analyzed for repeat size and GFP expression level. Correct splicing of the GFP

mRNA was determined as described earlier (Campuzano, Montermini, Molto, Pianese,

Cossee, Cavalcanti, Monros, Rodius, Duclos, Monticelli, Zara, Canizares, Koutnikova,

Bidichandani, Gellera, Brice, Trouillas, De Michele, Filla, De Frutos, Palau, Patel, Di

Donato, Mandel, Cocozza, Koenig and Pandolfo, 1996). All constructs were sequenced

prior to as well as after establishing the stable cell lines. The GFP_GAA lines were

selectively maintained in Dulbecco's Modified Eagle's Medium with 10% FBS, 100

U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 supplemented with

hygromycin (200 μg/ml) and blasticidin (5 μg/ml). To induce transcription of the GFP

minigene in the GFP_GAA system, cells were treated with 0.1 μg/ml tetracycline for 24h.

Polymerase chain reaction (PCR)

The size of the hyperexpanded tract of GAA repeats in lymphoid cell lines were

determined by conventional PCR using primers 2500F and 629 under previously

described conditions (Campuzano, Montermini, Molto, Pianese, Cossee, Cavalcanti,

Monros, Rodius, Duclos, Monticelli, Zara, Canizares, Koutnikova, Bidichandani, Gellera,

Brice, Trouillas, De Michele, Filla, De Frutos, Palau, Patel, Di Donato, Mandel, Cocozza,

Koenig and Pandolfo, 1996). The insertion of the GAA repeats at an intron of the *GFP*

gene in the GFP_GAA system was detected by the conventional PCR using the primers

listed in Table 1. The PCR products were analyzed on 0.8% agarose gels. To perform

quantitative reverse transcriptase PCR (qRT-PCR), total RNAs from lymphoid cell lines

and the GFP_GAA system were isolated using the RNeasy Mini Kit (Qiagen). DNase I

(TURBO DNA-free; Ambion) was added to remove genomic DNA contamination from

isolated RNAs. The qRT-PCR was conducted using the Power SYBR Green RNA-to-C_T

1-Step Kit (7500 Fast Real Time-PCR System, Applied Biosystems). As a control,

reactions were also performed without reverse transcriptase to confirm a removal of

genomic DNA. To calculate levels of *FXN* expression, the delta Ct value was generated

by subtraction of the Ct value of GAPDH from the Ct value of either *FXN* mRNA or 4

different region transcripts of each cell line. Levels of *FXN* mRNA from each cell line

were normalized to the delta Ct of GM15851. For levels of *FXN* pre-mRNA expression,

each delta Ct value of 4 different regions at the *FXN* gene was normalized to the delta Ct

value of the region upstream of the GAA repeat from GM15851. All primers used in this

study are listed in Tables 1 and 2.

Chromatin immunoprecipitation (ChIP)

ChIP was performed according to the EZ ChIP instructions (Upstate Biotechnology Inc.).

Briefly, proteins and DNA were cross-linked with 1% formaldehyde for 10min (15min in

the case of RNA pol II ChIP) at room temperature. The cross-linking reaction was

quenched with 125mM glycine for 5min (10min in the case of RNA pol II ChIP). Whole

lysates were prepared by using a cell lysis buffer (50mM Tris-HCl at pH8.0, 10mM

EDTA, and 1% SDS) supplemented with protease inhibitor (Mini EDTA-free protease

inhibitor cocktail, Roche) and sonicated to obtain 100 to 300 bp DNA fragments using a

Bioruptor Sonicator (Diagenode). The fragmented chromatin was diluted 10 times with

dilution buffer (16.7mM Tris-HCl at pH8.0, 167mM NaCl, 1.2mM EDTA, 1.1% Triton

X-100, and 0.01% SDS). The chromatin fragments were pre-cleared for 1h using Protein

A agarose (Millipore) pre-blocked with BSA and salmon sperm DNA. The equivalent of

5×10^6 (1×10^7 cells in the case of RNA pol II ChIP) cells was immunoprecipitated

overnight with 5μg of antibodies recognizing histone modifications (15μg of RNA pol II

antibody in the case of RNA pol II ChIP). For total histone H3 and H4, the equivalent of

5×10^6 cells was immunoprecipitated with 3μg of antibodies recognizing histone H3 and

H4 after estimating saturation point between antibody for antibody and antigen. The

immunoprecipitates were immobilized on the pre-blocked protein A agarose beads for 1h.

Subsequently, the beads were washed with buffers containing low salt, high salt, and

LiCl, chromatin was eluted from the beads with elution buffer (100mM $NaHCO_3$ and 1%

SDS) at room temperature for 15min twice and subsequently subjected to the reverse

cross-linking reaction using 5M NaCl at 65°C for at least 5h. The DNAs from chromatin

complexes were isolated with Tris-EDTA buffer (80mM Tris-HCl at pH6.5, and 20mM

EDTA) supplemented with Protienase K and RNase A at 37°C for 30min followed by

42°C for 1h. DNA fragments were purified using phenol/chloroform extraction before

quantitative real-time PCR (qPCR). The qPCR was conducted using the Power SYBR

Green-C_T Kit (7500 Fast Real Time-PCR System, Applied Biosystems). The qPCR was

carried out as follows: 10 min at 94°C, 50 cycles of 30s at 94°C followed by 60s at 60°C.

Since changes in nucleosome occupancy is associated with gene activation and gene

silencing (Bryant, Prabhu, Floer, Wang, Spagna, Schreiber and Ptashne, 2008), it is

worthy to notice that the changes of histone modifications (referred to as the "relative

fold over total H3") determined by qPCR was analyzed by normalizing the quantity of the immunoprecipitated sample to the quantity of total histone H3 (total H4 in the case of H4K20me3) after normalization to inputs.

Antibodies

The antibodies used in this study were anti-rabbit IgG as a negative control (Cell Signaling), anti-total H3 (Cell Signaling or Active Motif) and anti-total H4 (Abcam), anti-H3K9/14ac (Upstate), anti-H3K4me2 (Active Motif), anti-H3K4me3 (Abcam or Active Motif), anti-H3K9me3 (Upstate or Active Motif), anti-H3K27me3 (Abcam), anti-H3K36me3 (Upstate), anti-H3K79me2 (Upstate or Active Motif), anti-H4K20me3 (Active Motif), and an antibody against the large subunit of RNA polymerase II (N20; Santa Cruz Biotechnology).

D-ribofuranosylbenzimidazole (DRB) treatments

To optimize condition of DRB treatments in the lymphoid cell lines, various concentration, 1, 50, and 100 μM of DRB was applied to 1 x 10^6 cells for 5 hr. H3K4me3 distribution was determined using ChIP assay upon inhibition of transcription elongation by DRB in the lymphoid cell lines as described in the ChIP section of Materials and Methods.

Statistics

A 2-way analysis of variance followed by the Bonferroni post-hoc test were performed to determine the statistical significance of the results of qRT-PCR and ChIP analysis between unaffected and FRDA cell lines.

Table 1. Primers used for analyses in the GFP_GAA reporter system.

		Primer (5' to 3')
Repeat	Forward	CTTCCCTTTACACAACGTTTGGGTT
	Reverse	GTACTGTTTGGATTCAGTGAGGGACT
Ex1/Ex2	Forward	GCGACGTAAACGGCCACAAGTT
	Reverse	ATGCCCTTCAGCTCGATGCGGT
Ex1/Int	Forward	GACGACGGCAACTACAAGACC
	Reverse	CTAGGACAAAGGTGCCTAAGACC
Up	Forward	AATAGCCTCCTGACCACAGATCCTT
	Reverse	CCATGTGACATCTAGCCCCGCA
Down	Forward	CCCACAGGCCTGAAACACT
	Reverse	TTCATGCGTGCTAGGGTAAA
Int/Ex2	Forward	CCCTAGCACGCATGAACC
	Reverse	ATGCCCTTCAGCTCGATGCGGT*

* This primer is the same as the GFP Ex1/Ex2 reverse primer.

Table 2. Primers used for analyses in the lymphoid cell lines.

		Primer (5' to 3')
Ex3/Ex4	Forward	CCTTGCAGACAAGCCATACA
	Reverse	GGTCCACTGGATGGAGAAGA
In1Ex2	Forward	AGCACTCGGTTACAGGCACT
	Reverse	GCCCAAAGTTCCAGATTTCC
–242	Forward	CGCATTTTATAAACAAGGCACA
	Reverse	GTATGTGGGGCCAGGAGAC
Pro (or -133)	Forward	CCCCACATACCCAACTGCTG
	Reverse	GCCCGCCGCTTCTAAAATTC
+48	Forward	AAGCAGGCTCTCCATTTTTG
	Reverse	CCGCAGGCACTCTTCTGT
Up (or +1231)	Forward	GAAACCCAAAGAATGGCTGTG
	Reverse	TTCCCTCCTCGTGAAACACC
+1394	Forward	GGTACGCCGCATGTATTAGG
	Reverse	GCAACCAATCCCAAAGTTTC
Down	Forward	CTGGAAAAATAGGCAAGTGTGG
	Reverse	CAGGGGTGGAAGCCCAATAC
In2Ex3	Forward	GGTAATCATGTTTTGGGTTTTGTGC
	Reverse	AGTCCTCAAACGTGTATGGCTTGTC

Number on the name of primers denotes the first base of forward primer on the target region with respect to the TSS, +1.

Chapter 3. Defining of chromatin structure in the vicinity of the hyperexpanded GAA repeats at the *FXN* gene

Some parts of this dissertation were modified from the following journal article:

Eunah Kim, Marek Napierala, Sharon Y.R. Dent. Hyperexpansion of GAA repeats affects post-initiation steps of *FXN* transcription in Friedreich's ataxia, Nucleic Acids Research, (2011) 39(19): 8366-8377.

GAA repeat expansion and *FXN* deficiency in the lymphoid cell lines

In order to discriminate lengths of the GAA repeats at the *FXN* gene in the lymphoid cell lines from unaffected (GM15851, GM03928 and GM05152) and FRDA patients (GM15850, GM16798 and GM16209), conventional PCR was carried out using a primer set that recognizes sequences surrounding where the GAA repeats at the *FXN* gene as previously reported (Campuzano, Montermini, Molto, Pianese, Cossee, Cavalcanti, Monros, Rodius, Duclos, Monticelli, Zara, Canizares, Koutnikova, Bidichandani, Gellera, Brice, Trouillas, De Michele, Filla, De Frutos, Palau, Patel, Di Donato, Mandel, Cocozza, Koenig and Pandolfo, 1996). A band around 500 bp is detected in unaffected controls, whereas FRDA lymphoid cell lines exhibit expanded GAA repeats up to 3Kbp (Figure 6). This pattern is consistent with number of the GAA repeats reported in NIGMS Human Genetic Cell Repository at The Coriell Institute for Medical Research as described in Materials and Methods.

To confirm insufficient levels of *FXN* transcription in FRDA cell lines, *FXN* mature RNA was masured by qRT–PCR using a primer set recognizing exon 3 as a forward primer and exon 4 as a reverse primer (Figure 7). Clinically affected FRDA patients express as little as 5% and as much as 35% of *FXN* relative to unaffected individuals (Campuzano, Montermini, Molto, Pianese, Cossee, Cavalcanti, Monros, Rodius, Duclos, Monticelli, Zara, Canizares, Koutnikova, Bidichandani, Gellera, Brice, Trouillas, De Michele, Filla, De Frutos, Palau, Patel, Di Donato, Mandel, Cocozza, Koenig and Pandolfo, 1996; Pianese, Turano, Lo Casale, De Biase, Giacchetti, Monticelli, Criscuolo, Filla and

Cocozza, 2004). In order to establish a common epigenetic profile in FRDA cell lines, I selected three cell lines expressing low (GM16798), medium (GM15850), and high (GM16209) levels of the *FXN* mRNA corresponding to 8, 17 and 29% of the *FXN* mRNA found in the GM15851, respectively.

Figure 6. Hyperexpanded GAA repeats at the *FXN* gene in the lymphoid cell lines.

Hyperexpanded tract of GAA repeats (indicated by the asterisk) was confirmed by conventional PCR in lymphoid cell lines derived from patients with FRDA but not in cell lines derived from unaffected individuals.

Figure 6
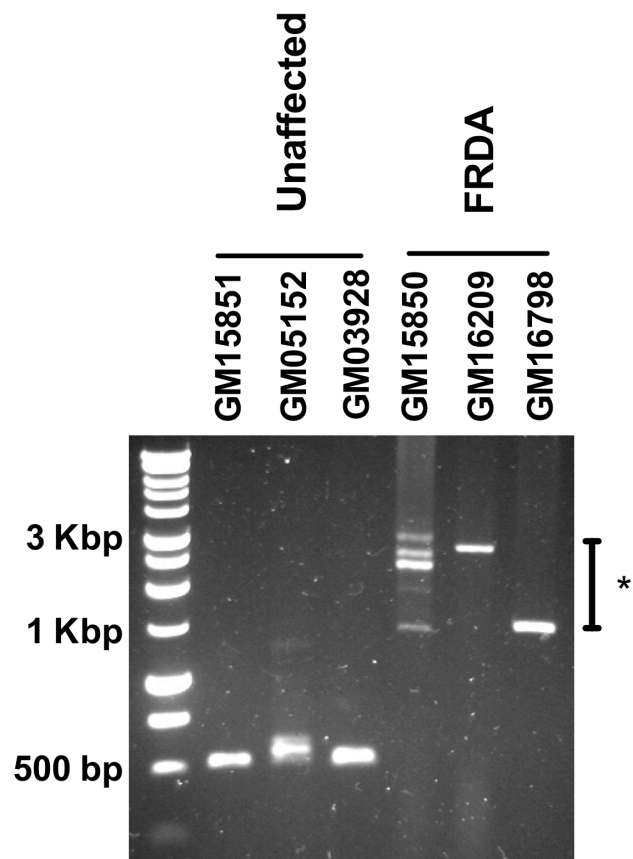
Figure 7. *FXN* transcription is impaired in FRDA lymphoid cell lines.

FXN mRNA was analyzed in 3 control and 3 FRDA cell lines by qRT-PCR with primers complementary to the exons 3 and 4. White bars designate control cell lines, while gray bars designate FRDA cell lines. Each cell line has different textures. *FXN* expression was normalized to the expression level determined for control GM15851 cells. The experiment was conducted in triplicate. All data are expressed as mean ± SD. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 7
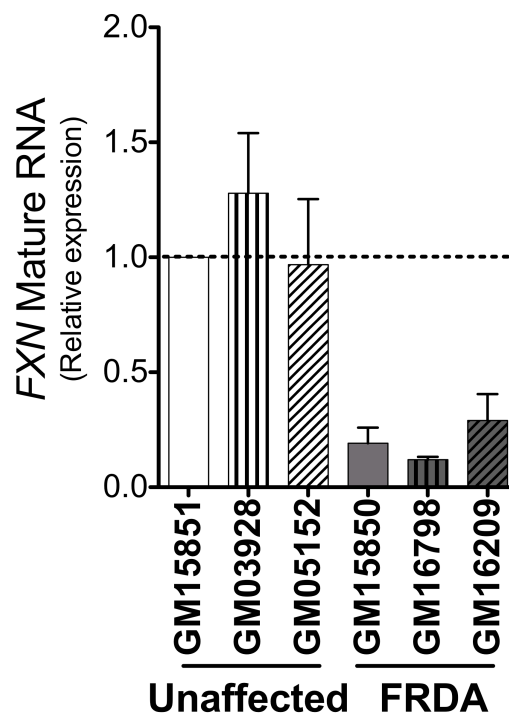
Distribution of histone modification at the *FXN* gene in the lymphoid cell lines

To determine if the hyperexpanded GAA repeats might influence chromatin structures, chromatin immunoprecipitation (ChIP) was used to examine histone modifications in the lymphoid cell lines. Chromatin from the lymphoid cell lines was fragmented to obtain a majority of chromatin fragment under 500 bp (Figure 8). All six lymphoid cell lines, including three unaffected cells and three FRDA, were applied to examine each histone modification in the following text and figures. Additionally, to demonstrate that the hyperexpanded GAA repeats is sufficient to induce locally repressive chromatin structure, I took advantage of the GFP_GAA reporter system that is engineered to harbor 560 GAA repeats at the *GFP* gene introduced into HEK293 cells. Each value in the ChIP experiments discussed below is represented as the relative fold over total H3 of DNA recovery against each histone modifications when normalized to total H3. Occupancy of total H3 is not affected by the presence of the hyperexpanded GAA repeats in either the lymphoid cell lines or the GFP_GAA system (Figure 9).

H3K9/14ac

To confirm the decreased levels of acetylation of histones at the *FXN* gene in FRDA observed in other reports, I performed ChIP for H3K9/14ac in the lymphoid cell lines. H3K9/14ac is well-known to be acetylated by GCN5 and p300/CBP-associated factor (PCAF), chromatin remodelers, in eukaryotes (Bhaumik, 2011). Four primer sets were used to define the distribution of H3K9/14ac at the *FXN* gene. I observed that the levels of H3K9/14ac, when normalized for nucleosomal occupancy, were decreased at the *FXN*

Figure 8. Fragmented chromatin extracted from the lymphoid cell lines and the GFP_GAA reporter system.

Fragmented chromatin was detected using a 0.8% agarose gel. The majority of resulting fragmentation is between 100 and 500 bp in length.

Figure 8
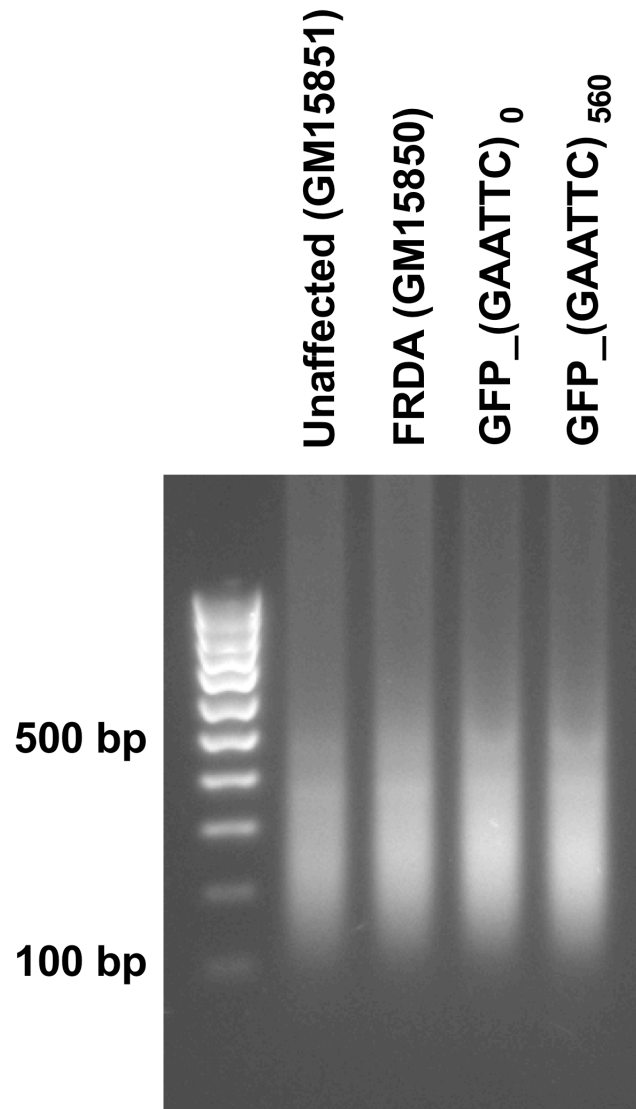
Figure 9. Hyperexpanded GAA repeats have no association with distribution of total histone H3.

Total histone H3 in lymphoid cell lines and the GFP_GAA reporter system was determined by ChIP. DNA from chromatin immunoprecipitated using antibodies specific for total histone H3 was subjected to qPCR using primers amplifying **(A)** promoter region, fragments upstream (Up) and downstream (Down) of the GAA repeats and junction between intron 2 and exon 3 (In2Ex3) in the lymphoid cell liens, and **(B)** Ex1/Int, a junction between exon 1 and the intron; Up, region upstream of the GAA repeats; Down, region downstream of the GAA repeats; Int1/Ex2, a junction between the intron and exon2 for the GFP_GAA reporter system. Total histone H3 levels are normalized to 1% of the input (5×10^6 cells). White bars designate control cell lines, while gray bars designate FRDA cell lines. Each cell line has different textures. No major differences of total histone H3 distribution are observed at the entire *FXN* gene in the lymphoid cell lines.

