Long intronic GAA repeats causing Friedreich ataxia impede transcription elongation

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Friedreich ataxia is a degenerative disease caused by deficiency of the protein frataxin (FXN). An intronic expansion of GAA triplets in the FXN-encoding gene, FXN, causes gene silencing and thus reduced FXN protein levels. Although it is widely assumed that GAA repeats block transcription via the assembly of an inaccessible chromatin structure marked by methylated H3K9, direct proof for this is lacking. In this study, we analysed different histone modification patterns along the human FXN gene in FRDA patient-derived lymphoblastoid cell lines. We show that FXN mRNA synthesis, but not turnover rates are affected by an expanded GAA repeat tract. Importantly, rather than preventing transcription initiation, long GAA repeat tracts affect transcription at the elongation step and this can occur independently of H3K9 methylation. Our data demonstrate that finding novel strategies to overcome the transcription elongation problem may develop into promising new treatments for FRDA.

INTRODUCTION

Friedreich ataxia (FRDA) is an autosomal recessive degenerative disease that primarily affects the nervous system and the heart (Campuzano et al, 1996). FRDA is the most common inherited ataxia across most of Europe, with an estimated prevalence of 3–4 cases per 100,000 individuals. The disease is caused by deficiency of the protein frataxin (FXN), which plays a crucial role in many aspects of mitochondrial iron metabolism. As a result of the FXN insufficiency, patients develop neurodegeneration of the large sensory neurons and spinocerebellar tracts, cardiomyopathy and diabetes mellitus (Schulz et al, 2009).

The genetic basis for the FXN protein deficiency lies in the structure of the FXN gene. The majority of FRDA patients have a pathogenic expansion of a trinucleotide GAA repeat within the first intron of the FXN gene. Whereas normal individuals have up to 38 GAA repeats, FRDA patients have approximately 70 to more than 1000 triplets, most commonly 600–900 GAA triplets on both alleles of the FXN gene (Pandolfo, 2009). Interestingly, the larger the number of GAAs, the earlier is the disease onset and the quicker the decline of the patient. As a functional consequence of GAA repeat hyperexpansion, the FRDA patients have a marked deficiency of FXN mRNA and thus protein levels, thought to be due to reduced FXN gene transcription (Campuzano et al, 1996, 1997).

Two molecular models have been put forward to explain silencing of FXN gene expression at the level of gene transcription. Based on *in vitro* experiments, it has been proposed that expanded GAA repeats adopt an unusual DNA structure, which impedes the progress of RNA polymerase and thus reduces gene transcription (Bidichandani et al, 1998; Ohshima et al, 1998; Sakamoto et al, 1999). More recently, it has been suggested that expanded GAA repeats induce the assembly of heterochromatin *in vivo*. Gene silencing at expanded FXN alleles is accompanied by hypoacetylation of histones H3 and H4 and methylation of histone H3 at Lys9 (H3K9), which is supposed to negatively regulate FXN expression (Al-Mahdawi et al, 2008; Greene et al, 2007; Herman et al, 2006; Rai et al, 2008). Furthermore, GAA repeats can exert position effect variegation (PEV) on a heterochromatin-sensitive reporter gene (Saveliev et al, 2003). PEV occurs when a gene lies in close proximity to heterochromatin, and results in the silencing of the affected gene. Importantly, this silencing is enhanced upon over-expression of the heterochromatin protein HP1 (Saveliev et al, 2003). These data supported the model that the expanded GAA repeat structure induces and/or maintains a repressive heterochromatin structure, which is generally thought to inhibit access of transcription factors and thus leads to transcriptional silencing of the respective gene (Dion & Wilson, 2009; Pandolfo, 2009).

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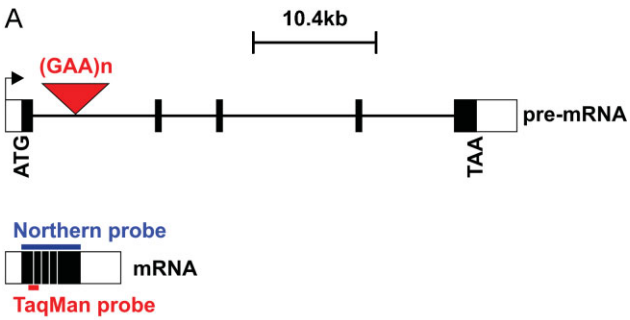
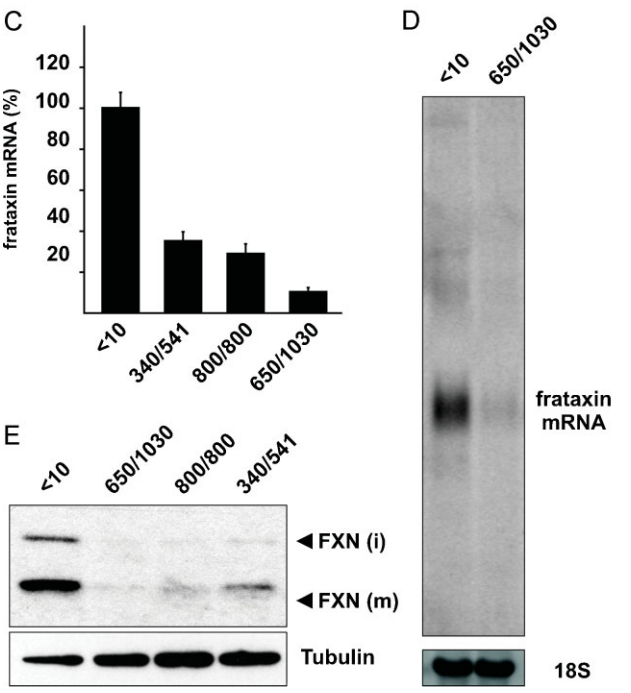
In this study, we confirm previous results that the expanded GAA repeat tract in FRDA patient-derived lymphoblastoid cell lines is associated with methylated H3K9, a characteristic mark for heterochromatin. However, we find that H3K9 methylation does not spread into the 5′ and 3′ parts of the FXN gene when studied in its genomic context. Consistent with the H3K9 methylation pattern, our data demonstrate that transcription initiation is not affected by expanded GAA repeat tracts. Rather, transcription of pathogenic FXN alleles is affected at the elongation step. Importantly, we show that H3K9 methylation is dispensable for this elongation block, supporting a model in which the DNA structure adopted by the expanded GAA repeats itself impedes the progress of RNA polymerase.