Figure 9

A.

B.
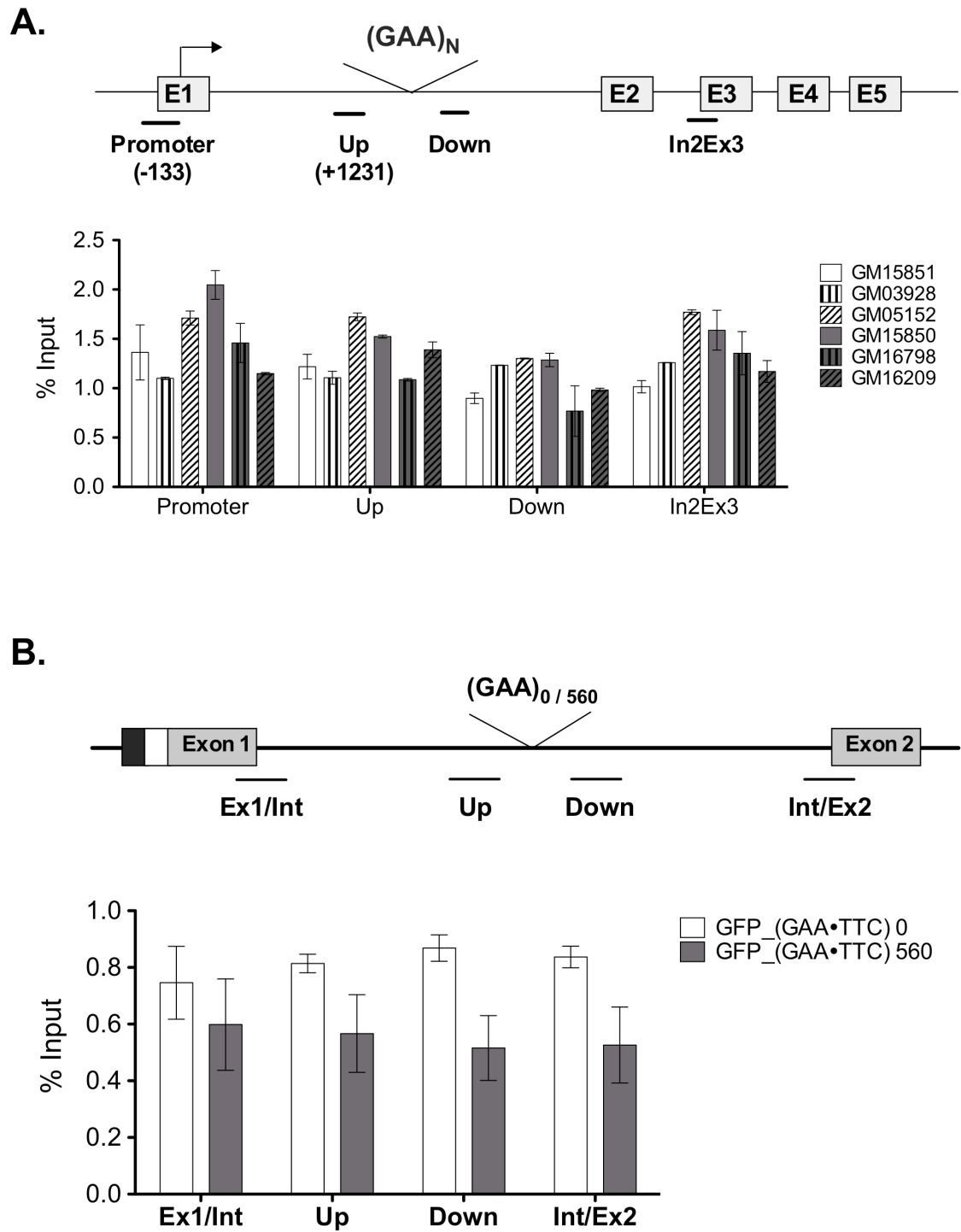
gene in FRDA cell lines as expected. Specifically the levels of H3K9/14ac were the

lowest at the region upstream of the GAA repeats in FRDA cells when compared with

unaffected cells (Figure 10). On the contrary, in promoter region of the *FXN* gene, the

levels of H3K9/14ac were similar in both groups. This suggests that the hyperexpanded

GAA repeats are associated with repressive chromatin structure, but this structure dose

not extend to the promoter in FRDA cell lines.

H3K9me3

To add more evidence that the *FXN* gene in patients with FRDA has a repressive

chromatin structure, I examined levels of H3K9me3 at the *FXN* gene by ChIP (Figure

11). High levels of H3K9me3 are observed within heterochromatin such as is found at

centromeres (Wang, Schones and Zhao, 2009). This methylation is most likely associated

with silencing of gene expression. At the *FXN* gene, H3K9me3, when normalized for

nucleosomal occupancy, was greatly enriched in the region upstream of the GAA repeats

where lower levels of H3K9/14ac were observed in FRDA cell lines.

H4K20me3

Although H3K9me3 is preferentially enriched at heterochromatin, this histone

modification is also detected in the coding regions of transcriptionally active genes

(Vakoc, Mandat, Olenchock and Blobel, 2005). In centromeric region, enrichment of

H4K20me3 is accompanied by enrichment of H3K9me3

Figure 10. H3K9/14ac is significantly repressed at the *FXN* gene in FRDA cells.

Distribution of H3K9/14ac at the *FXN* gene was defined by ChIP assay. DNA from chromatin immunoprecipitated using antibodies specific for H3K9/14ac was subjected to qPCR using primers amplifying promoter region, fragments upstream (Up) and downstream (Down) of the GAA repeats and junction between intron 2 and exon 3 (In2Ex3). The experiment was conducted in triplicate using three FRDA and three control cell lines. Data are expressed as mean ± SEM. *P* values were calculated by comparing the averages of the relative fold over total H3 between unaffected and FRDA lines. Levels of H3K9/14ac was significantly decreased in at the region upstream of the GAA repeats in FRDA cells. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 10

H3K9/14ac
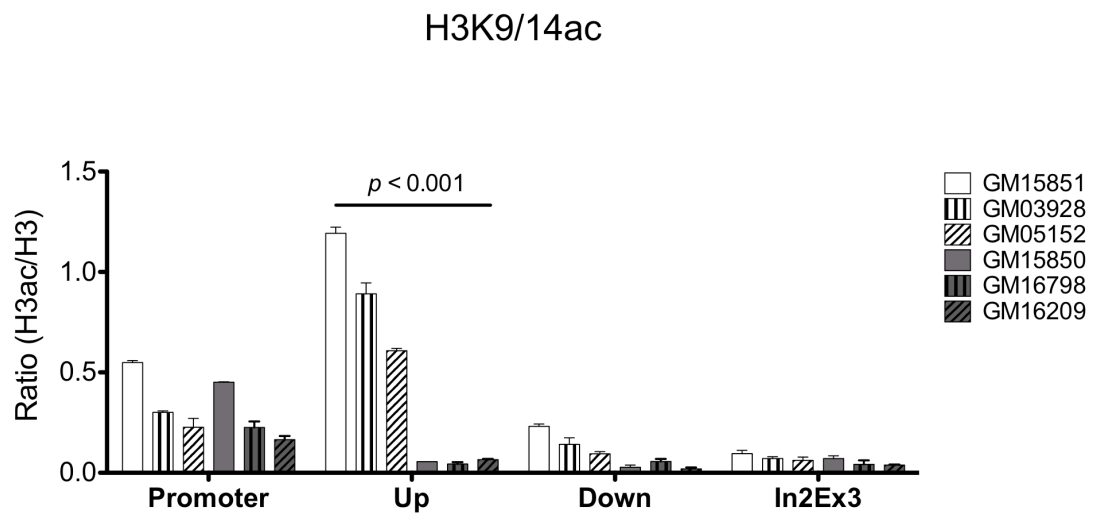
Figure 11. H3K9me3 is significantly increased at the *FXN* gene in FRDA cells.

Distribution of H3K9me3 at the *FXN* gene was defined by ChIP assay. The ChIP experiment was conducted in triplicate using three FRDA and three control cell lines. Data are expressed as mean ± SEM. The region upstream of the GAA repeats in FRDA cells is enriched with levels of H3K9me3. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 11

H3K9me3

Figure 12. H4K20me3 is significantly increased at the *FXN* gene in FRDA cells.

Distribution of H4K20me3 at the *FXN* gene was defined by ChIP assay. The ChIP

experiment was conducted in triplicate using three FRDA and three control cell lines.

Data are expressed as mean ± SEM. The region upstream of the GAA repeats in FRDA

cells is enriched with levels of H4K20me3. Permission to re-use by Oxford University

Press on behalf of the Nucleic Acids Research.

Figure 12
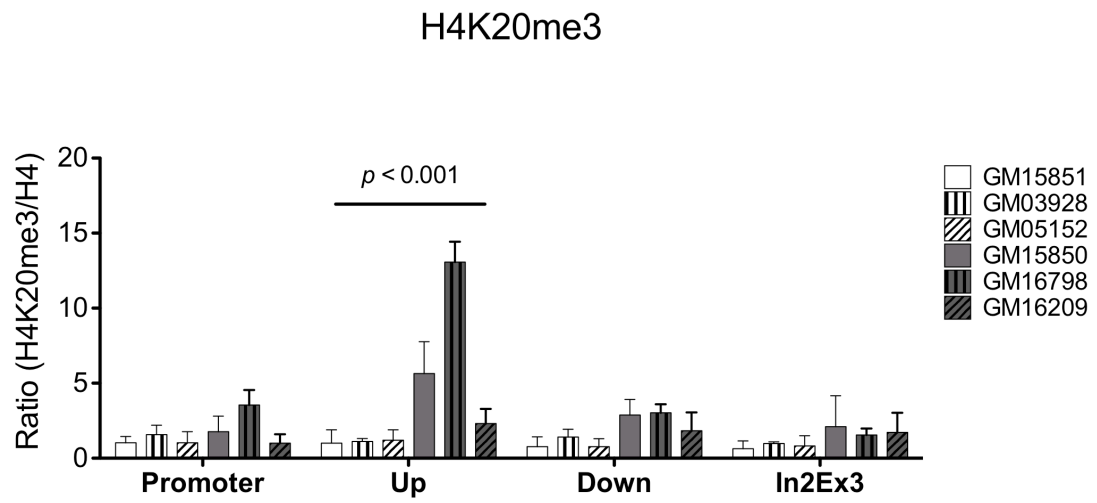
H4K20me3

(Kourmouli, Jeppesen, Mahadevhaiah, Burgoyne, Wu, Gilbert, Bongiorni, Prantera, Fanti, Pimpinelli, Shi, Fundele and Singh, 2004; Schotta, Lachner, Sarma, Ebert, Sengupta, Reuter, Reinberg and Jenuwein, 2004). To ascertain whether high levels of H3K9me3 at the *FXN* gene in FRDA cells are associated with the heterochromatin-like structure induced by proximate regions of the hyperexpanded GAA repeats, I determined the status of H4K20me3 at the *FXN* gene in FRDA and control cell lines (Figure 12). The levels of H4K20me3, when normalized for nucleosomal occupancy, were the highest at the region upstream of the GAA repeats in FRDA cell lines. The results of ChIP experiments indicate that enrichment of H3K9me3 was associated with those of H4K20me3 at the region upstream of the hyperexpanded GAA repeats in FRDA cells. These data demonstrate that the region upstream of the hyperexpanded GAA repeats facilitate a heterochromatin-like structure in FRDA cell lines.

H3K27me3

Repressive chromatin structures, such as the inactive X chromosome and silenced inducible genes such as the bivalent gene, are frequently associated with enrichment of H3K27me3 (Plath, Fang, Mlynarczyk-Evans, Cao, Worringer, Wang, de la Cruz, Otte, Panning and Zhang, 2003; Seenundun, Rampalli, Liu, Aziz, Palii, Hong, Blais, Brand, Ge and Dilworth, 2010). To further define change of chromatin configuration in the presence of the hyperexpanded GAA repeats, I examined distribution of H3K27me3 at the *FXN* gene in the lymphoid cell lines (Figure 13). ChIP assay revealed over representation of H3K27me3, when normalized for nucleosomal occupancy, in the region upstream of the

GAA repeats in FRDA cell lines, co-localizing with both H3K9me3 and H4K20me3 enrichment.

Taken together, I found that the expansion of GAA repeats induces a particular constellation of histone modifications associated with heterochromatin-like structures at the *FXN* gene in FRDA cells.
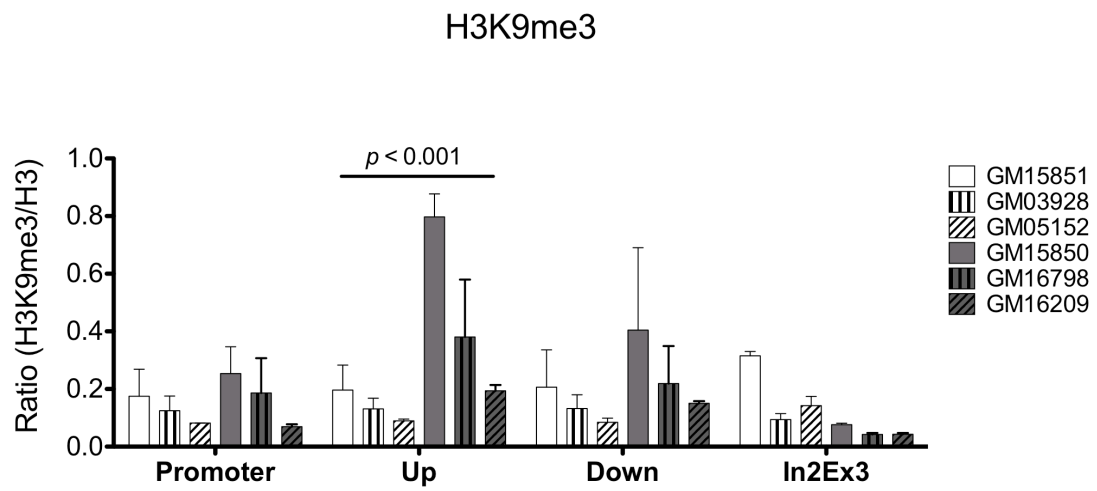
Figure 13. H3K27me3 is enriched at the *FXN* gene in FRDA cells.

Distribution of H3K27me3 at the *FXN* gene was defined by ChIP assay. The ChIP

experiment was conducted in triplicate using three FRDA and three control cell lines.

Data are expressed as mean ± SEM. The region upstream of the GAA repeats in FRDA

cells is enriched with levels of H3K27me3. Permission to re-use by Oxford University

Press on behalf of the Nucleic Acids Research.

Figure 13

H3K27me3
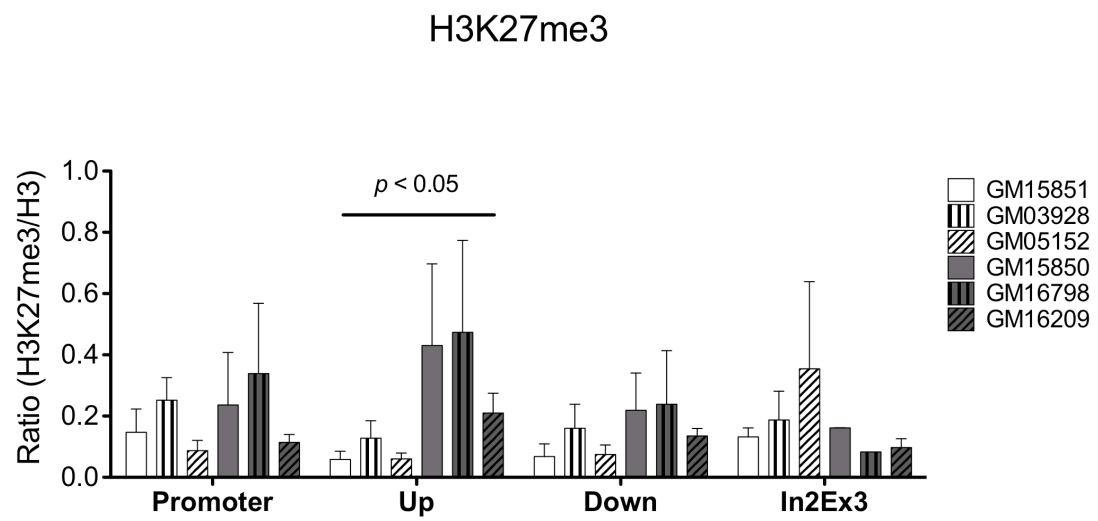
Distribution of histone modification in the GFP_GAA reporter system

To investigate whether the alteration of histone modifications at the *FXN* gene results from either inherent characteristics of the *FXN* gene or the hyperexpanded GAA repeats *per se*, I used a GFP_GAA reporter system that harbors a single copy of the GFP reporter gene containing 560 or 0 GAA repeats in the intron (Figure 14). I determined an effect of integrated tract of the GAA repeats on transcription using qRT-PCR. Mature RNA of *GFP* is decreased in the GFP_(GAA•TTC) $_{560}$ cells compared to the GFP_(GAA•TTC) $_0$ cells under the tetracycline treatment (Figure 15), indicating that the GFP_GAA system genetically resembles FRDA.

To assess changes in chromatin structures caused by presence of longer GAA repeats in the context of the *GFP* gene, I analyzed the distribution of histone modifications in the vicinity of the GAA repeats in the GFP_GAA reporter system.

H3K9/14ac and H3K9me3

In order to validate that chromatin structure flanking the hyperexpanded GAA repeats has characteristics of a heterochromatin-like structure, I examined distribution of H3K9/14ac as a mark for open chromatin (Figure 16) and H3K9me3 as a mark for closed chromatin in the GFP_GAA system (Figure 17). To further define whether the tract containing 560 GAA repeats has ability to bidirectionally spread across the *GFP* gene as characteristics of heterochromatin, 2 more primer sets, a junction between exon1 and the intron and a junction between the intron and exon 2 were used to perform qPCR following

Figure 14. Validation of the presence of 560 GAA repeats in the GFP_GAA system.

The presence of 560 GAA repeats in the GFP_GAA reporter system repeats was

confirmed by conventional PCR. The region flanking 560 GAA repeats was amplified by

primer sets, Up and Down for the *GFP* gene listed in Table 1. The GFP_(GAA•TCC) $_{560}$

cells exhibit approximately 3Kb of PCR product, indicating that appropriate interstion

and maintenance of 560 GAA repeats at the *GFP* gene in the GFP_(GAA•TCC) $_{560}$ cells.

Figure 14
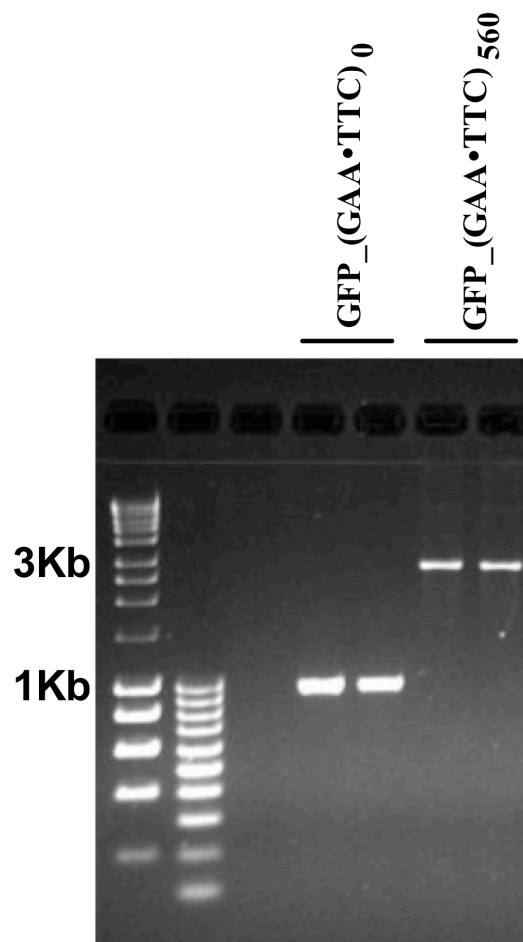
Figure 15. *GFP* transcription is impaired in the GFP_(GAA•TCC) $_{560}$ cells.

GFP mRNA was analyzed in the GFP_GAA reporter system with primers complementary to the exons 1 and exon 2. White bars designate control cell lines (the GFP_(GAA•TCC) $_{0}$ cells), while gray bars designate the GFP_(GAA•TCC) $_{560}$ cells. Each cell line has different textures. The experiment was conducted in triplicate. All data are expressed as mean ± SD. Asterisk indicates statistical significance ($p < 0.05$). The longer GAA repeats is associated with *GFP* deficiency in the GFP_(GAA•TCC) $_{560}$ cells.

Figure 15
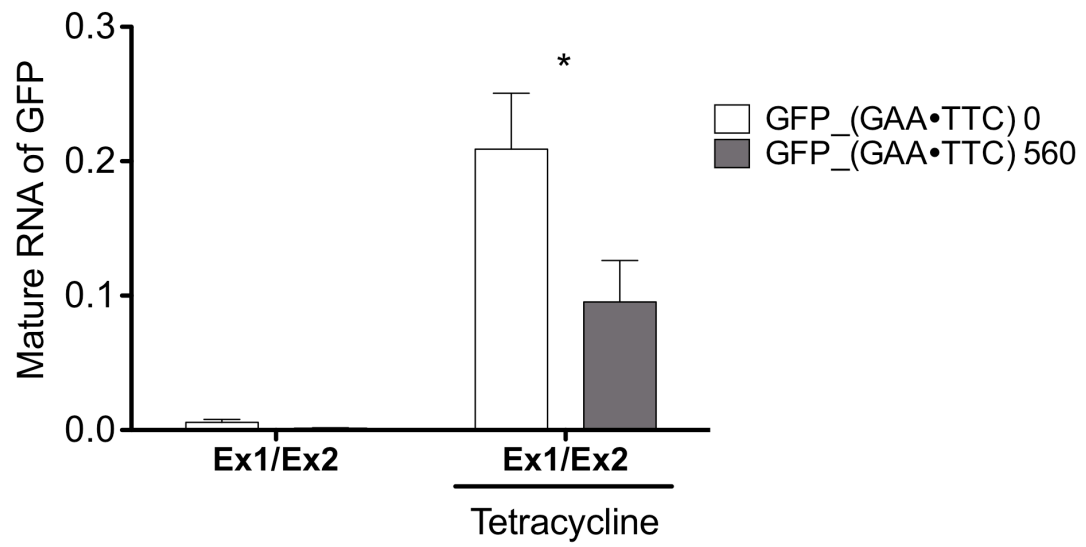
immunoprecipitation of chromatin against each histone modification. I confirmed

decreased levels of H3K9/14ac and increased levels of H3K9me3, when normalized for

nucleosomal occupancy, in the vicinity of the GAA repeats at the *GFP* gene in the

GFP_(GAA•TTC) $_{560}$ cells as shown in previous study (Soragni, Herman, Dent,

Gottesfeld, Wells and Napierala, 2008). Furthermore, I observed that both H3K9/14ac

and H3K9me3, when normalized for nucleosomal occupancy, extended up to the junction

between exon 1 and the intron as well as the junction between the intron and exon 2 of

the *GFP* gene in the GFP_(GAA•TTC) $_{560}$ cells. These results demonstrate that the

introduced GAA repeat induces condensed chromatin structures in the proximity of the

GAA repeats. Moreover, these results suggest that the hyperexpanded tract of GAA

repeats *per se* has the ability to induce spreading of condensed chromatin structures.

Figure 16. H3K9/14ac is significantly decreased at the *GFP* gene in the presence of GAA repeats.

Upon tetracycline treatment, the levels of H3K9/14ac in the GFP_GAA reporter system were determined by ChIP assay. White bars represent the GFP_(GAA•TTC) $_0$ cells. Gray bars represent the GFP_(GAA•TTC) $_{560}$ cells. Data are expressed as mean ± SEM. Asterisk indicates statistical significance (one: $p < 0.05$, two: $p < 0.01$, three: $p < 0.001$). The abundance of histone modifications is shown relative to input DNA in the PCR and normalized to a total H3 for each region. Levels of H3K9/14 are less enriched at the entire GFP gene in the GFP_(GAA•TTC) $_{560}$ cells relative to the GFP_(GAA•TTC) $_0$ cells. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 16

H3K9/14ac

Bar chart titled "H3K9/14ac" with y-axis labeled "H3K9K14ac/H3" ranging from 0 to 8. X-axis categories: Ex1/Int, Up, Down, Int/Ex2. Legend: GFP_(GAA•TTC) 0 (white bars), GFP_(GAA•TTC) 560 (gray bars). Significance markers: Ex1/Int **, Up ***, Down ***, Int/Ex2 **.
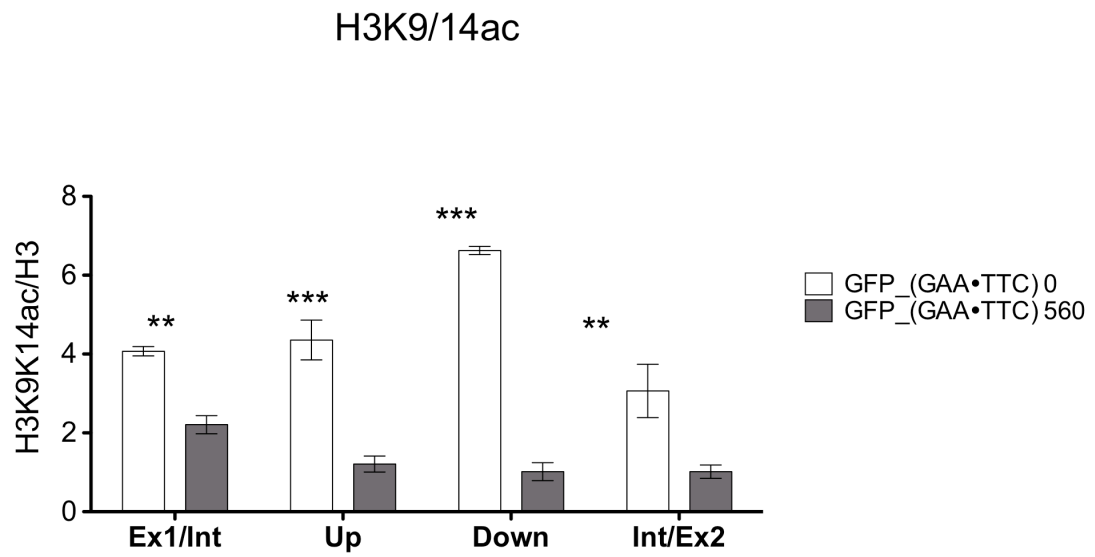
Figure 17. H3K9me3 is enriched at the *GFP* gene in the presence of longer GAA repeats.

The levels of H3K9me3 in the GFP_GAA reporter system were determined in active transcription. White bars represent the GFP_(GAA•TTC) $_0$ cells. Gray bars represent the GFP_(GAA•TTC) $_{560}$ cells. Data are expressed as mean ± SEM. Asterisk indicates statistical significance (one: $p < 0.05$, two: $p < 0.01$, three: $p < 0.001$). The abundance of histone modifications is shown relative to input DNA in the PCR and normalized to a total H3 for each region. H3K9me3 are significantly increased in the GFP_(GAA•TTC) $_{560}$ cells. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 17

H3K9me3
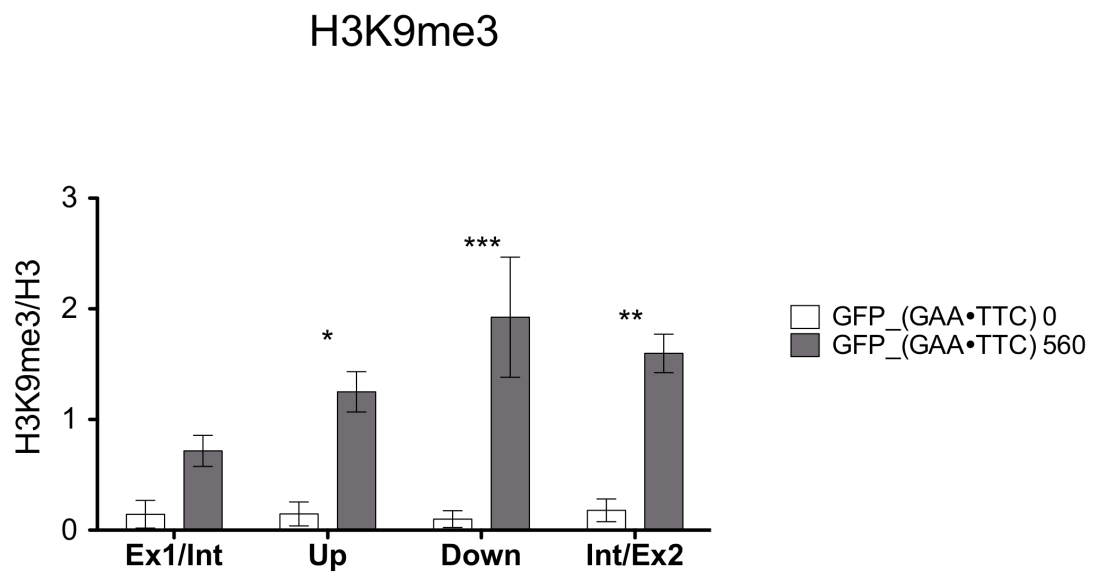
Chapter 4. Determination of the effect of the GAA repeats in transcription in the context of chromatin structure

In lymphoid cell lines

FXN transcription in lymphoid cells

In order to assess the effect of the hyperexpanded GAA repeats on the transcription

progression at the *FXN* gene, I examined levels of *FXN* pre-mRNA in one upstream

(from +1231 to +1344 within the intron 1) and in three regions downstream (variable

positions, depending on repeat tract length) of the GAA repeats (Figure 18). Importantly,

all primers pairs used in this experiment recognize *FXN* pre-mRNA to avoid a bias from

the *FXN* mRNA. I found that levels of *FXN* pre-mRNA at the region upstream of the

hyperexpanded GAA repeats have no differences in all three unaffected control cell lines

and two of the three FRDA lymphoid cell lines. The GM16798 cell line exhibited a lower

level of the *FXN* pre-mRNA upstream of the GAA repeats as compared to the remaining

five cell lines. The GM16798 also exhibited the lowest expression of *FXN* mRNA

(Figure 7). Additionally, FRDA patient from whom the GM16798 cell line was derived

reported the earliest onset of the disease (5 yr vs. 10 and 11 yrs for the other FRDA

individuals). In contrast to the similar levels of *FXN* pre-mRNA upstream of the GAA

repeats, levels of *FXN* pre-mRNA downstream of the GAA repeats were reduced

approximately 4-fold in all three FRDA lymphoid cell lines compared to unaffected cell

lines. This result suggests that levels of the *FXN* pre-mRNA downstream expression are

tightly associated with the pathology of FRDA.

Figure 18. *FXN* deficiency appears at the region downstream of the hyperexpanded GAA repeats in FRDA cells.

Relative level of *FXN* pre-mRNA was determined in various regions of the transcript. One 'Up' and three 'Down' primers amplify regions in the immediate vicinity of the GAA repeats. In1Ex2 and In2Ex3 anneal to the junctions between the corresponding introns and exons of *FXN* pre-mRNA. White bars designate control cell lines, while gray bars designate FRDA cell lines. Each cell line has different textures. *FXN* pre-mRNA expression was normalized to the level of the fragment amplified upstream of the GAA region in the control GM15851 cells. The experiment was performed in triplicate. The region downstream transcript is significantly decreased in FRDA cells compared to unaffected cells. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 18
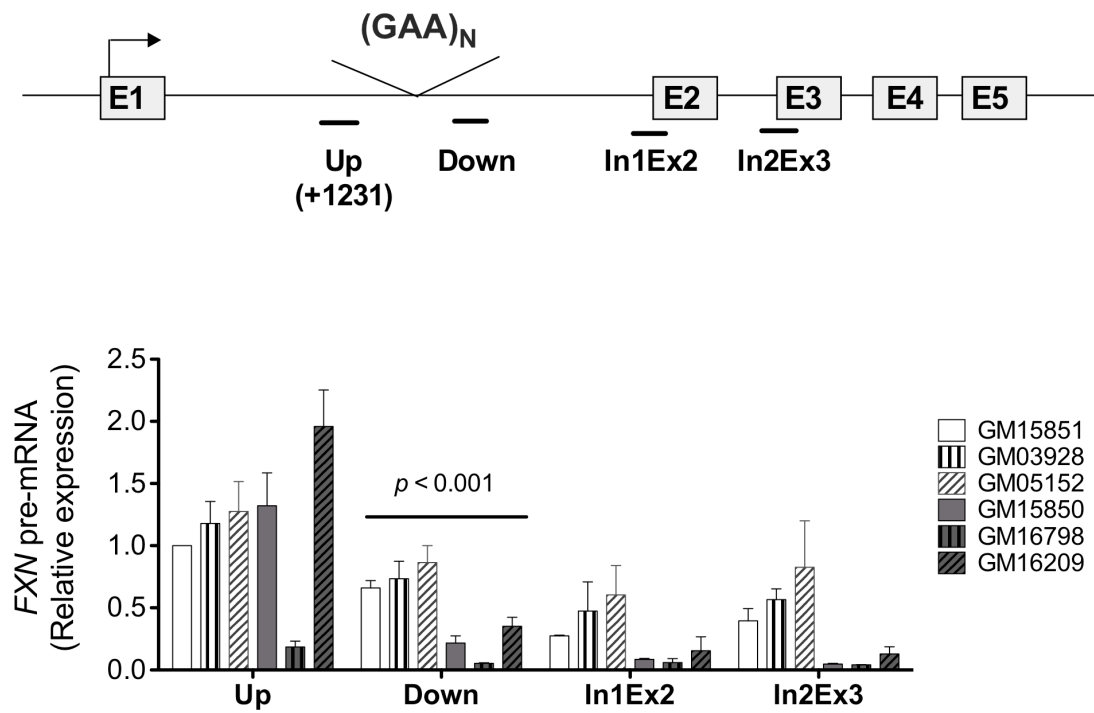
**Distribution of total RNA pol II at the *FXN* gene**

Gene expression is dependent on the accessibility of the DNA sequences to RNA polymerase, transcription factors, and chromatin remodeling complexes. In an effort to further investigate how the hyperexpanded GAA repeats influence progression of *FXN* transcription, I analyzed the distribution of total RNA pol II at the *FXN* gene by ChIP assay (Figure 19). Immunoprecipitation of total RNA pol II include both unphosphorylated and phosphorylated forms of the large subunit of RNA pol II. Seven primer sets were used to map the distribution of RNA Pol II in the *FXN* gene. Based on prior studies showing transcriptional pausing in the proximity of the long GAA tracts in *in vitro* and in cell culture system (Grabczyk and Usdin, 2000b; Krasilnikova, Kireeva, Petrovic, Knijnikova, Kashlev and Mirkin, 2007), I expected that RNA pol II progression would be inhibited in the region upstream of the GAA repeats, resulting in accumulation of transcriptional machinery in the region upstream of the GAA repeats in FRDA cell lines. However, I detected repressed levels of RNA pol II across the entire *FXN* gene in FRDA cell lines relative to unaffected cell lines. Importantly, levels of RNA pol II were not significantly changed at promoter region (from -242 to -12) in FRDA cell lines compared to unaffected cell lines, suggesting that *FXN* promoter activity is not affected in FRDA cell lines. These results correlate with similar levels of *FXN* pre-mRNA upstream of the GAA repeats observed in both FRDA and unaffected control cell lines as shown in Figure 17.

Interestingly, RNA pol II is mostly enriched at a *FXN* promoter-proximal region (from +48 to +150) in unaffected cell lines, even though a number of the GAA repeats is short

Figure 19. Distribution of total RNA pol II is repressed at the *FXN* gene in FRDA cells.

Level of total RNA pol II was determined by immunoprecipitating the large subunit of RNA pol II in FRDA and unaffected cells. Average occupancy of RNA pol II across the *FXN* gene in all three FRDA and three control cells is shown using gray circles and black squares, respectively. The position of the first nucleotide for the forward primer relative to transcription start site (TSS) is indicated below the X axis. The specificity of RNA pol II antibody for ChIP was verified by RNA pol II pausing at the +55 bp of the *GAPDH* gene. Error bars represent standard error of the mean. The experiment was conducted in triplicate using three FRDA and three control cell lines. The *P* value was generated by comparing the average % Input of total RNA pol II between unaffected and FRDA lymphoid cell lines. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 19
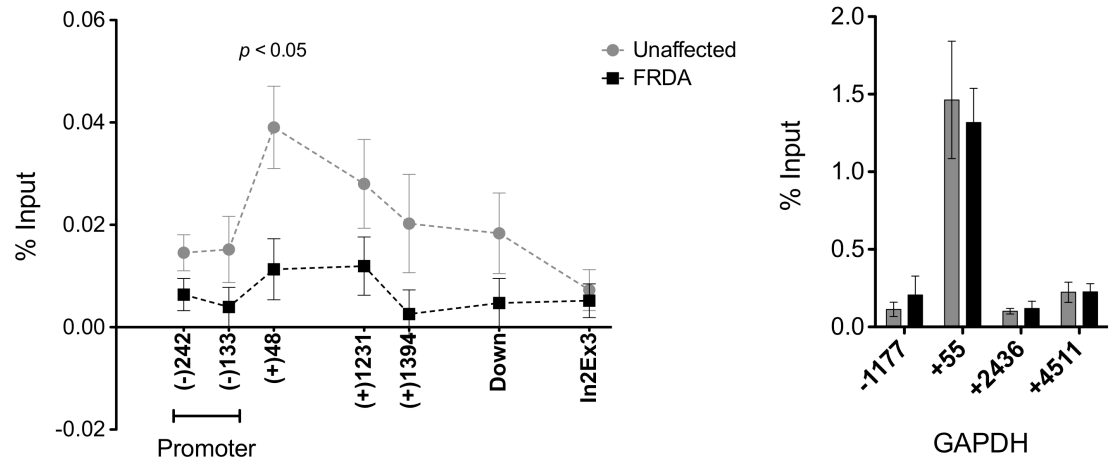
in these cells. The accumulation at (+) 48 bp is significant as compared to either (-)242 bp or (-)133 bp ($p < 0.05$). This phenomenon is also observed in some genes as shown at the *GAPDH* gene (Guenther, Levine, Boyer, Jaenisch and Young, 2007), where RNA pol II is known to pause. No RNA pol II Pausing was detected in the FRDA cell lines. These results suggest that defective transcriptional in FRDA cells originate at a post-initiation step of *FXN* transcription, which result in decreased enrichment of the transcription machinery at the promoter-proximal region as well as throughout the *FXN* gene.

H3K4me2 and H3K4me3

To investigate changes of active chromatin structure caused by the hyperexpanded GAA repeat, histone modifications relative to active chromatin structures was examined by ChIP in the lymphoid cell lines. As indicators of active transcription initiation, levels of both H3K4me2 and H3K4me3 exhibit a peak at transcription start site (TSS) of a given gene that is recently transcribed. Although H3K4me2 and H3K4me3 levels varied among lymphoid cell lines, levels of H3K4me2 and H3K4me3, when normalized for nucleosomal occupancy, were not changed at the promoter region (from −138 to −12) of the *FXN* gene in FRDA cell lines relative to unaffected controls (Figure 20 and Figure 21). These results are correlated with our quantitative *FXN* pre-mRNA analyses showing similar levels of *FXN* pre-mRNA upstream of GAA repeats in both unaffected and FRDA cell lines. Interestingly, H3K4me2 and H3K4me3 were significantly diminished at a region upstream of the GAA repeats (from +1228 to +1344) of the *FXN* gene in FRDA cell lines, while levels of H3K4me2 and H3K4me3 cells, when normalized for nucleosomal occupancy, were increased at this region in the unaffected cells. Enrichment

Figure 20. H3K4me2 is decreased at the region upstream of the GAA repeat, not promoter region in FRDA cells.