B

Phenotype	(GAA)n	Age	Sex	Coriell ID
wt	<10	14y	male	GM15851
FRDA	650/1030	13y	male	GM15850
FRDA	800/800	41y	female	GM16209
FRDA	340/541	30y	male	GM04079



RESULTS

An expanded GAA repeat tract causes silencing of the FXN gene in FRDA patient-derived B-lymphoblasts

In most cases of FRDA, a GAA repeat expansion leads to downregulation of the FXN gene and the protein it encodes for, FXN (Campuzano et al, 1996, 1997). In recent years, work from several laboratories has demonstrated that FXN silencing is associated with changes in post-translational histone modifications, suggesting that epigenetic mechanisms may be involved in FXN silencing (Al-Mahdawi et al, 2008; Greene et al, 2007; Herman et al, 2006; Rai et al, 2008). However, little is known about the epigenetic mechanisms that either establish these histone modifications or silence the pathogenic FXN allele. Previous studies also focused mainly on the repeat tract and did not directly address the influence of long GAA repeats on the transcriptional status of the FXN gene. Information about histone modifications beyond the GAA repeat tract at the FXN gene locus and their possible effects on the transcription machinery is not available. Therefore, we set out to investigate these issues in more detail.

To study silencing of the FXN locus in its natural genomic context (Fig 1A), we decided to work with several FRDA patient-derived lymphoblastoid cell lines (Coriell Cell Repositories), which have between 340 and 1030 GAA triplets on both FXN alleles (Fig 1B). As a control, a lymphoblastoid cell line from a healthy sibling ('wild type, GM15851') of one of the FRDA patients ('FRDA', GM15850) was used. In line with previous reports (Burnett et al, 2006; Herman et al, 2006), we observed an inverse correlation between GAA repeat length and FXN protein levels (Fig 1E). Both quantitative reverse-transcription real-time polymerase chain reaction (qRT-PCR) and Northern blot analysis revealed a strong reduction in FXN mRNA levels in the FRDA cell lines (Fig 1C and D). Interestingly, FXN silencing was strongest in the cell line with the most GAA triplets (GM15850, 650/1030 GAA triplets). Importantly, no alternatively spliced FXN transcripts could be observed in this FRDA cell line (Fig 1D). This suggests that long GAA repeat tracts at the FXN gene locus are unlikely to affect pre-mRNA splicing but affect either mRNA half-life or transcription of the FXN gene.

Figure 1. Pathogenic GAA repeat expansions reduce FXN mRNA and protein levels.

A. Schematic view of the human FXN gene (ENSG00000165060). Exons and untranslated regions are depicted as black and white boxes, respectively. The red arrowhead indicates localization of the GAA repeats. Northern blot and TaqMan probes annealing to the FXN mRNA are indicated as blue and red lines, respectively.

B. Cell lines used throughout this study. All cell lines were obtained from Coriell Cell Repositories.

C. Reduced accumulation of FXN mRNA in FRDA cells as determined by qRT-PCR. Values for FXN mRNA were normalized to 18S rRNA.

D. Northern blot showing that no major alternatively spliced FXN isoforms exist and that mRNA levels are reduced in FRDA cells. The Northern blot was probed with a human FXN cDNA probe. 18S served as a loading control.

E. Western blot showing reduced accumulation of FXN protein in FRDA cells. Intermediate (i) and mature (m) forms of the FXN proteins are indicated.

FXN silencing occurs on a transcriptional level

Despite extensive research on FRDA, no kinetic data on FXN RNA turnover is available. We therefore tested whether FXN mRNA turnover kinetics are different in wild type and FRDA cells by measuring FXN mRNA levels at different time points after blocking transcription with Actinomycin D (Sobell, 1985). This experiment revealed that the half-life of FXN mRNA is ~11 h in both wild type and FRDA cells (Fig 2B). As a control, we also measured the short-lived c-myc mRNA in the same samples and calculated a half-life of ~20 min (Fig 2A). These results suggested that FXN mRNA levels are regulated on a transcriptional, rather than post-transcriptional level. Therefore, we employed metabolic labelling to assess synthesis rates for FXN mRNA (Dolken et al, 2008). In these experiments, after addition of 4-thiouridine (4sU) to the cell culture media, newly synthesized RNA is labelled by incorporation of 4sU. This allows a biochemical separation of unlabelled 'old' RNA from labelled 'new' RNA (Fig 2C). We measured newly synthesized FXN mRNA at different time points after 4sU addition to the medium by qRT-PCR and found that FXN mRNA accumulated faster in wild type than in FRDA cells (Fig 2D). From the same experiment we isolated unlabelled FXN mRNA ('old RNA', Fig 2C) and used it to define the FXN mRNA decay rates. Consistent with the results obtained from Actinomycin D treatments shown in Fig 2B, the FXN mRNA half-life was the same in both wild type and FRDA cells (Fig 2E). Taken together, these results showed that the FXN gene is silenced on a transcriptional level and that post-transcriptional gene silencing mechanisms are not significantly contributing to its repression.

H3K9 methylation is locally confined in FRDA cells

Our results showing that transcription of the FXN gene is affected by a long GAA repeat tract would be consistent with

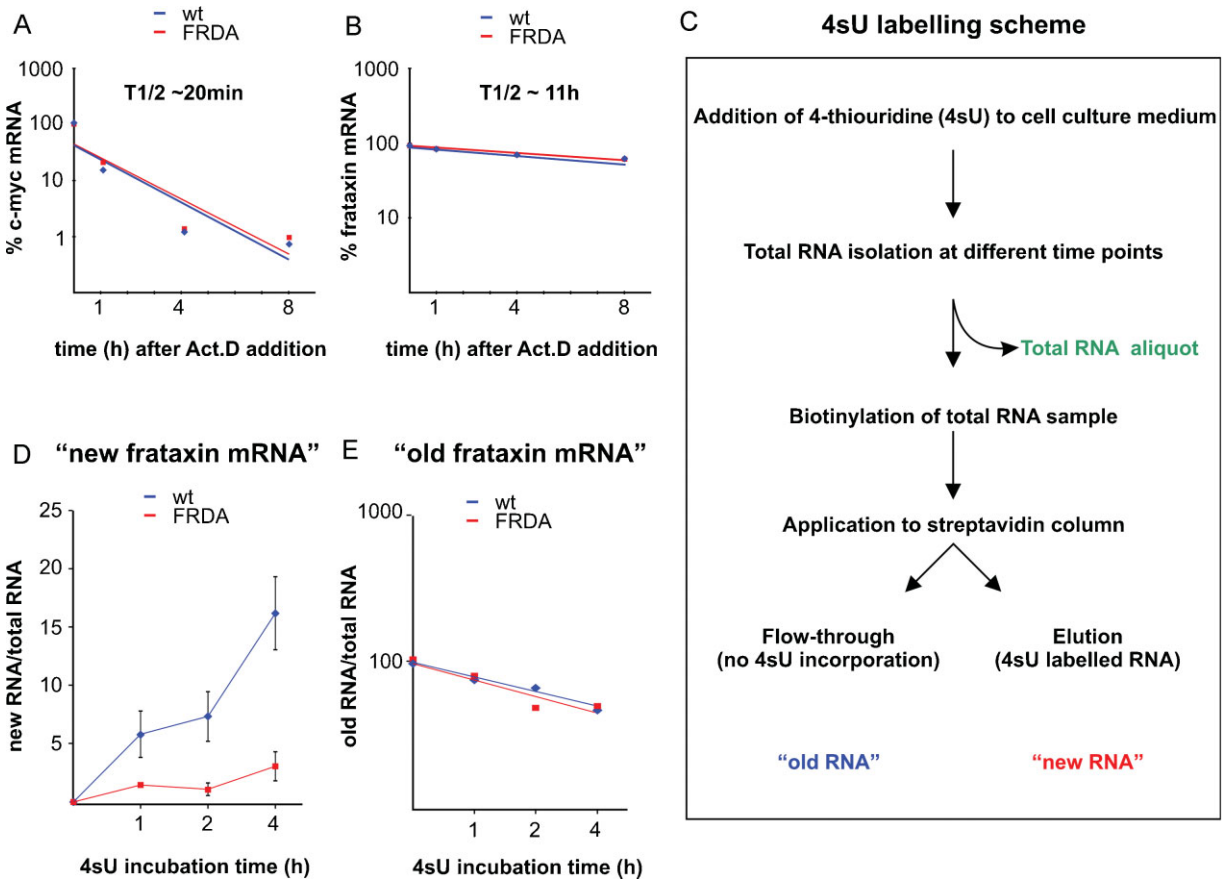


Figure 2. Synthesis but not decay rate of FXN mRNA is different in wild type and FRDA cells.