Distribution of H3K4me2 at the *FXN* gene was defined by ChIP assay. The ChIP experiment was conducted in triplicate using three FRDA (gray bars) and three control cell lines (white bars). Each cell line has different texture. Data are expressed as mean ± SEM. Levels of H3K4me2 are decreased at the region upstream of the GAA repeat in FRDA cell, while unaffected show great levels of H3K4me2. No differences of H3K4me2 were detected in both FRDA and unaffected cells. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 20
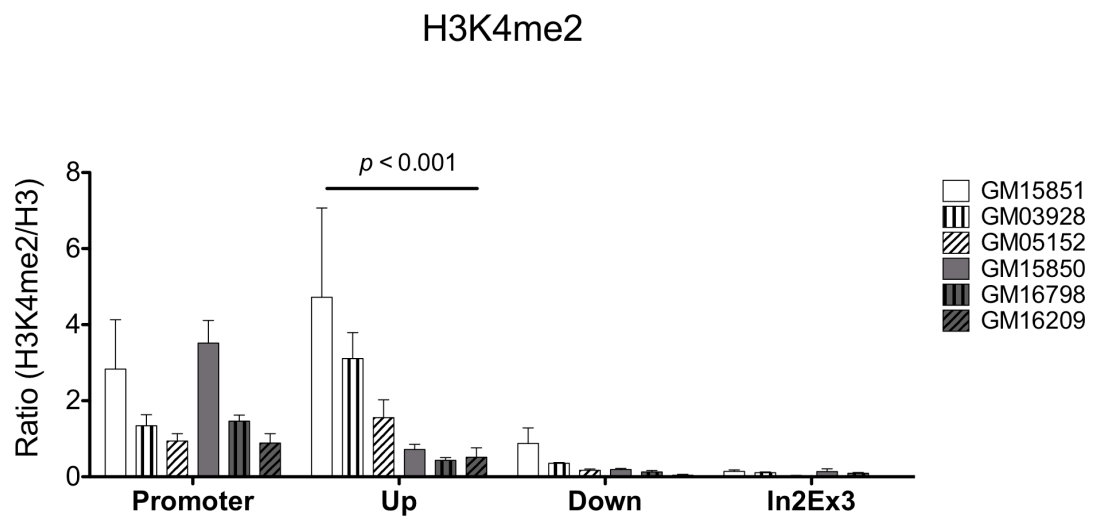
H3K4me2

Figure 21. Levels of H3K4me3 are not affected at promoter region in FRDA cells.

Distribution of H3K4me3 at the *FXN* gene was defined by ChIP assay. The ChIP experiment was conducted in triplicate using three FRDA (gray bars) and three unaffected cell lines (white bars). Each cell line has different texture. Data are expressed as mean ± SEM. Levels of H3K4me3 are not changed at promoter region in FRDA cells compared to unaffected cells. However, H3K4me3 is decreased at the region upstream of the GAA repeats in FRDA cells compared to unaffected cells. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 21
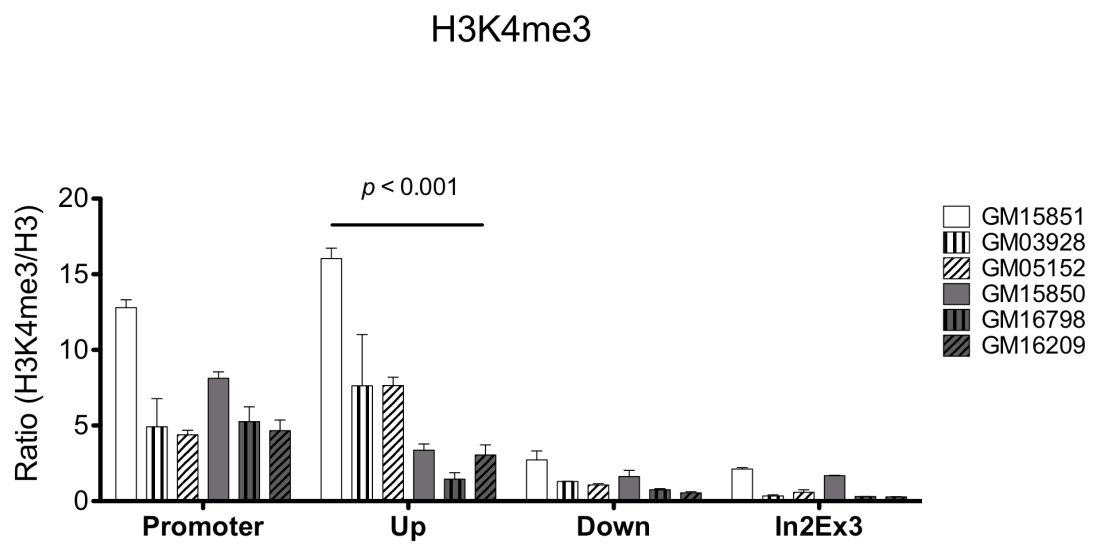
H3K4me3

of H3K4me2 has been associated with non-methylated CpG residues more tightly than transcriptional activity (Okitsu and Hsieh, 2007; Okitsu, Hsieh and Hsieh). In previous studies from other groups, increased levels of DNA methylation in the region upstream of the GAA repeats have been reported in FRDA cell lines relative to unaffected cells (Castaldo, Pinelli, Monticelli, Acquaviva, Giacchetti, Filla, Sacchetti, Keller, Avvedimento, Chiariotti and Cocozza, 2008; Greene, Mahishi, Entezam, Kumari and Usdin, 2007). Thus, the lower levels of H3K4me2 at the region upstream of the GAA repeats in FRDA cell lines could imply hyper DNA methylation, particularly at this region in FRDA patients. Enrichment of H3K4me3 in coding regions is associated with the efficient transition between initiation and elongation of transcription during ongoing transcription (Sims, Millhouse, Chen, Lewis, Erdjument-Bromage, Tempst, Manley and Reinberg, 2007). Therefore, the decreased levels of H3K4me3 in FRDA cell may reflect that the hyperexpanded GAA repeats influence the efficiency of post-initiation of *FXN* transcription.

To further examine whether levels of H3K4me3 at the region upstream of GAA repeats in unaffected cells is associated with the activity of transcription elongation rather than transcription initiation, I measured levels of H3K4me3 upon inhibition of transcription elongation by D-ribofuranosylbenzimidazole (DRB) (Figure 22). DRB, a pharmacologic inhibitor of CDK9, decreases RNA pol II phosphorylation at Serine-2, a hallmark feature of the elongating form of RNA pol II during transcription. Following DRB treatment, the control cell line (GM15851) exhibited a significant decrease of H3K4me3 at the region upstream of the GAA repeats, which was very similar to that observed in FRDA cell line

Figure 22. H3K4me3 at the region upstream of the GAA repeats in FRDA cells is associated with impared *FXN* transcription elongation.

H3K4me3 distribution were determined by ChIP assay upon inhibition of transcription elongation (50 μM DRB for 5 hr). The ChIP experiment was conducted in twice using one FRDA cell (gray bars: GM15850) and one unaffected cell line (white bars: GM15851). Data are expressed as mean ± SEM. H3K4me3 is decreased at the region upstream of the GAA repeats in FRDA cells upon inhibition of transcription elongation.

Figure 22
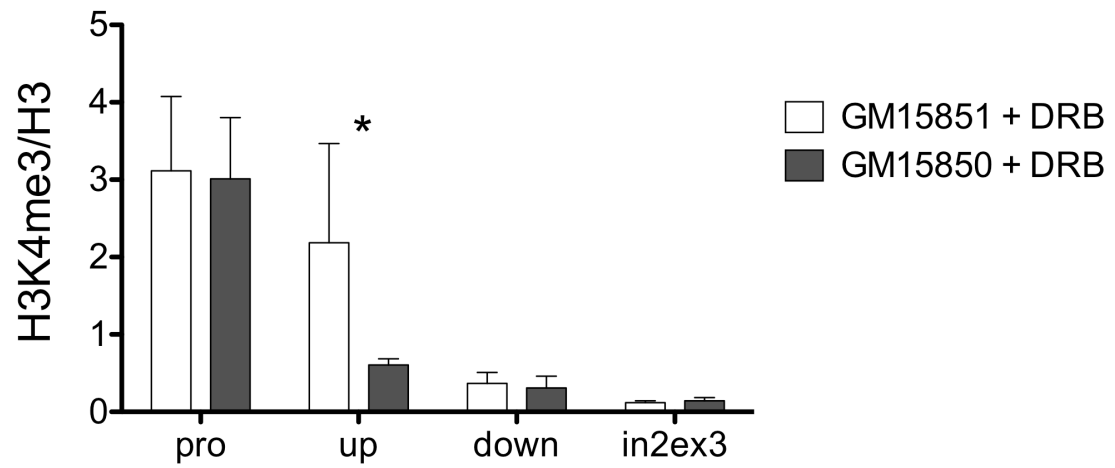
(GM15850), when normalized for nucleosomal occupancy. This result suggests that the levels of H3K4me3 at the region upstream of the GAA repeats are associated with transcription elongation rather than transcription initiation. Taken together, these results suggest that the hyperexpanded GAA repeat does not affect chromatin structures at the *FXN* promoter region but does result in impaired elongation of *FXN* transcription in FRDA.

H3K36me3 and H3K79me2

To further understand the effect of the hyperexpanded GAA repeats on the transcription process throughout the *FXN* gene, I examined the levels of H3K36me3 and H3K79me2, indicators of transcription elongation (Figure 23 and Figure 24). Increasing enrichment of H3K36me3 to the 3'-end of a gene is indicating ongoing transcription elongation. In unaffected cell lines, H3K36me3 levels, when normalized for nucleosomal occupancy, progressively increased toward the 3'-end of the *FXN* gene. However, enrichment of H3K36me3 was repressed across the *FXN* gene in FRDA cell lines. The distribution of H3K36me3 is consistent with total RNA pol II progression shown in Figure 19. These results indicate defective transcription elongation of the *FXN* gene in FRDA cell lines. A genome-wide study revealed that enrichment H3K79me2 at regions downstream of the TSS accompanying with H3K4me3 at the TSS is associated with full-length transcripts of genes (Kanhere, Viiri, Araujo, Rasaiyaah, Bouwman, Whyte, Pereira, Brookes, Walker, Bell, Pombo, Fisher, Young and Jenner, 2010). In contrast, enrichment of H3K4me3 alone is feature for genes that experience transcription initiation alone, without elongation (Rahl, Lin, Seila, Flynn, McCuine, Burge, Sharp and Young, 2010). I found that

H3K79me2, when normalized for nucleosomal occupancy, were dramatically decreased

throughout the *FXN* gene in FRDA cells, while H3K79me2 is pronounced in the vicinity

of the GAA repeats in the unaffected control cells. As shown in Figure 20, since

enrichment of H3K4me3 has no differences at the *FXN* promoter region in FRDA cell

lines compared to unaffected cells, these results further support the that *FXN* deficiency

in FRDA cells results from defective elongation step of *FXN* transcription after

successful initiation.

Figure 23. H3K36me3 are repressed at the entire *FXN* gene in FRDA cells relative to unaffected cells.

Distribution of H3K36me3 at the *FXN* gene was defined by ChIP assay. The ChIP experiment was conducted in triplicate using three FRDA (gray bars) and three unaffected cell lines (white bars). Each cell line has different texture. Data are expressed as mean ± SEM. Levels of H3K36me3 are decreased at the entire *FXN* gene in FRDA cells, while they are gradually increased toward 3' end of the *FXN* gene in unaffected cells. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 23

H3K36me3
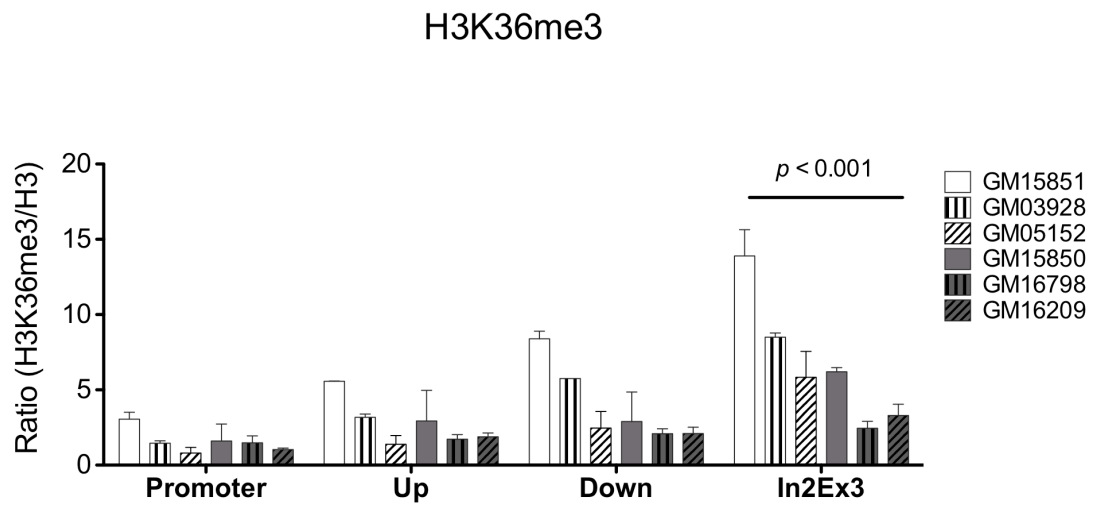
Figure 24. H3K79me2 is significantly decreased in FRDA cells.

Distribution of H3K79me2 at the *FXN* gene was defined by ChIP assay. The ChIP experiment was conducted in triplicate using three FRDA (gray bars) and three unaffected cell lines (white bars). Each cell line has different texture. Data are expressed as mean ± SEM. Levels of H3K79me2 are decreased at the entire *FXN* gene in FRDA cells, while they are enriched in the vicinity of the GAA repeats at the *FXN* gene in unaffected cells. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 24
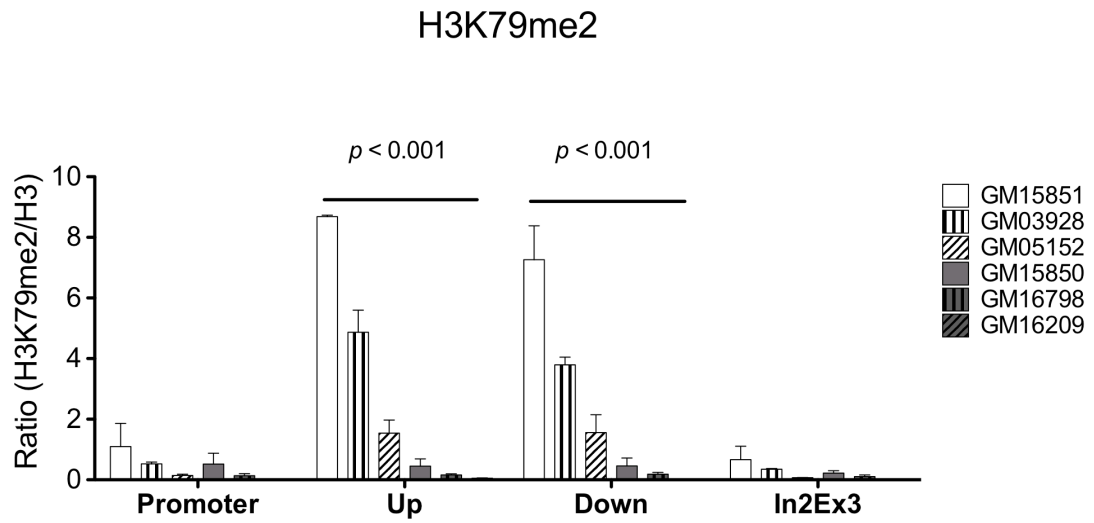
H3K79me2

In the GFP_GAA system

H3K4me3 and H3K79me2

To further investigate the effect of the presence of the GAA repeats in progression of transcription independently of *FXN* promoter or *FXN* intronic sequences, I examined enrichments of H3K4me3 (transcription initiation) and H3K79me2 (transcription elongation) at the *GFP* gene in the GFP_GAA reporter system using ChIP assay when normalized for nucleosomal occupancy (Figure 25 and Figure 26). Levels of H3K4me3 were not changed at the region upstream of the GAA repeats including a junction between exon 1 and the intron and the region upstream of the GAA repeats (named 'Up') in the GFP_(GAA•TTC)$_{560}$ cells compared to the GFP_(GAA•TTC)$_{0}$ cells that lack the GAA repeats. However, H3K4me3 levels were significantly decreased in the region downstream of the GAA repeats in the GFP_(GAA•TTC)$_{560}$ cells. This distribution of H3K4me3 at the *GFP* gene in GFP_(GAA•TTC)$_{560}$ cells is comparable to that found at the *FXN* gene in the FRDA cell lines. In addition, I found that enrichment of H3K79me2 was significantly decreased at the region downstream of the GAA repeats at the *GFP* gene in the GFP_(GAA•TTC)$_{560}$ cells when compared to the corresponding region in the GFP_(GAA•TTC)$_{0}$ cells. These results clearly show that the presence of the hyperexpanded GAA repeats not only stimulate epigenetic changes in the proximate chromatin independently of the DNA context, but also are sufficient to impede transcription elongation. More importantly, the observations of histone modification showing impediment of transcription elongation in the GFP_(GAA•TTC)$_{560}$ cells, without affecting initiation, is consistent with alteration of histone modifications

100

Figure 25. H3K4me3 is affected at the region downstream of the GAA repeats in the presence of the GAA repeats.

The levels of H3K4me3 in the GFP_GAA reporter system were determined by ChIP assay. White bars represent the GFP_(GAA•TTC)$_0$ cells. Gray bars represent the GFP_(GAA•TTC)$_{560}$ cells. Data are expressed as mean ± SEM. Asterisk indicates statistical significance ($p < 0.05$). The abundance of histone modifications is shown relative to input DNA in the PCR and normalized to a total H3 for each region. Upon tetracycline treatment, H3K4me3 are affected at the region downstream of the GAA repeats in the GFP_(GAA•TTC)$_{560}$ cells compared to the GFP_(GAA•TTC)$_0$ cells. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 25

H3K4me3
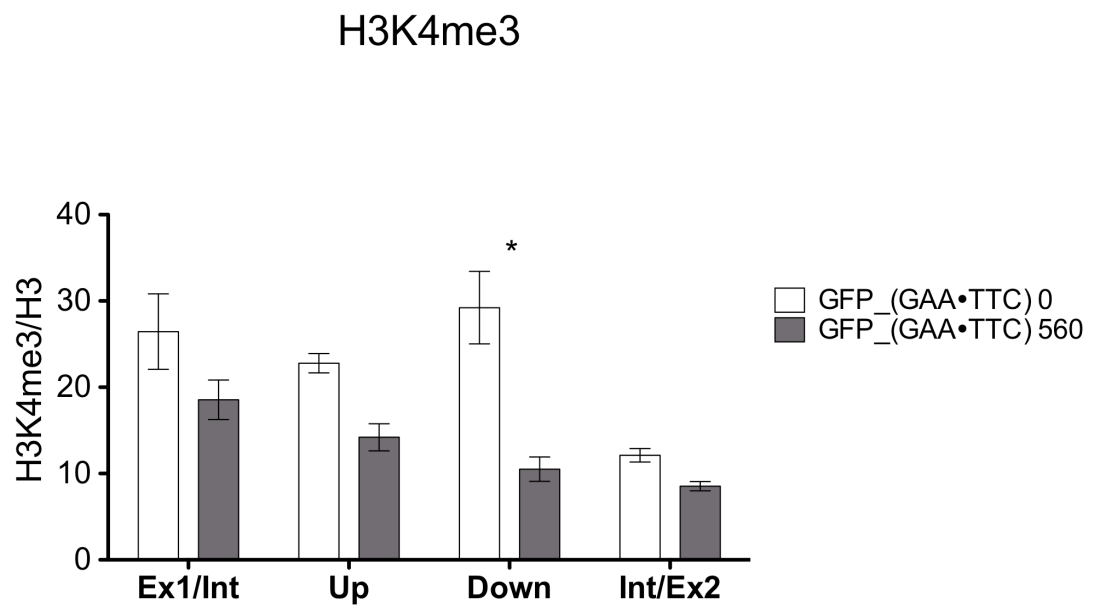
Figure 26. H3K79me2 is affected at the region downstream of the GAA repeats in the GFP_(GAA•TTC) $_{560}$ cells.