A. Decay kinetics of c-myc mRNA.

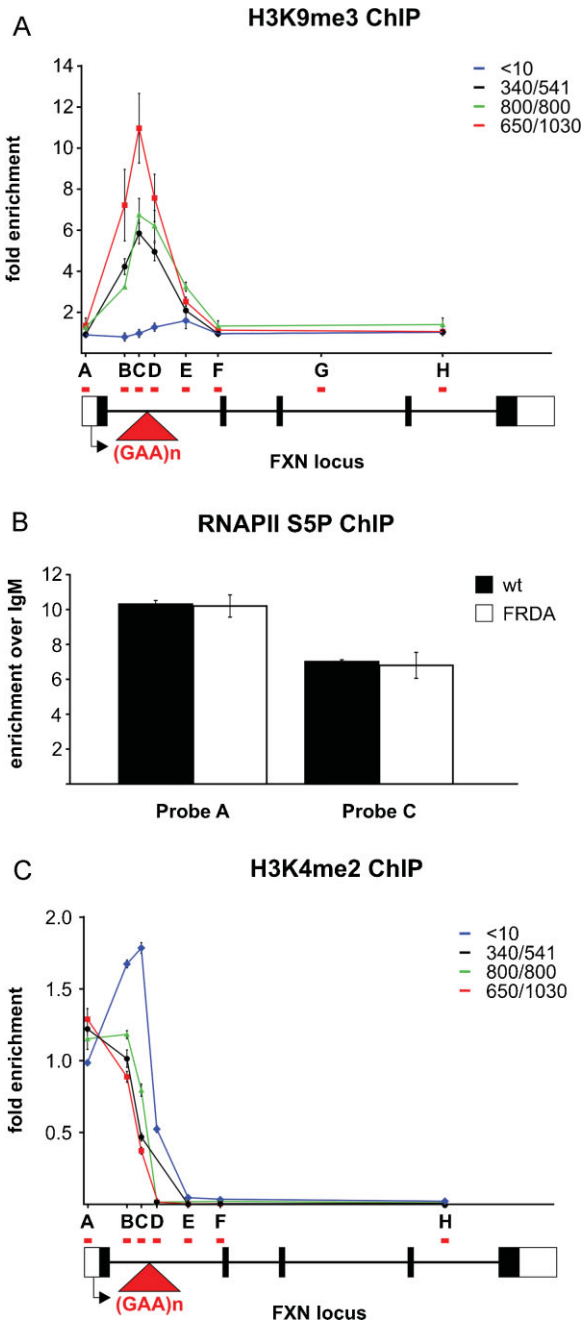
B. FXN mRNA decay rate is similar in WT (GM15851) and FRDA (GM15850) cells. (A, B) WT (GM15851) and FRDA (GM15850) cells were treated with Actinomycin D (10 μg/ml) and total RNA was isolated at the indicated time-points. Relative changes in c-myc or FXN mRNA levels were analysed by qRT-PCR and the data were normalized to 18S rRNA.

C. 4-thiouridine (4sU) labelling scheme.

D. Synthesis rates for FXN mRNA in 4sU labelled wild type and FRDA cells are different. The values for biotinylated RNAs were normalized to total RNA

E. Similar decay rates for FXN mRNA in wild type and FRDA cells. The values for unlabelled RNAs were normalized to total RNA.

earlier findings that long GAA repeat tracts can adopt features characteristic of heterochromatin. GAA repeats confer variegation of expression on a linked transgene in mice, which is characteristic for heterochromatic gene silencing (Saveliev et al, 2003). More recent studies revealed that hypoacetylation of specific residues in histones H3 and H4 and methylation of H3K9, a hallmark of heterochromatin, can be found at expanded GAA repeats in FRDA patient-derived cells (Al-Mahdawi et al, 2008; Greene et al, 2007; Herman et al, 2006). However, little is known about the spreading of H3K9 methylation along the FXN gene and direct evidence for methylated H3K9 being responsible for FXN silencing is lacking.



To examine this, we performed chromatin immunoprecipitation (ChIP) assays with antibodies against methylated H3K9 (H3K9me2/3) in wild type and FRDA cells. We analysed the relative enrichment of the H3K9me2/3 mark compared to background levels using different primer pairs covering the ~30,000 bp FXN gene locus (Fig 3A). In accordance with previously published data, FRDA cells showed accumulation of H3K9me2/3 signals in the proximity of the GAA repeats when compared to wild-type cells (Herman et al, 2006; Fig 3A, probes B–F, Fig 5A and B, Supporting Information Fig S1A). However, we found that H3K9me2/3 was confined to a region spanning from ~1400 bp upstream to ~5000 bp downstream of the GAA repeat expansion (Fig 3A and Supporting Information Fig S1). Interestingly, GAA repeats can exert PEV on a linked transgene, demonstrating spreading of heterochromatin (Saveliev et al, 2003). In contrast, although the expanded GAA repeat tract plus ~6 kb of flanking sequences in the first intron of the FXN gene is associated with methylated H3K9 in FRDA cells, spreading of this heterochromatic mark towards both ends of the gene seems to be prevented by a mechanism that has yet to be determined.

Transcription initiation is not affected by GAA repeat expansion

It has been suggested that long GAA repeat-mediated heterochromatin assembly affects transcription initiation at the FXN gene promoter (Greene et al, 2007; Saveliev et al, 2003). However, our results that H3K9 methylation does not spread into the promoter region (Fig 3A and Supporting Information Fig S1) suggested that transcription initiation might not be affected in FRDA cells. Therefore, we set out to investigate transcription initiation at the FXN gene promoter more carefully.

The largest subunit of the RNA polymerase II (RNAPII) holoenzyme has a very long and unstructured C-terminal domain (CTD). The CTD is made up of 52 heptad repeats in humans and undergoes various post-translational modifications that are

Figure 3. H3K9 methylation is locally confined in FRDA cells and does not affect marks of transcription initiation.