The levels of H3K79me2 in the GFP_GAA reporter system were determined by ChIP assay. White bars represent the GFP_(GAA•TTC) $_0$ cells. Gray bars represent the GFP_(GAA•TTC) $_{560}$ cells. Data are expressed as mean ± SEM. Asterisk indicates statistical significance (one: $p < 0.05$, three: $p < 0.001$). The abundance of histone modifications is shown relative to input DNA in the PCR and normalized to a total H3 for each region. Upon tetracycline treatment, H3K79me3 are decreased at the region upstream of the GAA repeats in the GFP_(GAA•TTC) $_{560}$ cells compared to the GFP_(GAA•TTC) $_0$ cells. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 26

H3K79me2
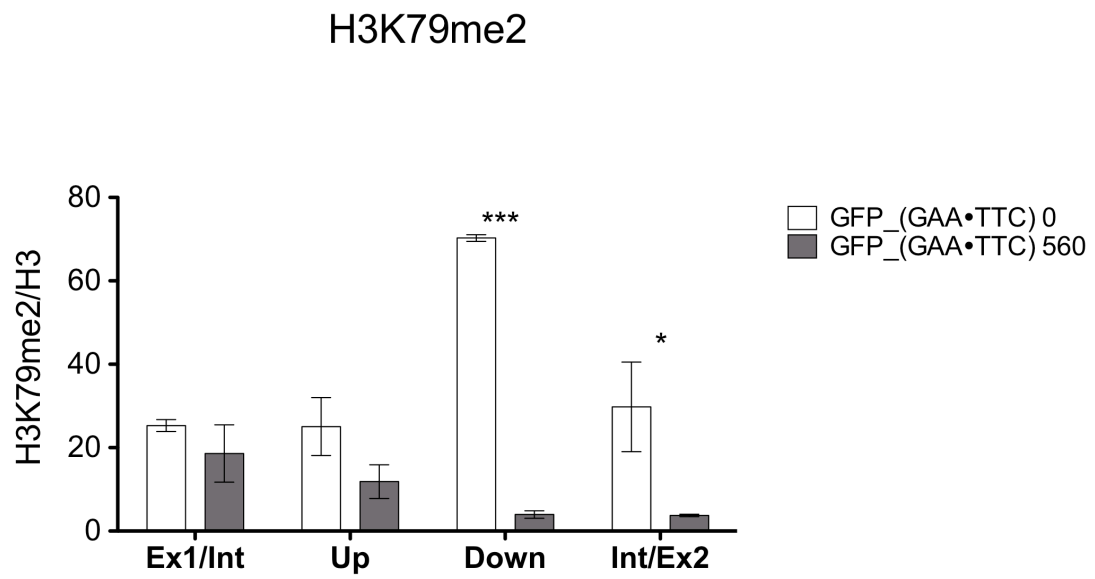
observed in FRDA cell lines. These results strongly suggest that elongation is the major step hampered by the hyperexpanded GAA repeats in FRDA patients.

Longer GAA repeats is associated with inhibiting progression of RNA polymerases in *in vitro* and *in vivo* systems (Grabczyk and Usdin, 2000b; Krasilnikova, Kireeva, Petrovic, Knijnikova, Kashlev and Mirkin, 2007). Moreover, transcription arrest is associated with alteration of histone modifications (Balakrishnan and Milavetz, 2007; Li, Carey and Workman, 2007; Nagata, Ito, Arimitsu, Koyama and Sekimizu, 2009). To assess whether RNA pol II arrest associated with the presence of the GAA repeats can prompt the cascade of silencing events, I used the GFP_GAA reporter system allowing us to precisely control initiation of transcription using tetracycline regulated operator/repressor (Figure 27). Generally, the GFP_GAA reporter system has been cultured in the absence of tetracycline to maintain cell lines. Both the GFP_(GAA•TTC)$_{560}$ and the GFP_(GAA•TTC)$_0$ cells were sub-cultured for less than 10 passages prior to the tetracycline treatment. In order to investigate the effect of transcription in alteration of histone modifications by the hyperexpanded GAA repeats, the cells were prepared in parallel in the absence or presence of the tetracycline. Upon the absence of tetracycline to inhibit transcription, levels of H3K9me3 were pronounced in regions both upstream and downstream of the GAA repeats in the GFP_(GAA•TTC)$_{560}$ cells, not the GFP_(GAA•TTC)$_0$ cells. After initiation of transcription by the tetracycline treatment, no significant changes of H3K9me3 levels were observed in the proximity of the GAA repeats in the GFP_(GAA•TTC)$_{560}$ cells when compared to the GFP_(GAA•TTC)$_{560}$ cells that is not treated with tetracycline. However, enrichments of H3K79me2 were

dramatically reduced at the region downstream of the GAA repeats in the

GFP_(GAA•TTC) $_{560}$ cells upon inhibition of transcription as I expected. This result

strongly suggests that the hyperexpanded GAA repeats *per se* are the underlying cause of

the heterochromatin-like structure formation and the arrest of RNA pol II is unlikely to

serve as a signal instigating epigenetic changes associated with the longer GAA repeats.

Taken together, my results demonstrate that insufficient levels of *FXN* expression in

FRDA patients are a consequence of impeded transcription elongation through the

heterochromatin-like structure induced by the hyperexpanded GAA repeats.

Figure 27. The tract of GAA repeats is sufficient to form the heterochromatin-like structure in the absence of transcription, which lead to inhibition of transcription elongation.

Effect of transcription on the formation of the heterochromatin-like structure was analyzed using ChIP assay in the GFP_GAA reporter system. Enrichment of H3K9me3 and H3K79me2 at the region upstream (Up) and the region downstream (Down) were determined in the absence and presence of tetracycline (0.1 μg/mL for 24 h). White bars represent results obtained from cell line lacking the GAA repeats in intron of the *GFP* gene while gray bars representing cells harboring intronic 560 of GAAs. Data are expressed as mean ± SEM. The experiment was conducted in triplicate and *P* values were calculated from the average of all three determinations for the GFP_(GAA•TTC)$_{560}$ and the GFP_(GAA•TTC)$_0$ cells. H3K9me3 is enriched in the GFP_(GAA•TTC)$_{560}$ in the absence and presence of tetracycline. H3K79me2 are impaired in the GFP_(GAA•TTC)$_{560}$ upon tetracycline treatment to resume transcription. Permission to re-use by Oxford University Press on behalf of the Nucleic Acids Research.

Figure 27
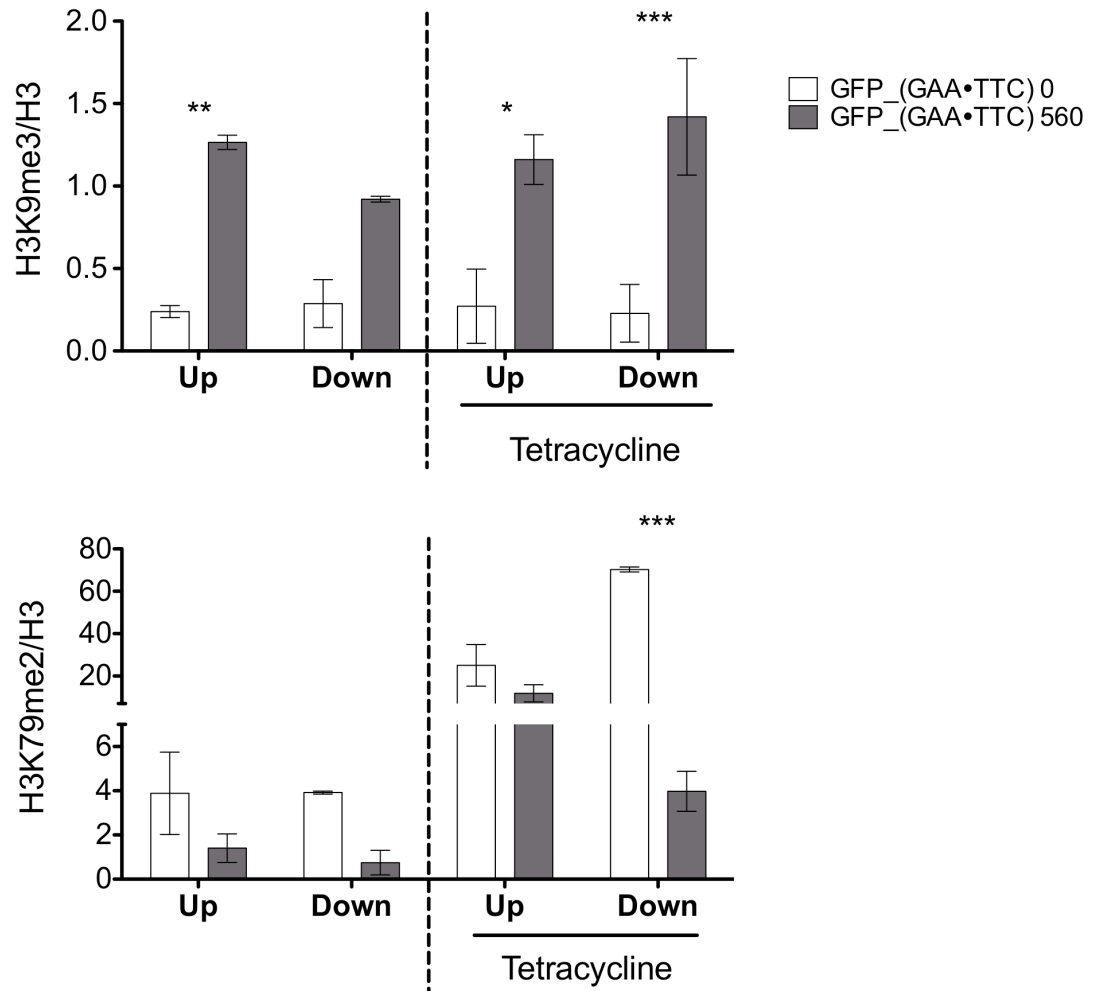
Chapter 5. Discussion

Some parts of this dissertation were modified from the following journal article:

Eunah Kim, Marek Napierala, Sharon Y.R. Dent. Hyperexpansion of GAA repeats affects post-initiation steps of *FXN* transcription in Friedreich's ataxia, Nucleic Acids Research, (2011) 39(19): 8366-8377.

In my study, defining altered histone modifications and repressive RNA pol II distribution at the *FXN* gene in FRDA cell lines provides strong evidence that *FXN* insufficiency in FRDA cell lines begin at a post-initiation step of *FXN* transcription. I also detected an accumulation of total RNA pol II at the promoter-proximal region of the *FXN* gene in unaffected cells, but not in FRDA cells. Several studies using genome-wide analysis have suggested that post-initiation events are rate-limiting to regulate transcription of some genes (Guenther, Levine, Boyer, Jaenisch and Young, 2007; Saunders, Core and Lis, 2006). This phenomenon is well-described for a heat shock–inducible gene, *Hsp70,* in *Drosophila* (Gilmour and Lis, 1986). Increased levels of RNA pol II are observed at the promoter of the *Hsp70* gene upon uninduced condition, and this RNA pol II is released from the promoter by heat shock stimuli, leading to launching of *Hsp70* transcription (Gilmour and Lis, 1986). Genome-wide analysis of transcripts using nuclear run-on assay reveals paused Pol II on a large number of genes in human cells (Core, Waterfall and Lis, 2008). This phenomenon prompts me to hypothesize that *FXN* gene may be categorized as an inducible gene that is regulated by tissue-specific factors. The *FXN* gene is positively regulated by two transcription factors, peroxisome proliferator-activated receptor (PPAR) gamma and hypoxia-inducible factor-2 alpha (Marmolino, Acquaviva, Pinelli, Monticelli, Castaldo, Filla and Cocozza, 2009; Oktay, Dioum, Matsuzaki, Ding, Yan, Haller, Szweda and Garcia, 2007). PPAR gamma is more highly expressed in FRDA cells compared to unaffected individuals (Marmolino, Acquaviva, Pinelli, Monticelli, Castaldo, Filla and Cocozza, 2009). Imbalance of iron as detected in FRDA patients may also affect the induction of FXN expression, as proposed in a previous study (Li, Besse, Ha, Kovtunovych and Rouault, 2008). The longer GAA

repeats at the *FXN* gene in FRDA patient may recruit particular factors that inhibit

induction of FXN expression. In contrast, non-B DNA structure adopted by the longer

GAA repeat *per se* may inhibit to recruit particular factors that induce of FXN

expression.

According to my results, paused RNA pol II is unlikely to reflect ongoing transcription,

at least at the *FXN* gene. Although   *FXN* pre-mRNA levels are similar in both

unaffected and FRDA cells, total RNA pol II, as measured by ChIP, only accumulates at

the promoter-proximal region of the *FXN* gene in FRDA cells. Moreover, this

accumulation is restricted to the promoter-proximal region, not other regions including

promoter. Therefore, *FXN* pre-mRNA seems to reflect FXN promoter activity more than

chromatin structure at the region   This finding is consistent with those of a previous

study that indicated promoter activity is not affected at the *FXN* gene in FRDA cells,

although altered histone modifications were observed at the promoter-proximal region

(Kumari, Biacsi and Usdin, 2011b). In an effort to confirm correlation between the levels

of RNA pol II and FXN transcription, I tried to measure pre-mature FXN RNA at the

promoter-proximal region (+48bp). Unfortunately, the *FXN* promoter-proximal region is

quite CG-rich, so that qRT-PCR assays were not successful. Both H3K4me2 and

H3K4me3 are greatly decreased at this region in FRDA cells when compared to

unaffected cells. This result suggests that the paused RNA pol II at the promoter-

proximal region in unaffected cells may facilitate chromatin remodeling for euchromatin

as shown in my results to trigger additional transcription progression. The paused RNA

pol II may help to stabilize the binding of transcription machinery assisting further

rounds of transcription initiation. Indeed, the accumulated RNA pol II in the promoter-proximal region is associated with the enrichment of active histone marks such as H3K9/14ac and H3K4me3 in the human genome, regardless of transcription status (Guenther, Levine, Boyer, Jaenisch and Young, 2007). FRDA cells exhibit a slow rate of *FXN* mRNA synthesis when compared to unaffected cell lines (Punga and Buhler, 2010).

The decreased levels of RNA pol II in the coding region of genes is associated with decreased levels of H3K4me2 and H3K4me3, hyper-methylated CpG residues, and increased levels of H3K9me (Okitsu and Hsieh, 2007; Okitsu, Hsieh and Hsieh, 2010). Consistently, levels of total RNA pol II is repressed in the region upstream of the GAA repeats in FRDA cells compared to unaffected cells. Moreover, the region upstream of the GAA repeats display less enrichment of H3K4me3 and H3K4me2 in FRDA cells compared to unaffected cells. Enrichment of H3K4me3 in the coding region of genes is associated with the efficiency of transition between initiation and elongation in transcription (Okitsu and Hsieh, 2007; Okitsu, Hsieh and Hsieh, 2010), and contributes to recruit chromatin-remodeling complexes (Li, Ilin, Wang, Duncan, Wysocka, Allis and Patel, 2006; Sims, Millhouse, Chen, Lewis, Erdjument-Bromage, Tempst, Manley and Reinberg, 2007). Therefore, the decreased levels of H3K4me3 at the region upstream of GAA repeats in FRDA cells may impair recruitment of chromatin-modifying complexes or lead to *FXN* deficiency resulting from impediment of *FXN* transcription elongation in FRDA patients.

Histone methylation status represents both an important regulatory element of gene

expression and an indicator of the transcriptional status of a gene. Enzymes that add or remove methyl groups on specific sites of histones regulate the balance between methylation and demethylation in the cells. Activity of a large group of histone demethylases containing Jumonji C-terminal domain depends on the presence of non-heme iron (Hahn, Bose, Edler and Lengeling, 2008). Several reports show that low levels of FXN, an iron chaperone, results in accumulated irons in the mitochondria and depleted iron in the other cellular compartments leading to a severe imbalance of iron in FRDA cells (Adinolfi, Iannuzzi, Prischi, Pastore, Iametti, Martin, Bonomi and Pastore, 2009; Bulteau, O'Neill, Kennedy, Ikeda-Saito, Isaya and Szweda, 2004). This suggests that the imbalance of iron in FRDA patients may be associated with the activity of iron-dependent demethylases responsible for levels of H3K27me3. In FRDA cells, compared to controls, I detected significant increase of H3K27me3, histone methylation marks potentially affected by iron-dependent histone demethylases such UTX. Indeed, over expression of UTX increases expression of *FXN* mRNA in KYSE180 cancer cells (van Haaften, Dalgliesh, Davies, Chen, Bignell, Greenman, Edkins, Hardy, O'Meara, Teague, Butler, Hinton, Latimer, Andrews, Barthorpe, Beare, Buck, Campbell, Cole, Forbes, Jia, Jones, Kok, Leroy, Lin, McBride, Maddison, Maquire, McLay, Menzies, Mironenko, Mulderrig, Mudie, Pleasance, Shepherd, Smith, Stebbings, Stephens, Tang, Tarpey, Turner, Turrell, Varian, West, Widaa, Wray, Collins, Ichimura, Law, Wong, Yuen, Leung, Tonon, DePinho, Tai, Anderson, Kahnoski, Massie, Khoo, Teh, Stratton and Futreal, 2009). Moreover, genome-wide approach reveals that FXN deficiency is associated with down regulation of genes involved in DNA packaging and nucleosome assembly (Huang, Becker, Whitnall, Rahmanto, Ponka and Richardson, 2009).

In an effort to alleviate transcriptional block facilitated by the GAA repeat expansions in FRDA patients, dissecting the mechanism of heterochromatin-like structure induced by the hyperexpanded GAA repeats is critical for logical design of therapeutic approaches. Currently, HDACis are the most promising small molecules that restore *FXN* transcription and alter the chromatin status in the proximity of the GAA repeats, but not the promoter region of the *FXN* gene in FRDA cells (Herman, Jenssen, Burnett, Soragni, Perlman and Gottesfeld, 2006). However, the question of either the initial event or the cascade of epigenetic changes in the GAA repeats region remains to be addressed. Perhaps the hyperexpanded GAA repeats *per se* or non-B DNA structures facilitated by the hyperexpanded GAA repeats employ particular proteins that affect modifications on histones in the chromatin environment in the vicinity of the GAA repeats. Moreover, paused RNA pol II at the c-*Myc* gene is observed in the vicinity of a triplex forming repeat structure (Belotserkovskii, De Silva, Tornaletti, Wang, Vasquez and Hanawalt, 2007), suggest that non-canonical DNA structures induced by the hyperexpanded GAA repeats might be a primary origin of deficient *FXN* transcription in FRDA.

In fission yeast and Drosophila, and possibly other eukaryotes, the RNA interference (RNAi) pathway is involved in heterochromatin formation (Wassenegger, 2005). Endogenous short interfering RNA (siRNA) originates from repetitive sequences such as centromeric repeats, transposable elements or rDNA and act as a trigger the RNAi pathway (Fagegaltier, Bouge, Berry, Poisot, Sismeiro, Coppee, Theodore, Voinnet and Antoniewski, 2009). A genome-wide approach revealed that there are antisense

transcripts at the *FXN* locus in humans (He, Vogelstein, Velculescu, Papadopoulos and Kinzler, 2008). Recently, more antisense transcripts (*FAST*-1) were observed in the proximity of TSS of the FXN gene in FRDA cells (De Biase, Chutake, Rindler and Bidichandani, 2009). Antisense *FXN* transcripts can be involved in prompting silencing of *FXN* transcription via RNAi-mediated heterochromatin formation through double stranded RNA generated from repetitive DNA sequences. In an effort to assess the relative contribution of antisense *FXN* transcripts in regulation of *FXN* expression, we also performed strand-specific RT-PCR to detect antisense transcription in the *FXN* locus. Unfortunately, we were unable to detect antisense transcripts at the regions specified in the previous study, the proximity of TSS of the *FXN* gene as well as region upstream of the GAA repeats (data not shown). Therefore, all *FXN* transcripts examined in this study represent sense FXN RNA transcripts.