A. ChIP assay to profile H3K9me3 along the FXN gene. The profile for wild type is shown in blue (<10) and the profiles for different FRDA cells are shown in black (340/541), green (800/800) and red (650/1030). The digits after each colour line indicate the reported GAA repeat numbers in the corresponding cell lines as shown in Fig 1B. The fold enrichment was determined by qPCR and is relative to the value obtained with probe A in wild-type cells. Exons and untranslated regions are depicted as black and white boxes, respectively. The red arrowhead indicates localization of the GAA repeats. Amplified DNA fragments A-H by qPCR are indicated in red. The exact genomic location of amplicons is indicated in Supporting Information Table 1.

B. ChIP assay using an antibody against the phosphorylated CTD (serine 5) of RNAPII. Data are represented as fold enrichment over IgM antibody background. Primer pairs A and C were used for FXN (see schematic in (A)). Detailed information about the primers is included in Supporting Information Table 1.

C. ChIP assay to profile H3K4me2 along the FXN gene. The profile for wild type is shown in blue (<10) and the profiles for different FRDA cells are shown in black (340/541), green (800/800) and red (650/1030). Location of qPCR probes and fold enrichment is presented as in Fig 3A.

important for the regulation of transcription. Initiation of transcription coincides with phosphorylation of the fifth serine in the heptad repeats of the CTD (S5P). S5P levels remain high as RNAPII transcribes the first few hundred nucleotides of genes but declines further downstream (Komarnitsky et al, 2000; Phatnani & Greenleaf, 2006). Using an S5P-specific RNAPII antibody, we found that the region 5′ to the GAA repeat was highly enriched for S5P-RNAPII (Fig 3B). Importantly, even the longest repeats (650/1030) did not affect the S5 phosphorylation status of RNAPII at the 5′ end of the FXN gene (Fig 3B), demonstrating that early events in the transcription of the FXN gene are not influenced by long GAA repeats. CTD phosphorylation of RNAPII has been implicated in the recruitment of histone methyltransferases (HMTs) which can methylate lysine 4 of histone H3 (H3K4), a histone modification which is characteristic of actively transcribed genes and found at the 5′ end of genes (Barski et al, 2007; Buratowski, 2009; Guenther et al, 2007; Pokholok et al, 2005; Schubeler et al, 2004). Consistent with our S5P RNAPII results, the FXN promoter region showed very similar dimethylated H3K4 (H3K4me2) signal accumulation in the wild type and FRDA cell lines (Figs 3C and 5C). In contrast, the H3K4me2 signal was reduced at expanded GAA repeats in all FRDA cell lines (Fig 3C, probes B–E). These results show that transcription at the FXN promoter is initiated equally well in both wild type and FRDA cells and suggest that transcription is rather affected at the elongation step.

An expanded GAA repeat tract affects H3K36 methylation

Our results that RNA synthesis rates are lower in FRDA cells, even though long GAA repeat tracts do not affect transcription initiation, strongly suggested that FXN silencing in FRDA cells may result from an elongation block induced by the long GAA repeat tract. This hypothesis is consistent with earlier work that long GAA repeat tracts block transcription elongation (Krasilnikova et al, 2007; Ohshima et al, 1998). However, this has never been directly addressed for the FXN gene in its genomic context. Therefore, we set out to investigate RNAPII elongation at the FXN gene in the FRDA patient-derived cell lines.

A commonly used indicator for transcription elongation is trimethylated lysine 36 of histone H3 (H3K36me3). Importantly, trimethylation of H3K36 occurs co-transcriptionally and thus is one of the best indicators for elongating RNAPII (Carrozza et al, 2005; Krogan et al, 2003). H3K36me3 is associated most prominently with 3′ ends of actively transcribed genes (Bannister et al, 2005; Barski et al, 2007; Bell et al, 2007). Consistent with this, we found H3K36me3 in the central and 3′ coding region of the FXN gene in wild-type cells (Fig 4A and C). In order to demonstrate that H3K36 trimethylation also depends on elongating RNAPII at the FXN gene locus, we treated wild-type cells with 5,6-dichloro-1-B-D-ribofuranosylbenzimidazole (DRB). DRB is a classical transcription inhibitor which affects RNAPII at the elongation step (Yamaguchi et al, 1998). As expected, this treatment resulted in reduced H3K36me3 signal along the FXN gene. Concomitantly, FXN mRNA levels were reduced in the same cells (Fig 4A and B). Thus, these experiments confirmed that H3K36 trimethylation at the FXN gene depends on elongating RNAPII. Importantly, H3K36me3 ChIP experiments with the FRDA cell lines revealed an inverse correlation with GAA triplet repeat length and FXN expression, *i.e.* the longer the repeat tract, the lower the H3K36me3 and FXN mRNA levels (Figs 1C and 4C). This demonstrates that transcription elongation is affected at pathogenic FXN alleles.

H3K9 methylation is dispensable for FXN silencing

Chromatin marked by methylated H3K9 has long been thought to be an inert and transcriptionally inactive structure. Therefore, one might have concluded that the local H3K9 methylation hinders transcription at pathogenic FXN alleles (Festenstein, 2006; Herman et al, 2006). However, recent findings have challenged this longstanding belief (Buhler & Moazed, 2007; Vakoc et al, 2005) and led us to test to what extent the H3K9 methylated chromatin found at the GAA repeat tract impacts transcription elongation in FRDA.