, Most diseases assocated with repetitive DNA sequences exhibit distinct patterns of repetitive element instability in neuronal as compared to non-neuronal cells (Pearson, Nichol Edamura and Cleary, 2005). Indeed, although GAA contractions occur in all tissues, expansions occur only in the brain of FRDA patients (Cleary and Pearson, 2003). The tissue specificity could contribute to disease progression (Swami, Hendricks, Gillis, Massood, Mysore, Myers and Wheeler, 2009). DNA metabolism such as replication, repair, recombination and transcription, is important for instability of the repetitive DNA unit. Notably, abnormal function of mismatch repair proteins, such as MSH2, is associated with GAA instability in yeast (Shishkin, Voineagu, Matera, Cherng, Chernet, Krasilnikova, Narayanan, Lobachev and Mirkin, 2009) and in inducible pluripotent stem

(iPS) cells from FRDA patients (Ku, Soragni, Campau, Thomas, Altun, Laurent, Loring, Napierala and Gottesfeld, 2010). Similar to FRDA, base excision repair factors are associated with CTG repeat stability (Liu, Prasad, Beard, Hou, Horton, McMurray and Wilson, 2009). Tissue specific levels of these *trans*-acting factors may contribute to increased instability of repetitive DNA sequence in neuronal cells of the patients. Also, *cis*-elements, such as the CTCF DNA binding motif, are associated with tissue and developmental specificity of increased repeat instability (Pearson, Nichol Edamura and Cleary, 2005). CTCF DNA binding motifs flank numerous genes that contain repetitive DNA units and are known to modulate chromatin structure and transcription in several neurological disorders (Chang, Zhang, Heath, Galjart, Wang and Milbrandt, 2010; Pearson, Nichol Edamura and Cleary, 2005). Methylation of cytosine or mutation of CTCF-binding motif at the *SCA7* locus inhibits CTCF binding, thereby increasing tissue specific repeat instability in SCA7 (CAG repeats) (Libby, Hagerman, Pineda, Lau, Cho, Baccam, Axford, Cleary, Moore, Sopher, Tapscott, Filippova, Pearson and La Spada, 2008). In FRDA, more CTCF occupancy are accompanied with heterochromatinized structures at the *FXN* genes, which lead to transcribe antisense *FXN* RNAs (De Biase, Chutake, Rindler and Bidichandani, 2009). However, distinct levels of CTCF in neuronal cells have not yet been reported in neurodegenerative diseases affected by repetitive DNA sequences. Moreover, occupancy of CTCF is not correlated with overt transcription in mammals (Splinter, Heath, Kooren, Palstra, Klous, Grosveld, Galjart and de Laat, 2006). This suggests that upstream tissue-specific factors that regulate CTCF may contribute to tissue specificity of repeat instability. Methylations at the promoter of the *BDNF* gene, a growth factor that plays important roles in regulating neurogenesis,

synaptic plasticity, and neuronal survival, are associated with neurodegenerative disorders, including Huntingtons disease (Zuccato and Cattaneo, 2009). Hypermethylated CpG residues in the BDNF gene in the cortical neuron in neurodegenerative conditions inhibits CTCF binding leading to silence of BDNF expression (Chang, Zhang, Heath, Galjart, Wang and Milbrandt, 2010). This report may shed light on the mechanistic relationship between pathology of neurodegenerative diseases and repeat instability in neuronal cell types.

Moreover, CTCF is associated with chromatin looping and altered histone modifications (Splinter, Heath, Kooren, Palstra, Klous, Grosveld, Galjart and de Laat, 2006). I found that pausing of RNA pol II occurred at the promoter-proximal region, not the region upstream of the GAA repeat at the *FXN* gene. This phenomenon might suggest looping of the chromosome from the TSS to a region upstream of the GAA repeats at the *FXN* gene in FRDA cells. The hyperexpanded GAA repeats influence the looping in FRDA cells, thereby affect not only chromatin structure but also *FXN* transcription. In reconstitution assays, nucleosomes are less well assembled into double stranded DNA sequences containing longer GAA repeats (Ruan and Wang, 2008). The hyperexpanded GAA repeat that have less nucleosome assembly might have higher opportunity to be looped between TSS and the region immediately upstream of the GAA repeats, thereby affecting configuration of chromatin structure and distribution of RNA pol II at the *FXN* gene in FRDA cells.

Expansions of repetitive DNA sequences are correlated with human neurological and neuromuscular diseases. To date, at least 15 neurodegenerative disorders are identified as the repeats-expansion diseases (Wells, 1996). These lengths of repetitive DNA unit are dynamic in among individuals, generations, and tissues of the patients. There are many evidences that these repeat expansion diseases have common epigenetic features such as heterochromatin associated histone modifications, the presence of short/antisense RNA, and hypermethylated CpG residue, and CTCF binding in the proximity of the repeats (Al-Mahdawi, Pinto, Ismail, Varshney, Lymperi, Sandi, Trabzuni and Pook, 2008; Cho, Thienes, Mahoney, Analau, Filippova and Tapscott, 2005; Filippova, Thienes, Penn, Cho, Hu, Moore, Klesert, Lobanenkov and Tapscott, 2001; Pietrobono, Tabolacci, Zalfa, Zito, Terracciano, Moscato, Bagni, Oostra, Chiurazzi and Neri, 2005; Verdel, Jia, Gerber, Sugiyama, Gygi, Grewal and Moazed, 2004). These similarities in epigenetic pathologies on silenced alleles of each repeat expansion diseases suggest similar therapeutic approaches to enhance the gene expression in an effort to alleviate disease symptom. However, some of features are excluded when epigenetic pathology of each repeat expansion disease defines, suggesting that distinct mechanisms responsible for repeat-mediated chromatin remodeling in each repeat expansion disease may be present. Therefore, my study that carefully define chromatin structures adopted by the hyperexpanded GAA repeat will help not only understand shared epigenetic features in repeat expansion diseases but also distinguish epigenetic features of FRDA from other repeats expansion diseases.

Chapter 6. Significance and Future Directions

My studies define comprehensive distribution of histone modification at the *FXN* gene in multiple lymphoid cell lines derived from FRDA patients and unaffected controls (Figure 28). I found that the hyperexpanded GAA repeats influence chromatin structure in the regions flanking the GAA repeats, but these differences do not spread to the promoter. On the contrary, the levels of RNA pol II and histone methylations representing transcription elongation were significantly decreased at the *FXN* gene in FRDA cell lines. These results strongly demonstrate that the transcription machinery is inhibited after successful initiating *FXN* transcription in FRDA cells. Moreover, I first show that RNA pol II is predominant at the *FXN* promoter-proximal region, appears to be paused, in unaffected cell lines, but not FRDA cell lines. Comparison of altered H3K4me3 and H3K79me2 levels at the *FXN* gene revealed that *FXN* deficiency is associated with defective efficiency of transition from initiation to elongation of *FXN* transcription. Taken together, my study provides strong indication that *FXN* insufficiency in FRDA patients is caused by a defective transition at a post-initiation step of *FXN* transcription. Perhaps heterochromatin-like structures in the vicinity of the hyperexpanded GAA repeats act as an obstacle to produce full length *FXN* mRNA.

Regulatory elements located in the first intron affect expression of several genes (De Jaco, Camp and Taylor, 2005; Lee, Dahi, Mahimkar, Tulloch, Alfonso-Jaume, Lovett and Sarkar, 2005). Studies using deletion mapping of the *FXN* intron 1 revealed that the intron 1 is capable of regulating *FXN* expression (Greene, Mahishi, Entezam, Kumari and Usdin, 2007). Results of ChIP assay in this study demonstrate that the intronic region upstream of the hyperexpanded GAA repeats exhibit the most significant differences of

Figure 28. A summary of distributed histone modifications at the human *FXN* gene.

In FRDA cells, the *FXN* gene enriched with constellation of H3K9me3, H3K27me3, and H4K20me3, strongly supporting that the *FXN* gene in FRDA cells has heterochromatin-like structures due to possibly the hyperexpanded tract of GAA repeats. This phenomenon demonstrated in the GFP_GAA reporter system. H3K4me2, H3K36me3, and H3K79me2 associated with euchromatin are decreased at the *FXN* gene in FRDA cells as well as the *GFP* gene in the GFP_(GAA•TTC) $_{560}$ cells of the GFP_GAA reporter system. Distribution of RNA pol II is repressed at the entire FXN gene in FRDA cells compared to unaffected cells. RNA pol II seems to be poised at the downstream of the transcription start site (TSS) of the *FXN* gene in unaffected cells, not FRDA cells. Although the detailed mechanisms is unclear, heterochromatin-like structure at the vicinity of the GAA repeats induced by the hyperexpanded GAA repeats lead to consequence of *FXN* deficiency at the region downstream of the GAA repeats in FRDA cells.

Figure 28
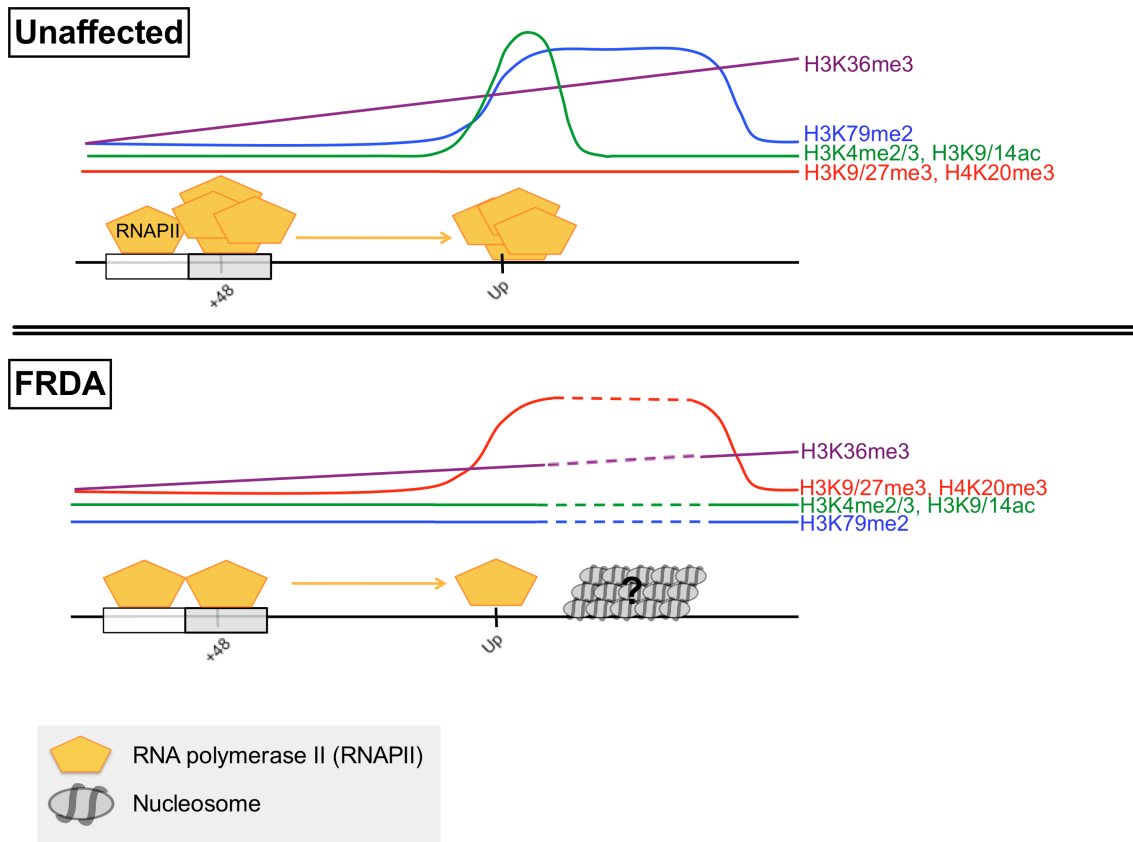
histone modifications between FRDA and control cell lines. The cytosine residue in this region is preferentially methylated in FRDA patients (Greene, Mahishi, Entezam, Kumari and Usdin, 2007). The levels of DNA methylation proportionally correlate with the length of the GAA repeats (Castaldo, Pinelli, Monticelli, Acquaviva, Giacchetti, Filla, Sacchetti, Keller, Avvedimento, Chiariotti and Cocozza, 2008). One of the methylated cytosine is localized into an E-box motif (CANNTG). The c-Myc, a bHLH transcription factor that recognize the E-box motif, interacts with the E-box resided in the region upstream of the repeats in P493 cells (Fernandez, Frank, Wang, Schroeder, Liu, Greene, Cocito and Amati, 2003). Moreover, the induction of c-Myc is associated with enhancement of *FXN* transcription (Schuhmacher, Kohlhuber, Holzel, Kaiser, Burtscher, Jarsch, Bornkamm, Laux, Polack, Weidle and Eick, 2001). Myc proteins allow the paused RNA pol II to be released from promoter to activate gene expression (Rahl, Lin, Seila, Flynn, McCuine, Burge, Sharp and Young, 2010). c-Myc physically interact with components from chromatin modifying/remodeling complexes such as GCN5 to activate gene expression (Flinn, Wallberg, Hermann, Grant, Workman and Wright, 2002; Kenneth, Ramsbottom, Gomez-Roman, Marshall, Cole and White, 2007). In the unaffected cells, enrichments of H3K4me3, H3K4me2 and H3K79me2 in the region upstream of the GAA repeats encompassing E-box motif potentially facilitate binding of myc proteins in the E-box. Therefore, hypermethylated DNA sequences in the region upstream of the GAA repeats in FRDA cells can influence the interactions between myc proteins and the E-box motif (Guccione, Martinato, Finocchiaro, Luzi, Tizzoni, Dall' Olio, Zardo, Nervi, Bernard and Amati, 2006; Perini, Diolaiti, Porro and Della Valle, 2005). My study here emphasizes the region upstream of the repeats is valuable as the

regulatory element leading to appropriate *FXN* transcription. It is likely that epigenetic changes in the intron 1 of the *FXN* gene induced by the hyperexpanded GAA repeats interfere with transcriptional enhancers. Subsequently, reduced progression of transcriptional machinery result in defective *FXN* expression in FRDA patients. Additionally, c-Myc has been shown to interact with GCN5, an acetyltransferase responsible for H3K9/14ac in eukaryotes (Flinn, Wallberg, Hermann, Grant, Workman and Wright, 2002; Kenneth, Ramsbottom, Gomez-Roman, Marshall, Cole and White, 2007). The decreased levels of H3K9/14ac at the region upstream of the GAA repeats in FRDA cells might result from failure of GCN5 recruitment at the region upstream of the GAA repeats in FRDA cells. Reduced occupancy (or interaction) of C-Myc and GCN5 in FRDA cells may lead to reduced recruitment of transcription machinery containing RNAPII. This impaired recruitment may result in particular constellation of histone methylations marking repressive chromatin structures at *FXN* gene in FRDA patients as I observed. In order to investigate this possibility, distribution of c-Myc and GCN5 could be defined by ChIP-seq in the lymphoid cell lines. Unfortunately, there are no appropriate antibodies for GCN5 and c-Myc ChIP. Alternatively, distribution of histone modification at the *FXN* gen can be determined in either the absence or over expression of either c-Myc or GCN5 in lymphoid cell lines. However, the lymphoid cell lines exhibit technically low efficiency of transfection. It would take longer time to set a condition to optimize transfection activity. Alternatively, other cell lines such as K562 and HEK293T cells as general cell lines can be utilized to investigate effect of c-Myc or GCN5 in *FXN* transcription. However, this system will exclude effect of the hyperexpanded GAA repeats to understand the roles of c-Myc or GCN5 in *FXN* transcription. The GFP_GAA

system will be a suitable system. However, this system also has limitations that the artificial condition in the GFP_GAA system may not be correlated with actual cellular function to the heterochromatin-like structure at the *FXN* gene in FRDA patients. Moreover, since several studies suggest that intronic region of the *FXN* gene have regulatory function in *FXN* transcription, the GFP_GAA system has weakness that have non-*FXN* intronic sequence to regulate either GFP transcription and chromatin structure change.

During transcription consisting of series of phase such as initiation, elongation, and termination of RNA transcript by RNA pol II, the promoter-proximal pausing of RNAPII is a rate-limiting step to produce full-length transcription(Guenther, Levine, Boyer, Jaenisch and Young, 2007). I found that levels of RNA pol II is impaired at the promoter-proximal region of the *FXN* gene in FRDA cells. More interestingly, the SAGA complex that contains GCN5 and the Mediator complex have important roles in inducing appropriate levels of gene expression from poised promoters in budding yeast (Lee, Fletcher, Zhang, Chen, Fischbeck and Stargell, 2010). Moreover, c-Myc is involved in the release of paused RNA pol II from the promoter-proximal region of a given gene (Rahl, Lin, Seila, Flynn, McCuine, Burge, Sharp and Young, 2010). These results raise the possibilities that c-Myc bound to the E-box in the region upstream of the GAA repeats might be involved in release of paused RNA pol II at the promoter-proximal region of the *FXN* gene.

To further confirm that paused RNA pol II regulates FXN expression, I would examine

additional transcription factors, DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) as well as P-TEFb using conventional ChIP technique. DSIF and NELF are sufficient to stall RNA pol II at the promoter-proximal region of genes (Wu, Yamaguchi, Benjamin, Horvat-Gordon, Washinsky, Enerly, Larsson, Lambertsson, Handa and Gilmour, 2003). The paused RNA pol II is released from the promoter-proximal region by phosphorylation of NELF by P-TEFb (Rahl, Lin, Seila, Flynn, McCuine, Burge, Sharp and Young, 2010). The P-TEFb is necessary to release the paused RNA pol II by phosphorylation of NELF (Peterlin and Price, 2006). Inhibition of P-TEFb induces accumulated RNA pol II at the most of genes, suggesting that majority of gene is regulated by pausing of RNA pol II (Cheng and Price, 2007; Peterlin and Price, 2006). C-myc is associated with activation of P-TEFb to release paused RNA pol II (Rahl, Lin, Seila, Flynn, McCuine, Burge, Sharp and Young, 2010). Successful release of the paused RNA pol II from the promoter-proximal region of genes is necessary to elongate full-length transcripts (Saunders, Core and Lis, 2006). Artificial recruitment of P-TEFb at the *Hsp70* gene under uninduced conditions increases basal Hsp70 expression (Lis, Mason, Peng, Price and Werner, 2000). This result suggests that the *FXN* gene in FRDA may have less P-TEFb compared to unaffected controls. To investigate this possibility, I could first treat pharmacological inhibitor of P-TEFb in lymphoid cell lines followed by ChIP for total RNA pol II at the *FXN* gene.

Phosphorylation at serine 2 in the CTD of RNA pol II by P-TEFb is indicator of ongoing elongation of full-length transcripts (Peterlin and Price, 2006). Unfortunately, I couldn't detect appreciative levels of phosphorylated serine 2 of RNA pol II by conventional

ChIP. Instead, nuclear run on assay is possibly able to detect elongating full-length *FXN* transcripts. If paused RNA pol II is associated with regulation of FXN transcription, I would detect increased phosphorylated RNA pol II at serine 2 upon the treatment of pharmacological P-TEFb inhibitor in FRDA cells.

Levels of H3K9me3 have been less correlated with *FXN* transcription, suggesting that levels of H3K9me3 are not the primary means to induce the heterochromatin-like structure facilitated by the GAA repeats. This suggestion allows me to hypothesize that hyper DNA methylation at the region upstream of the GAA repeats in FRDA cells is associated with altered histone modifications as is observed in my studies. Although the relationship between DNA methylation and histone modifications has not been fully understood, these two systems may play a part in regulating repressive chromatin structures. To investigate the effect of the DNA methylation in *FXN* transcription as well as altered chromatin structure at the *FXN* gene in FRDA cells, I would examine distribution of more histone modifications and total RNA pol II after removal of DNA methylation using 5-aza-2-deoxycytidine, an inhibitor of DNA methylation. If there is no change in histone modifications, it would further demonstrate that hyper DNA methylation in FRDA patients is involved in a different mechanism to silence *FXN* transcription in FRDA patients. If there are changes in histone modifications associated with the transcription process after 5-aza-2-deoxycytidine treatments, I will be able to determine which step of *FXN* transcription is affected by high levels of the DNA methylation at the region upstream of the GAA repeats in FRDA patients. To further demonstrate that the observations after removal of DNA methylation result from the

hyperexpanded GAA repeats *per se*, not either inherent characteristics of the *FXN* gene, I will take advantage of the GFP_GAA system. First of all, I will examine the levels of DNA methylation at the region upstream of the GAA repeats in the GFP_GAA cell lines to see the expanded GAA repeats induce DNA methylation in the vicinity of the repeats using either MS-PCR or bisulphate sequencing. If there are no DNA methylation changes in the GFP_GAA cells, one possibility is that the GAA repeats do not induce DNA methylations. The histone modification changes by the GAA repeats are primary factors to restore *FXN* transcription in FRDA. Another possibility is that 560 GAA repeats insufficient to induce DNA methylation to silence gene expression since the DNA methylation is correlated with the length of the GAA repeats in FRDA patients. If the GFP_(GAA•TTC) $_{560}$ cells have more DNA methylation compared to the GFP_(GAA•TTC) $_0$ cells, I should determine which factor between DNA methylation and histone modification can be more important/first targets to restore *FXN* transcription by additional experiments.

Although we detected no antisense *FXN* transcripts at the *FXN* locus, we cannot exclude possibility that there are double strand RNAs originating from the hyperexpanded GAA repeats to form RNAi-mediated heterochromatin-like structures at the *FXN* gene in FRDA cells. Moreover, the presence of H3K9me3 overlaps with RNAi-mediated heterochromatin formation in fission yeast (Cam, Sugiyama, Chen, Chen, FitzGerald and Grewal, 2005). Although I observed significant levels of H3K9me3 at the region upstream of the GAA repeat in FRDA cells, I could not detect any isoforms of HP1 protein. This suggest different pathway to form heterochromatin-like structure at the *FXN*

gene. The argonaute protein is a key player to participate in RNAi-mediated gene silencing as part of the RNA-Induced Transcriptional Silencing (RITS) complex mediating RNAi pathway to heterochromatin assembly (Buker, Iida, Buhler, Villen, Gygi, Nakayama and Moazed, 2007). The identifying small RNAs and proteins associated with the RITS complex at the *FXN* gene in FRDA will determine if the hyperexpanded GAA repeat is involved in RNAi-mediated *FXN* silencing via assembly of heterochromatin in FRDA.

Although expansion of the GAA repeats is the primary factor driving chromatin changes, the possibilities that metabolic changes resulting from frataxin deficiency in FRDA cells contribute to shaping of epigenetic landscape at the *FXN* gene has not been excluded. Frataxin is associated with protestation of cells against cytotoxic stress that might be involved in the fine-tuning regulation of FXN expression (Jiralerspong, Ge, Hudson and Pandolfo, 2001).

Moreover, HDACi treatments have no association of H3K9me3 levels at the *FXN* gene in FRDA cells in despite of enhancing *FXN* expression (Herman, Jenssen, Burnett, Soragni, Perlman and Gottesfeld, 2006; Punga and Buhler, 2010). This result suggests that inhibition of acetylation on non-histone is involved in the regulation of *FXN* transcription as well as chromatin structure changes at the *FXN* gene in FRDA cells. Therefore, global analyses of nucleosome position and histone modifications in FRDA are necessary to reveal the full spectrum of epigenetic consequences of reduced frataxin levels.

Overall, my study will help to clarify underlying mechanisms of how epigenetic changes observed in the vicinity of the hyperexpanded GAA repeats at the *FXN* gene are associated with *FXN* deficiency in FRDA patients. Furthermore, this study can potentially be developed to investigate therapeutic targets (*e.g.* methyltransferase or demethylase responsible for altered histone modifications observed in my studies) for curing FRDA.

Figure 29. A working model of heterochromatin-like structure formation at the

***FXN* gene in FRDA.**

HDACs are recruited to the *FXN* gene and remove acetyl group at the N-terminal tail of

the histones. Histone lysine methyltransferase add methyl group at the N-terminal tail of

the histones in the vicinity of the hyperexpanded GAA repeats in FRDA cells. According

to the distribution of RNA pol II in the lymphoid cell lines, the fate of RNA pol II is first

likely to be determined at the promoter-proximal region (+48 bp) to regulate either *FXN*

expression or chromatin structures. At the region upstream of the GAA repeats, RNA pol

II would be inhibited to elongate *FXN* transcription through one of mechanisms in

following, which lead to *FXN* deficiency in FRDA patients. One is that either non-B

DNA structure or heterochromatin-like structure induced by the hyperexpanded tract of

GAA repeats inhibit recruitment of chromatin modifying/remodeling complexes or DNA

methyltransferase. Another is that methylated CpG residue at the E-box (CACGTG) in

FRDA inhibits binding of transcription factors such as c-Myc, which lead to low levels of

RNA pol II and altered histone modifications resulting in *FXN* deficiency in FRDA.

Moreover, antisense *FXN* transcripts from the hyperexpanded GAA repeats in FRDA

patients can be involved in small RNA-mediated heterochromatin formation.

Figure 29
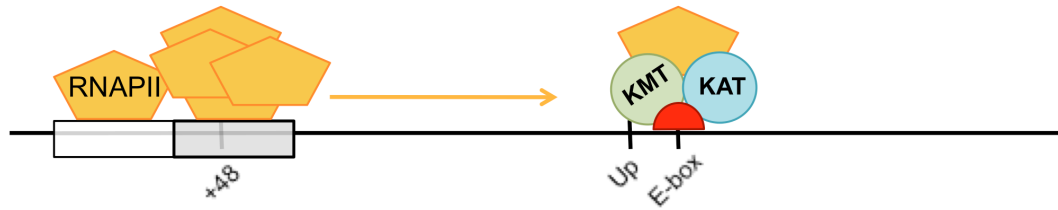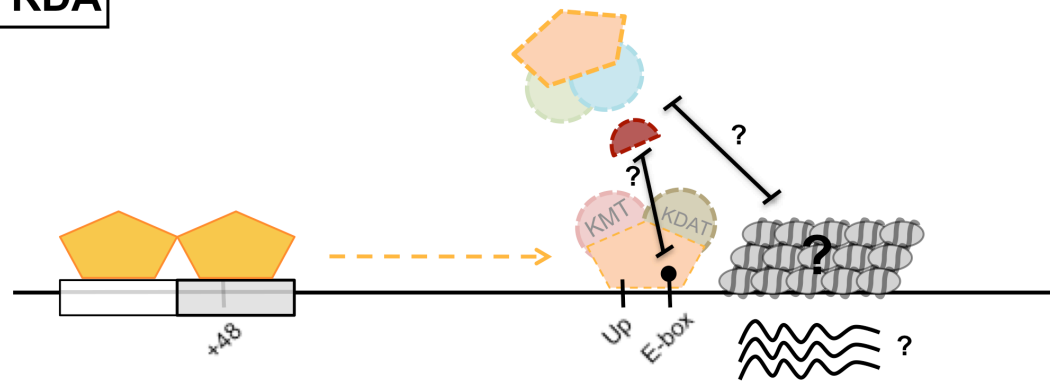
Bibliography

Adinolfi, S., Iannuzzi, C., Prischi, F., Pastore, C., Iametti, S., Martin, S.R., Bonomi, F., and Pastore, A. (2009). Bacterial frataxin CyaY is the gatekeeper of iron-sulfur cluster formation catalyzed by IscS. Nat Struct Mol Biol *16*, 390-396.

Al-Mahdawi, S., Pinto, R.M., Ismail, O., Varshney, D., Lymperi, S., Sandi, C., Trabzuni, D., and Pook, M. (2008). The Friedreich ataxia GAA repeat expansion mutation induces comparable epigenetic changes in human and transgenic mouse brain and heart tissues. Hum Mol Genet *17*, 735-746.

Al-Mahdawi, S., Pinto, R.M., Ruddle, P., Carroll, C., Webster, Z., and Pook, M. (2004). GAA repeat instability in Friedreich ataxia YAC transgenic mice. Genomics *84*, 301-310.

Al-Mahdawi, S., Pinto, R.M., Varshney, D., Lawrence, L., Lowrie, M.B., Hughes, S., Webster, Z., Blake, J., Cooper, J.M., King, R., and Pook, M.A. (2006). GAA repeat expansion mutation mouse models of Friedreich ataxia exhibit oxidative stress leading to progressive neuronal and cardiac pathology. Genomics *88*, 580-590.

Ayyanathan, K., Lechner, M.S., Bell, P., Maul, G.G., Schultz, D.C., Yamada, Y., Tanaka, K., Torigoe, K., and Rauscher, F.J., 3rd (2003). Regulated recruitment of HP1 to a euchromatic gene induces mitotically heritable, epigenetic gene silencing: a mammalian cell culture model of gene variegation. Genes Dev *17*, 1855-1869.

Babcock, M., de Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Pandolfo, M., and Kaplan, J. (1997). Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin. Science *276*, 1709-1712.

Balakrishnan, L., and Milavetz, B. (2007). Histone hyperacetylation in the coding region of chromatin undergoing transcription in SV40 minichromosomes is a dynamic process regulated directly by the presence of RNA polymerase II. J Mol Biol *365*, 18-30.

Barski, A., Cuddapah, S., Cui, K., Roh, T.Y., Schones, D.E., Wang, Z., Wei, G., Chepelev, I., and Zhao, K. (2007). High-resolution profiling of histone methylations in the human genome. Cell *129*, 823-837.

Belotserkovskii, B.P., De Silva, E., Tornaletti, S., Wang, G., Vasquez, K.M., and Hanawalt, P.C. (2007). A triplex-forming sequence from the human c-MYC promoter interferes with DNA transcription. J Biol Chem *282*, 32433-32441.

Bhaumik, S.R. (2011). Distinct regulatory mechanisms of eukaryotic transcriptional activation by SAGA and TFIID. Biochim Biophys Acta *1809*, 97-108.

Bidichandani, S.I., Ashizawa, T., and Patel, P.I. (1998). The GAA triplet-repeat expansion in Friedreich ataxia interferes with transcription and may be associated with an unusual DNA structure. Am J Hum Genet *62*, 111-121.

Bryant, G.O., Prabhu, V., Floer, M., Wang, X., Spagna, D., Schreiber, D., and Ptashne, M. (2008). Activator control of nucleosome occupancy in activation and repression of transcription. PLoS Biol *6*, 2928-2939.

Buker, S.M., Iida, T., Buhler, M., Villen, J., Gygi, S.P., Nakayama, J., and Moazed, D. (2007). Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat Struct Mol Biol *14*, 200-207.

Bulteau, A.L., O'Neill, H.A., Kennedy, M.C., Ikeda-Saito, M., Isaya, G., and Szweda, L.I. (2004). Frataxin acts as an iron chaperone protein to modulate mitochondrial aconitase activity. Science *305*, 242-245.

Cam, H.P., Sugiyama, T., Chen, E.S., Chen, X., FitzGerald, P.C., and Grewal, S.I. (2005). Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. Nat Genet *37*, 809-819.

Campuzano, V., Montermini, L., Lutz, Y., Cova, L., Hindelang, C., Jiralerspong, S., Trottier, Y., Kish, S.J., Faucheux, B., Trouillas, P., Authier, F.J., Durr, A., Mandel, J.L., Vescovi, A., Pandolfo, M., and Koenig, M. (1997). Frataxin is reduced in Friedreich ataxia patients and is associated with mitochondrial membranes. Hum Mol Genet *6*, 1771-1780.

Campuzano, V., Montermini, L., Molto, M.D., Pianese, L., Cossee, M., Cavalcanti, F., Monros, E., Rodius, F., Duclos, F., Monticelli, A., Zara, F., Canizares, J., Koutnikova, H., Bidichandani, S.I., Gellera, C., Brice, A., Trouillas, P., De Michele, G., Filla, A., De Frutos, R., Palau, F., Patel, P.I., Di Donato, S., Mandel, J.L., Cocozza, S., Koenig, M., and Pandolfo, M. (1996). Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. Science *271*, 1423-1427.

Castaldo, I., Pinelli, M., Monticelli, A., Acquaviva, F., Giacchetti, M., Filla, A., Sacchetti, S., Keller, S., Avvedimento, V.E., Chiariotti, L., and Cocozza, S. (2008). DNA methylation in intron 1 of the frataxin gene is related to GAA repeat length and age of onset in Friedreich ataxia patients. J Med Genet *45*, 808-812.

Cedar, H., and Bergman, Y. (2009). Linking DNA methylation and histone modification: patterns and paradigms. Nat Rev Genet *10*, 295-304.

Chang, J., Zhang, B., Heath, H., Galjart, N., Wang, X., and Milbrandt, J. (2010). Nicotinamide adenine dinucleotide (NAD)-regulated DNA methylation alters CCCTC-

binding factor (CTCF)/cohesin binding and transcription at the BDNF locus. Proc Natl

Acad Sci U S A *107*, 21836-21841.

Cheng, B., and Price, D.H. (2007). Properties of RNA polymerase II elongation

complexes before and after the P-TEFb-mediated transition into productive elongation. J

Biol Chem *282*, 21901-21912.

Cho, D.H., Thienes, C.P., Mahoney, S.E., Analau, E., Filippova, G.N., and Tapscott, S.J.

(2005). Antisense transcription and heterochromatin at the DM1 CTG repeats are

constrained by CTCF. Mol Cell *20*, 483-489.

Clark, R.M., De Biase, I., Malykhina, A.P., Al-Mahdawi, S., Pook, M., and Bidichandani,

S.I. (2007). The GAA triplet-repeat is unstable in the context of the human FXN locus

and displays age-dependent expansions in cerebellum and DRG in a transgenic mouse

model. Hum Genet *120*, 633-640.

Cleary, J.D., and Pearson, C.E. (2003). The contribution of cis-elements to disease-

associated repeat instability: clinical and experimental evidence. Cytogenet Genome Res

100, 25-55.

Core, L.J., Waterfall, J.J., and Lis, J.T. (2008). Nascent RNA sequencing reveals

widespread pausing and divergent initiation at human promoters. Science *322*, 1845-

1848.

Cossee, M., Puccio, H., Gansmuller, A., Koutnikova, H., Dierich, A., LeMeur, M.,

Fischbeck, K., Dolle, P., and Koenig, M. (2000). Inactivation of the Friedreich ataxia

mouse gene leads to early embryonic lethality without iron accumulation. Hum Mol

Genet *9*, 1219-1226.

Cossee, M., Schmitt, M., Campuzano, V., Reutenauer, L., Moutou, C., Mandel, J.L., and Koenig, M. (1997). Evolution of the Friedreich's ataxia trinucleotide repeat expansion: founder effect and premutations. Proc Natl Acad Sci U S A *94*, 7452-7457.

Davey, C., Pennings, S., and Allan, J. (1997). CpG methylation remodels chromatin structure in vitro. J Mol Biol *267*, 276-288.

De Biase, I., Chutake, Y.K., Rindler, P.M., and Bidichandani, S.I. (2009). Epigenetic silencing in Friedreich ataxia is associated with depletion of CTCF (CCCTC-binding factor) and antisense transcription. PLoS One *4*, e7914.

De Jaco, A., Camp, S., and Taylor, P. (2005). Influence of the 5' intron in the control of acetylcholinesterase gene expression during myogenesis. Chem Biol Interact *157-158*, 372-373.

Delatycki, M.B., Williamson, R., and Forrest, S.M. (2000). Friedreich ataxia: an overview. J Med Genet *37*, 1-8.

Dervan, P.B., and Edelson, B.S. (2003). Recognition of the DNA minor groove by pyrrole-imidazole polyamides. Curr Opin Struct Biol *13*, 284-299.

Dhe-Paganon, S., Shigeta, R., Chi, Y.I., Ristow, M., and Shoelson, S.E. (2000). Crystal structure of human frataxin. J Biol Chem *275*, 30753-30756.

Dormann, H.L., Tseng, B.S., Allis, C.D., Funabiki, H., and Fischle, W. (2006). Dynamic regulation of effector protein binding to histone modifications: the biology of HP1 switching. Cell Cycle *5*, 2842-2851.

Fagegaltier, D., Bouge, A.L., Berry, B., Poisot, E., Sismeiro, O., Coppee, J.Y., Theodore, L., Voinnet, O., and Antoniewski, C. (2009). The endogenous siRNA pathway is

involved in heterochromatin formation in Drosophila. Proc Natl Acad Sci U S A *106*, 21258-21263.

Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. (2003). Genomic targets of the human c-Myc protein. Genes Dev *17*, 1115-1129.

Festenstein, R., Sharghi-Namini, S., Fox, M., Roderick, K., Tolaini, M., Norton, T., Saveliev, A., Kioussis, D., and Singh, P. (1999). Heterochromatin protein 1 modifies mammalian PEV in a dose- and chromosomal-context-dependent manner. Nat Genet *23*, 457-461.

Filippova, G.N., Thienes, C.P., Penn, B.H., Cho, D.H., Hu, Y.J., Moore, J.M., Klesert, T.R., Lobanenkov, V.V., and Tapscott, S.J. (2001). CTCF-binding sites flank CTG/CAG repeats and form a methylation-sensitive insulator at the DM1 locus. Nat Genet *28*, 335-343.

Fleming, J., Spinoulas, A., Zheng, M., Cunningham, S.C., Ginn, S.L., McQuilty, R.C., Rowe, P.B., and Alexander, I.E. (2005). Partial correction of sensitivity to oxidant stress in Friedreich ataxia patient fibroblasts by frataxin-encoding adeno-associated virus and lentivirus vectors. Hum Gene Ther *16*, 947-956.

Flinn, E.M., Wallberg, A.E., Hermann, S., Grant, P.A., Workman, J.L., and Wright, A.P. (2002). Recruitment of Gcn5-containing complexes during c-Myc-dependent gene activation. Structure and function aspects. J Biol Chem *277*, 23399-23406.

Gilmour, D.S., and Lis, J.T. (1986). RNA polymerase II interacts with the promoter region of the noninduced hsp70 gene in Drosophila melanogaster cells. Mol Cell Biol *6*, 3984-3989.

Gomez-Sebastian, S., Gimenez-Cassina, A., Diaz-Nido, J., Lim, F., and Wade-Martins, R. (2007). Infectious delivery and expression of a 135 kb human FRDA genomic DNA locus complements Friedreich's ataxia deficiency in human cells. Mol Ther *15*, 248-254.

Gordon, D.M., Shi, Q., Dancis, A., and Pain, D. (1999). Maturation of frataxin within mammalian and yeast mitochondria: one-step processing by matrix processing peptidase. Hum Mol Genet *8*, 2255-2262.

Gottesfeld, J.M. (2007). Small molecules affecting transcription in Friedreich ataxia. Pharmacol Ther *116*, 236-248.

Gottesfeld, J.M., Turner, J.M., and Dervan, P.B. (2000). Chemical approaches to control gene expression. Gene Expr *9*, 77-91.

Grabczyk, E., Mancuso, M., and Sammarco, M.C. (2007). A persistent RNA.DNA hybrid formed by transcription of the Friedreich ataxia triplet repeat in live bacteria, and by T7 RNAP in vitro. Nucleic Acids Res *35*, 5351-5359.

Grabczyk, E., and Usdin, K. (2000a). Alleviating transcript insufficiency caused by Friedreich's ataxia triplet repeats. Nucleic Acids Res *28*, 4930-4937.

Grabczyk, E., and Usdin, K. (2000b). The GAA*TTC triplet repeat expanded in Friedreich's ataxia impedes transcription elongation by T7 RNA polymerase in a length and supercoil dependent manner. Nucleic Acids Res *28*, 2815-2822.

Greene, E., Mahishi, L., Entezam, A., Kumari, D., and Usdin, K. (2007). Repeat-induced epigenetic changes in intron 1 of the frataxin gene and its consequences in Friedreich ataxia. Nucleic Acids Res *35*, 3383-3390.

Grewal, S.I., and Jia, S. (2007). Heterochromatin revisited. Nat Rev Genet *8*, 35-46.

Guccione, E., Martinato, F., Finocchiaro, G., Luzi, L., Tizzoni, L., Dall' Olio, V., Zardo, G., Nervi, C., Bernard, L., and Amati, B. (2006). Myc-binding-site recognition in the human genome is determined by chromatin context. Nat Cell Biol *8*, 764-770.

Guenther, M.G., Levine, S.S., Boyer, L.A., Jaenisch, R., and Young, R.A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. Cell *130*, 77-88.

Hahn, P., Bose, J., Edler, S., and Lengeling, A. (2008). Genomic structure and expression of Jmjd6 and evolutionary analysis in the context of related JmjC domain containing proteins. BMC Genomics *9*, 293.

He, Y., Vogelstein, B., Velculescu, V.E., Papadopoulos, N., and Kinzler, K.W. (2008). The antisense transcriptomes of human cells. Science *322*, 1855-1857.

Herman, D., Jenssen, K., Burnett, R., Soragni, E., Perlman, S.L., and Gottesfeld, J.M. (2006). Histone deacetylase inhibitors reverse gene silencing in Friedreich's ataxia. Nat Chem Biol *2*, 551-558.