In contrast to H3K9me3, H3K9me2 appears to be of crucial importance in transcriptional control (Jenuwein, 2006). Reactivation of tumor suppressor genes in cancer cells can occur after removal of H3K9me2 without affecting H3K9me3 levels (McGarvey et al, 2006). In order to inhibit H3K9 dimethylation in the FRDA cell lines, we chose to use a recently identified HMT inhibitor, BIX-01294, which specifically inhibits the G9a HMT (Kubicek et al, 2007). Treatment of the FRDA cell lines with this compound removed the H3K9me2 mark from the GAA repeat tract very efficiently (Fig 5A). Interestingly, H3K9me3 levels were also greatly affected upon BIX-01294 treatment (Fig 5B). H3K4me2 was not affected by the BIX-01294 treatment, further corroborating the specificity of this compound towards H3K9 methyltransferases (Fig 5C). These results indicate that G9a might be the main HMT responsible for H3K9 dimethylation and that this is a prerequisite for H3K9 trimethylation at the FXN locus. Surprisingly, although H3K9me2 was erased from the expanded GAA repeats, H3K36me3 and FXN mRNA levels remained unaffected (Fig 5B and data not shown). Only in the FRDA cell lines with shorter repeats could a slight increase in FXN mRNA levels be observed. Therefore, H3K9 methylation plays, if at all, a redundant role in FXN silencing.

DISCUSSION

Despite many years of research, the precise FXN gene silencing mechanism underlying FRDA is not yet understood. The rapidly developing field of epigenetics has been providing new hints as to what mechanisms might contribute to FXN gene silencing. We have started an extended analysis of epigenetic changes at the FXN gene locus by comparing lymphoblastoid cell lines derived from patients and healthy individuals. Below we discuss the implications of our findings for the molecular understanding of FRDA.

Transcriptional *versus* post-transcriptional gene silencing

An effect of GAA repeats on transcription was first suggested by experiments performed with a reporter gene whose expression was inhibited by an intronic GAA repeat insertion (Ohshima et al, 1998). Those experiments did not reveal splicing abnormalities and provided evidence in favour of a transcription

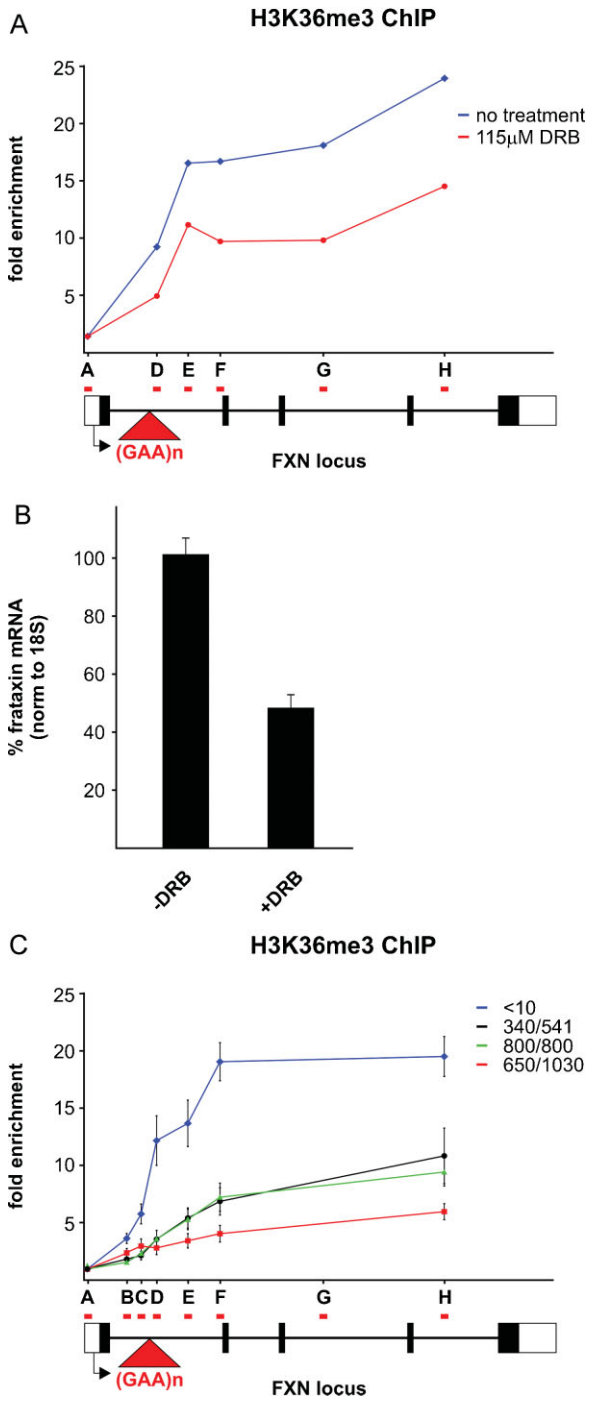


Figure 4. Expanded GAA repeats affect H3K36 trimethylation.

A. Treatment of wild-type cells with the transcription elongation inhibitor DRB reduces the H3K36me3 signal along the FXN gene locus. Wild-type (GM15851) cells were treated with 115 μM DRB for 5 h before ChIP with anti-H3K36me3 antibody. Location of qPCR probes and fold enrichment is presented as in Fig 3A.

B. Reduced accumulation of FXN mRNA after DRB treatment in wild-type (GM15851) cells. RNA was isolated from the same cells as used for the ChIP shown in (A).

C. ChIP assay to profile H3K36me3 along the FXN gene in wild type and different FRDA cell lines. The profile for wild type is shown in blue (<10) and the profiles for different FRDA cells are shown in black (340/541), green (800/800) and red (650/1030). The digits after each colour line indicate the reported GAA repeat numbers in the corresponding cell lines as shown in Fig 1B. The fold enrichment was determined by qPCR and is relative to the value obtained with probe A in wild-type cells. The red arrowhead indicates localization of the GAA repeats. Amplified DNA fragments A–H by qPCR are are indicated in red. The exact genomic location of amplicons is indicated in Supporting Information Table 1.

block. This is consistent with our findings that the synthesis rate, but not the half-life of FXN mRNA is affected in FRDA patient-derived lymphoblasts. Recently, it has been reported that a long intronic GAA repeat tract (100 × GAA) in hybrid minigenes negatively influences splicing of the repeat-containing pre-mRNA without affecting its transcription. This led the authors to conclude that inefficient FXN pre-mRNA splicing rather than a direct transcriptional block is likely to be the cause of lower levels of mature FXN mRNA in FRDA (Baralle et al, 2008). Unless alternatively spliced FXN RNAs are highly unstable, we should have been able to detect them by our Northern blot analysis. However, the only FXN RNA isoform which we were able to detect was correctly spliced mRNA. Therefore, we conclude that inefficient FXN pre-mRNA splicing, if at all, contributes only slightly to FXN gene silencing in FRDA. We suspect that the reason for this discrepancy lies in the design of the intron in the hybrid minigenes.

Consequence of H3K9 methylation

Our experiments revealed that transcription of pathogenic FXN alleles is affected at the elongation rather than initiation step, raising the question of whether it is the repeat DNA itself or the associated H3K9 methylation that impedes transcription elongation. The former possibility is strongly supported by earlier *in vitro* experiments which demonstrated that DNA triplex structures adopted by the GAA repeats might sequester RNA polymerases and therefore affect transcription (Mirkin, 2007; Sakamoto et al, 1999). In theory, the heterochromatin-like chromatin structure found at GAA repeats could also interfere with transcription elongation. However, our finding that FXN expression cannot be restored by preventing H3K9 methylation demonstrates that H3K9 methylation plays, if at all, a minor role in FXN silencing. Interestingly, GAA repeats can exert PEV on a heterochromatin-sensitive reporter gene (Saveliev et al, 2003). However, whether such GAA repeat-induced heterochromatin is refractory to the transcription machinery has not been tested. As recently demonstrated for PEV in fission yeast, it is possible that silencing could also be mediated by co-transcriptional RNA decay rather than solely switching off transcription, a possibility which needs to be addressed in future studies (Buhler, 2009; Buhler et al, 2007; Murakami et al, 2007). In conclusion, we propose that the unusual DNA structure adopted by long GAA repeat tracts constitutes the main obstacle for RNAPII. To what extent and how H3K9 methylation would contribute to silencing, if the problem of transcribing through GAA repeat DNA could be overcome, remains unknown.

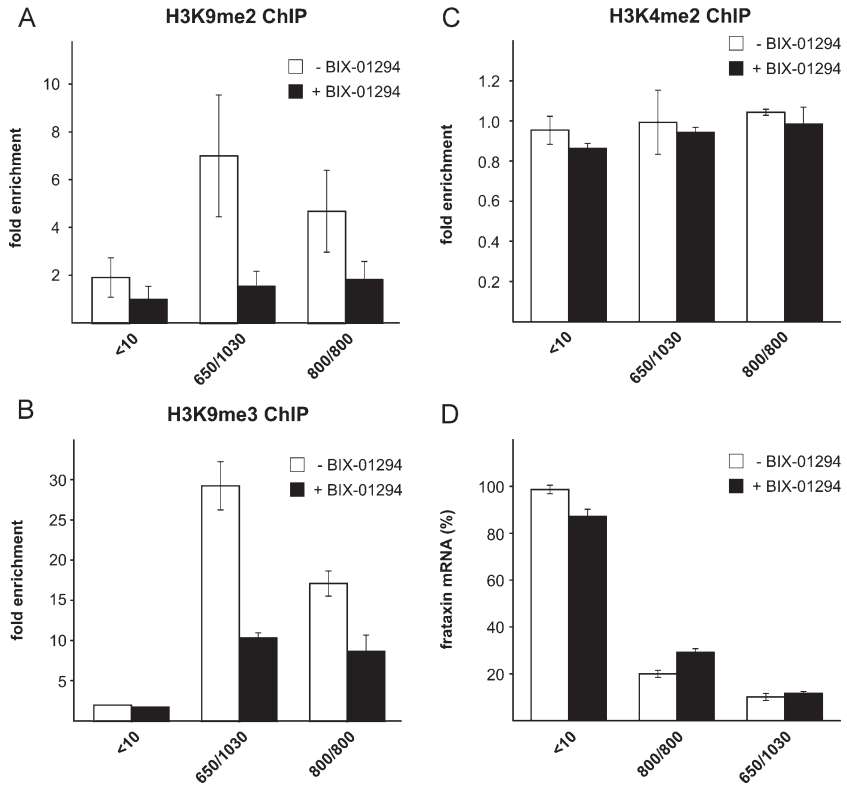


Figure 5. H3K9 methylation is dispensable for FXN gene silencing.