Huang, M.L., Becker, E.M., Whitnall, M., Rahmanto, Y.S., Ponka, P., and Richardson, D.R. (2009). Elucidation of the mechanism of mitochondrial iron loading in Friedreich's ataxia by analysis of a mouse mutant. Proc Natl Acad Sci U S A *106*, 16381-16386.

Jensen, L.T., Sanchez, R.J., Srinivasan, C., Valentine, J.S., and Culotta, V.C. (2004). Mutations in Saccharomyces cerevisiae iron-sulfur cluster assembly genes and oxidative stress relevant to Cu,Zn superoxide dismutase. J Biol Chem *279*, 29938-29943.

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science *293*, 1074-1080.

Jiralerspong, S., Ge, B., Hudson, T.J., and Pandolfo, M. (2001). Manganese superoxide dismutase induction by iron is impaired in Friedreich ataxia cells. FEBS Lett *509*, 101-105.

Jiralerspong, S., Liu, Y., Montermini, L., Stifani, S., and Pandolfo, M. (1997). Frataxin shows developmentally regulated tissue-specific expression in the mouse embryo. Neurobiol Dis *4*, 103-113.

Jones, P.L., Veenstra, G.J., Wade, P.A., Vermaak, D., Kass, S.U., Landsberger, N., Strouboulis, J., and Wolffe, A.P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet *19*, 187-191.

Kanhere, A., Viiri, K., Araujo, C.C., Rasaiyaah, J., Bouwman, R.D., Whyte, W.A., Pereira, C.F., Brookes, E., Walker, K., Bell, G.W., Pombo, A., Fisher, A.G., Young, R.A., and Jenner, R.G. (2010). Short RNAs are transcribed from repressed polycomb target genes and interact with polycomb repressive complex-2. Mol Cell *38*, 675-688.

Kenneth, N.S., Ramsbottom, B.A., Gomez-Roman, N., Marshall, L., Cole, P.A., and White, R.J. (2007). TRRAP and GCN5 are used by c-Myc to activate RNA polymerase III transcription. Proc Natl Acad Sci U S A *104*, 14917-14922.

Kostrzewa, M., Klockgether, T., Damian, M.S., and Muller, U. (1997). Locus heterogeneity in Friedreich ataxia. Neurogenetics *1*, 43-47.

Kourmouli, N., Jeppesen, P., Mahadevhaiah, S., Burgoyne, P., Wu, R., Gilbert, D.M., Bongiorni, S., Prantera, G., Fanti, L., Pimpinelli, S., Shi, W., Fundele, R., and Singh, P.B. (2004). Heterochromatin and tri-methylated lysine 20 of histone H4 in animals. J Cell Sci *117*, 2491-2501.

Krasilnikova, M.M., Kireeva, M.L., Petrovic, V., Knijnikova, N., Kashlev, M., and Mirkin, S.M. (2007). Effects of Friedreich's ataxia (GAA)n*(TTC)n repeats on RNA synthesis and stability. Nucleic Acids Res *35*, 1075-1084.

Ku, S., Soragni, E., Campau, E., Thomas, E.A., Altun, G., Laurent, L.C., Loring, J.F., Napierala, M., and Gottesfeld, J.M. (2010). Friedreich's ataxia induced pluripotent stem cells model intergenerational GAATTC triplet repeat instability. Cell Stem Cell *7*, 631-637.

Kumari, D., Biacsi, R.E., and Usdin, K. (2011a). Repeat expansion affects both transcription initiation and elongation in friedreich ataxia cells. J Biol Chem.

Kumari, D., Biacsi, R.E., and Usdin, K. (2011b). Repeat expansion affects both transcription initiation and elongation in friedreich ataxia cells. J Biol Chem *286*, 4209-4215.

Lee, J.G., Dahi, S., Mahimkar, R., Tulloch, N.L., Alfonso-Jaume, M.A., Lovett, D.H., and Sarkar, R. (2005). Intronic regulation of matrix metalloproteinase-2 revealed by in vivo transcriptional analysis in ischemia. Proc Natl Acad Sci U S A *102*, 16345-16350.

Lee, S.K., Fletcher, A.G., Zhang, L., Chen, X., Fischbeck, J.A., and Stargell, L.A. (2010). Activation of a poised RNAPII-dependent promoter requires both SAGA and mediator. Genetics *184*, 659-672.

Lehnertz, B., Ueda, Y., Derijck, A.A., Braunschweig, U., Perez-Burgos, L., Kubicek, S., Chen, T., Li, E., Jenuwein, T., and Peters, A.H. (2003). Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. Curr Biol *13*, 1192-1200.

LeProust, E.M., Pearson, C.E., Sinden, R.R., and Gao, X. (2000). Unexpected formation of parallel duplex in GAA and TTC trinucleotide repeats of Friedreich's ataxia. J Mol Biol *302*, 1063-1080.

Li, B., Carey, M., and Workman, J.L. (2007). The role of chromatin during transcription. Cell *128*, 707-719.

Li, H., Ilin, S., Wang, W., Duncan, E.M., Wysocka, J., Allis, C.D., and Patel, D.J. (2006). Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. Nature *442*, 91-95.

Li, K., Besse, E.K., Ha, D., Kovtunovych, G., and Rouault, T.A. (2008). Iron-dependent regulation of frataxin expression: implications for treatment of Friedreich ataxia. Hum Mol Genet *17*, 2265-2273.

Li, S., and Shogren-Knaak, M.A. (2009). The Gcn5 bromodomain of the SAGA complex facilitates cooperative and cross-tail acetylation of nucleosomes. J Biol Chem *284*, 9411-9417.

Libby, R.T., Hagerman, K.A., Pineda, V.V., Lau, R., Cho, D.H., Baccam, S.L., Axford, M.M., Cleary, J.D., Moore, J.M., Sopher, B.L., Tapscott, S.J., Filippova, G.N., Pearson, C.E., and La Spada, A.R. (2008). CTCF cis-regulates trinucleotide repeat instability in an epigenetic manner: a novel basis for mutational hot spot determination. PLoS Genet *4*, e1000257.

Lim, F., Palomo, G.M., Mauritz, C., Gimenez-Cassina, A., Illana, B., Wandosell, F., and Diaz-Nido, J. (2007). Functional recovery in a Friedreich's ataxia mouse model by frataxin gene transfer using an HSV-1 amplicon vector. Mol Ther *15*, 1072-1078.

Lis, J.T., Mason, P., Peng, J., Price, D.H., and Werner, J. (2000). P-TEFb kinase recruitment and function at heat shock loci. Genes Dev *14*, 792-803.

Liu, Y., Prasad, R., Beard, W.A., Hou, E.W., Horton, J.K., McMurray, C.T., and Wilson, S.H. (2009). Coordination between polymerase beta and FEN1 can modulate CAG repeat expansion. J Biol Chem *284*, 28352-28366.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251-260.

Lynch, D.R., Perlman, S.L., and Meier, T. (2010). A phase 3, double-blind, placebo-controlled trial of idebenone in friedreich ataxia. Arch Neurol *67*, 941-947.

Marmolino, D., Acquaviva, F., Pinelli, M., Monticelli, A., Castaldo, I., Filla, A., and Cocozza, S. (2009). PPAR-gamma agonist Azelaoyl PAF increases frataxin protein and mRNA expression: new implications for the Friedreich's ataxia therapy. Cerebellum *8*, 98-103.

Miranda, C.J., Santos, M.M., Ohshima, K., Smith, J., Li, L., Bunting, M., Cossee, M., Koenig, M., Sequeiros, J., Kaplan, J., and Pandolfo, M. (2002). Frataxin knockin mouse. FEBS Lett *512*, 291-297.

Mirkin, S.M. (2007). Expandable DNA repeats and human disease. Nature *447*, 932-940.

Monticelli, A., Giacchetti, M., De Biase, I., Pianese, L., Turano, M., Pandolfo, M., and Cocozza, S. (2004). New clues on the origin of the Friedreich ataxia expanded alleles from the analysis of new polymorphisms closely linked to the mutation. Hum Genet *114*, 458-463.

Musco, G., Stier, G., Kolmerer, B., Adinolfi, S., Martin, S., Frenkiel, T., Gibson, T., and Pastore, A. (2000). Towards a structural understanding of Friedreich's ataxia: the solution structure of frataxin. Structure *8*, 695-707.

Nagata, M., Ito, T., Arimitsu, N., Koyama, H., and Sekimizu, K. (2009). Transcription arrest relief by S-II/TFIIS during gene expression in erythroblast differentiation. Genes Cells *14*, 371-380.

Napierala, M., Dere, R., Vetcher, A., and Wells, R.D. (2004). Structure-dependent recombination hot spot activity of GAA.TTC sequences from intron 1 of the Friedreich's ataxia gene. J Biol Chem *279*, 6444-6454.

Ng, H.H., Xu, R.M., Zhang, Y., and Struhl, K. (2002). Ubiquitination of histone H2B by Rad6 is required for efficient Dot1-mediated methylation of histone H3 lysine 79. J Biol Chem *277*, 34655-34657.

Ng, H.H., Zhang, Y., Hendrich, B., Johnson, C.A., Turner, B.M., Erdjument-Bromage, H., Tempst, P., Reinberg, D., and Bird, A. (1999). MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. Nat Genet *23*, 58-61.

Ohshima, K., Montermini, L., Wells, R.D., and Pandolfo, M. (1998). Inhibitory effects of expanded GAA.TTC triplet repeats from intron I of the Friedreich ataxia gene on transcription and replication in vivo. J Biol Chem *273*, 14588-14595.

Ohshima, K., Sakamoto, N., Labuda, M., Poirier, J., Moseley, M.L., Montermini, L., Ranum, L.P., Wells, R.D., and Pandolfo, M. (1999). A nonpathogenic GAAGGA repeat in the Friedreich gene: implications for pathogenesis. Neurology *53*, 1854-1857.

Ohshima, K., and Wells, R.D. (1997). Hairpin formation during DNA synthesis primer realignment in vitro in triplet repeat sequences from human hereditary disease genes. J Biol Chem *272*, 16798-16806.

Okitsu, C.Y., and Hsieh, C.L. (2007). DNA methylation dictates histone H3K4 methylation. Mol Cell Biol *27*, 2746-2757.

Okitsu, C.Y., Hsieh, J.C., and Hsieh, C.L. (2010). Transcriptional activity affects the H3K4me3 level and distribution in the coding region. Mol Cell Biol *30*, 2933-2946.

Oktay, Y., Dioum, E., Matsuzaki, S., Ding, K., Yan, L.J., Haller, R.G., Szweda, L.I., and Garcia, J.A. (2007). Hypoxia-inducible factor 2alpha regulates expression of the mitochondrial aconitase chaperone protein frataxin. J Biol Chem *282*, 11750-11756.

Pandolfo, M. (1998). Molecular genetics and pathogenesis of Friedreich ataxia. Neuromuscul Disord *8*, 409-415.

Pearson, C.E., Nichol Edamura, K., and Cleary, J.D. (2005). Repeat instability: mechanisms of dynamic mutations. Nat Rev Genet *6*, 729-742.

Perini, G., Diolaiti, D., Porro, A., and Della Valle, G. (2005). In vivo transcriptional regulation of N-Myc target genes is controlled by E-box methylation. Proc Natl Acad Sci U S A *102*, 12117-12122.

Peterlin, B.M., and Price, D.H. (2006). Controlling the elongation phase of transcription with P-TEFb. Mol Cell *23*, 297-305.

Pianese, L., Turano, M., Lo Casale, M.S., De Biase, I., Giacchetti, M., Monticelli, A., Criscuolo, C., Filla, A., and Cocozza, S. (2004). Real time PCR quantification of frataxin mRNA in the peripheral blood leucocytes of Friedreich ataxia patients and carriers. J Neurol Neurosurg Psychiatry *75*, 1061-1063.

Pietrobono, R., Tabolacci, E., Zalfa, F., Zito, I., Terracciano, A., Moscato, U., Bagni, C., Oostra, B., Chiurazzi, P., and Neri, G. (2005). Molecular dissection of the events leading to inactivation of the FMR1 gene. Hum Mol Genet *14*, 267-277.

Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131-135.

Pook, M.A., Al-Mahdawi, S., Carroll, C.J., Cossee, M., Puccio, H., Lawrence, L., Clark, P., Lowrie, M.B., Bradley, J.L., Cooper, J.M., Koenig, M., and Chamberlain, S. (2001). Rescue of the Friedreich's ataxia knockout mouse by human YAC transgenesis. Neurogenetics *3*, 185-193.

Puccio, H., Simon, D., Cossee, M., Criqui-Filipe, P., Tiziano, F., Melki, J., Hindelang, C., Matyas, R., Rustin, P., and Koenig, M. (2001). Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and Fe-S enzyme deficiency followed by intramitochondrial iron deposits. Nat Genet *27*, 181-186.

Punga, T., and Buhler, M. (2010). Long intronic GAA repeats causing Friedreich ataxia impede transcription elongation. EMBO Mol Med *2*, 120-129.

Rahl, P.B., Lin, C.Y., Seila, A.C., Flynn, R.A., McCuine, S., Burge, C.B., Sharp, P.A., and Young, R.A. (2010). c-Myc regulates transcriptional pause release. Cell *141*, 432-445.

Rai, M., Soragni, E., Jenssen, K., Burnett, R., Herman, D., Coppola, G., Geschwind, D.H., Gottesfeld, J.M., and Pandolfo, M. (2008). HDAC inhibitors correct frataxin deficiency in a Friedreich ataxia mouse model. PLoS One *3*, e1958.

Ruan, H., and Wang, Y.H. (2008). Friedreich's ataxia GAA.TTC duplex and GAA.GAA.TTC triplex structures exclude nucleosome assembly. J Mol Biol *383*, 292-300.

Sakamoto, N., Chastain, P.D., Parniewski, P., Ohshima, K., Pandolfo, M., Griffith, J.D., and Wells, R.D. (1999). Sticky DNA: self-association properties of long GAA.TTC repeats in R.R.Y triplex structures from Friedreich's ataxia. Mol Cell *3*, 465-475.

Sarsero, J.P., Li, L., Wardan, H., Sitte, K., Williamson, R., and Ioannou, P.A. (2003). Upregulation of expression from the FRDA genomic locus for the therapy of Friedreich ataxia. J Gene Med *5*, 72-81.

Saunders, A., Core, L.J., and Lis, J.T. (2006). Breaking barriers to transcription elongation. Nat Rev Mol Cell Biol *7*, 557-567.

Saveliev, A., Everett, C., Sharpe, T., Webster, Z., and Festenstein, R. (2003). DNA triplet repeats mediate heterochromatin-protein-1-sensitive variegated gene silencing. Nature *422*, 909-913.

Schmucker, S., Argentini, M., Carelle-Calmels, N., Martelli, A., and Puccio, H. (2008). The in vivo mitochondrial two-step maturation of human frataxin. Hum Mol Genet *17*, 3521-3531.

Schotta, G., Lachner, M., Sarma, K., Ebert, A., Sengupta, R., Reuter, G., Reinberg, D., and Jenuwein, T. (2004). A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev *18*, 1251-1262.

Schuhmacher, M., Kohlhuber, F., Holzel, M., Kaiser, C., Burtscher, H., Jarsch, M., Bornkamm, G.W., Laux, G., Polack, A., Weidle, U.H., and Eick, D. (2001). The

transcriptional program of a human B cell line in response to Myc. Nucleic Acids Res *29*, 397-406.

Seenundun, S., Rampalli, S., Liu, Q.C., Aziz, A., Palii, C., Hong, S., Blais, A., Brand, M., Ge, K., and Dilworth, F.J. (2010). UTX mediates demethylation of H3K27me3 at muscle-specific genes during myogenesis. EMBO J *29*, 1401-1411.

Seluanov, A., Mittelman, D., Pereira-Smith, O.M., Wilson, J.H., and Gorbunova, V. (2004). DNA end joining becomes less efficient and more error-prone during cellular senescence. Proc Natl Acad Sci U S A *101*, 7624-7629.

Shishkin, A.A., Voineagu, I., Matera, R., Cherng, N., Chernet, B.T., Krasilnikova, M.M., Narayanan, V., Lobachev, K.S., and Mirkin, S.M. (2009). Large-scale expansions of Friedreich's ataxia GAA repeats in yeast. Mol Cell *35*, 82-92.

Sims, R.J., 3rd, Millhouse, S., Chen, C.F., Lewis, B.A., Erdjument-Bromage, H., Tempst, P., Manley, J.L., and Reinberg, D. (2007). Recognition of trimethylated histone H3 lysine 4 facilitates the recruitment of transcription postinitiation factors and pre-mRNA splicing. Mol Cell *28*, 665-676.

Soragni, E., Herman, D., Dent, S.Y., Gottesfeld, J.M., Wells, R.D., and Napierala, M. (2008). Long intronic GAA*TTC repeats induce epigenetic changes and reporter gene silencing in a molecular model of Friedreich ataxia. Nucleic Acids Res *36*, 6056-6065.

Splinter, E., Heath, H., Kooren, J., Palstra, R.J., Klous, P., Grosveld, F., Galjart, N., and de Laat, W. (2006). CTCF mediates long-range chromatin looping and local histone modification in the beta-globin locus. Genes Dev *20*, 2349-2354.

Sturm, B., Stupphann, D., Kaun, C., Boesch, S., Schranzhofer, M., Wojta, J., Goldenberg, H., and Scheiber-Mojdehkar, B. (2005). Recombinant human erythropoietin: effects on frataxin expression in vitro. Eur J Clin Invest *35*, 711-717.

Swami, M., Hendricks, A.E., Gillis, T., Massood, T., Mysore, J., Myers, R.H., and Wheeler, V.C. (2009). Somatic expansion of the Huntington's disease CAG repeat in the brain is associated with an earlier age of disease onset. Hum Mol Genet *18*, 3039-3047.

Vakoc, C.R., Mandat, S.A., Olenchock, B.A., and Blobel, G.A. (2005). Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. Mol Cell *19*, 381-391.

van Haaften, G., Dalgliesh, G.L., Davies, H., Chen, L., Bignell, G., Greenman, C., Edkins, S., Hardy, C., O'Meara, S., Teague, J., Butler, A., Hinton, J., Latimer, C., Andrews, J., Barthorpe, S., Beare, D., Buck, G., Campbell, P.J., Cole, J., Forbes, S., Jia, M., Jones, D., Kok, C.Y., Leroy, C., Lin, M.L., McBride, D.J., Maddison, M., Maquire, S., McLay, K., Menzies, A., Mironenko, T., Mulderrig, L., Mudie, L., Pleasance, E., Shepherd, R., Smith, R., Stebbings, L., Stephens, P., Tang, G., Tarpey, P.S., Turner, R., Turrell, K., Varian, J., West, S., Widaa, S., Wray, P., Collins, V.P., Ichimura, K., Law, S., Wong, J., Yuen, S.T., Leung, S.Y., Tonon, G., DePinho, R.A., Tai, Y.T., Anderson, K.C., Kahnoski, R.J., Massie, A., Khoo, S.K., Teh, B.T., Stratton, M.R., and Futreal, P.A. (2009). Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet *41*, 521-523.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. Science *303*, 672-676.

Wang, Z., Schones, D.E., and Zhao, K. (2009). Characterization of human epigenomes. Curr Opin Genet Dev *19*, 127-134.

Wassenegger, M. (2005). The role of the RNAi machinery in heterochromatin formation. Cell *122*, 13-16.

Wells, R.D. (1996). Molecular basis of genetic instability of triplet repeats. J Biol Chem *271*, 2875-2878.

Wilson, R.B. (2003). Frataxin and frataxin deficiency in Friedreich's ataxia. J Neurol Sci *207*, 103-105.

Wilson, R.B., and Roof, D.M. (1997). Respiratory deficiency due to loss of mitochondrial DNA in yeast lacking the frataxin homologue. Nat Genet *16*, 352-357.

Wu, C.H., Yamaguchi, Y., Benjamin, L.R., Horvat-Gordon, M., Washinsky, J., Enerly, E., Larsson, J., Lambertsson, A., Handa, H., and Gilmour, D. (2003). NELF and DSIF cause promoter proximal pausing on the hsp70 promoter in Drosophila. Genes Dev *17*, 1402-1414.

Zuccato, C., and Cattaneo, E. (2009). Brain-derived neurotrophic factor in neurodegenerative diseases. Nat Rev Neurol *5*, 311-322.

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