A. BIX-01294 treatment depletes H3K9me2 signals at expanded GAA repeats in FRDA cells. Wild type (<10) and two different FRDA cell lines were treated with 4 μM of BIX-01294 for 72 h. Probe C was used to determine the H3K9me2 levels which are shown relative to the value obtained with probe A in wild-type cells (see also Fig 3A).

B. BIX-01294 treatment depletes H3K9me2 signals at expanded GAA repeats in FRDA cells. Wild type (<10) and two different FRDA cell lines were treated with 4 μM of BIX-01294 for 72 h. Probe D was used to determine the H3K9me2 levels which are shown relative to the value obtained with probe A in wild-type cells (see also Fig 3A).

C. BIX-01294 treatment does not affect H3K4me2 signals at the FXN gene promoter. Wild type (<10) and two different FRDA cell lines were treated with 4 μM of BIX-01294 for 72 h. Probe A was used to determine the H3K9me2 levels which are shown relative to the value obtained in wild-type cells (see also Fig 3B).

D. BIX-01294 treatment does not reverse FXN gene silencing. Wild type (<10) and two FRDA cell lines (800/800, 650/1030) were treated with 4 μM of BIX-01294 for 72 h. Relative accumulation of FXN mRNA is shown after normalization to GAPDH mRNA levels.

Cause of H3K9 methylation

Irrespective of the consequence(s) of H3K9 methylation, our observations raise the interesting question as to how GAA repeats trigger H3K9 methylation and what prevents it from spreading into the promoter region. One possibility could be that stalled RNAPII itself recruits HMTs to methylate H3K9 locally. Based on our BIX-01294 results, this HMT is likely to be G9a. Alternatively, specific proteins might recognize triplex DNA structures formed by the repeats, or the GAA repeat sequence itself and recruit heterochromatin assembly factors which would methylate H3K9. Alternatively, such proteins could also be recruited by GAA-containing RNA rather than DNA. Furthermore, a role for the RNA interference (RNAi) pathway in the establishment of epigenetic histone modifications associated with noncoding repeat expansion diseases has been discussed recently (Dion & Wilson, 2009; Kumari & Usdin, 2009). However, we consider an involvement of the RNAi pathway in FRDA to be unlikely, as our deep-sequencing efforts did not reveal small interfering RNAs (siRNAs) that would match the FXN locus (T.P. and M.B., unpublished data).

Therapeutic intervention with FXN gene silencing

Since the expanded GAA repeats in FRDA reside in an intron and thus do not alter the amino acid sequence of the FXN protein, gene reactivation would be of therapeutic benefit. Efforts to find small molecules that reverse FXN gene silencing are therefore of prime medical importance. Encouraging progress in this endeavour has been made in recent years and it has been demonstrated that benzamide-based histone deacetylase inhibitors (HDACi) such as BML-210 and the related pimelic diphenylamides 4b and 106 have the ability to reactivate the silenced FXN gene in FRDA (Chou et al, 2008; Herman et al, 2006; Rai et al, 2008). These studies provided convincing evidence that the acetylation status of histone tails is an important regulator of transcription at the FXN locus. However, it is important to note that these

The paper explained

PROBLEM:

Friedreich ataxia (FRDA) is a degenerative disease caused by deficiency of the protein FXN. The genetic basis for this deficiency is an expansion of a GAA repeat within the first intron of the FXN encoding gene. The long repeats cause FXN gene silencing by a mechanism that is not well understood. Gene activation could be of therapeutic benefit and therefore it is important to fully understand the mechanisms by which FXN is silenced in FRDA patients.

RESULTS:

Long intronic GAA repeats can induce the methylation of histone H3 at lysine 9 (H3K9me) at the FXN locus in FRDA cells. This epigenetic modification is a hallmark of heterochromatin and it has therefore generally been assumed that the FXN locus is assembled into a chromatin structure that hinders transcription. However, we find that H3K9me is restricted to the GAA repeat tract and that initiation of transcription is not affected. Importantly, we demonstrate that FXN gene silencing can occur independently of H3K9 methylation. Therefore, the repeat DNA itself seems to be a major obstacle for RNA polymerase II progression at the FXN locus.

IMPACT:

The data demonstrate that long GAA repeats constitute an obstacle to the transcription machinery in FRDA patient-derived cells. The block appears to occur during transcription elongation, irrespectively of the H3K9 methylation status of the FXN gene. Novel therapeutic strategies designed to overcome this elongation problem may develop into promising new treatments for this devastating disease that has thus far remained incurable.

molecules increase FXN gene expression only moderately (T.P. and M.B., unpublished data; Herman et al, 2006; Xu et al, 2009). Moreover, FXN gene expression increases on both pathogenic and nonpathogenic alleles (Herman et al, 2006; Rai et al, 2008). Therefore, these HDAC inhibitors are not specifically reversing the silencing induced by long GAA repeats, but rather have a general positive impact on the expression of the FXN gene.

Interestingly, BIX-01294 treatment slightly increased FXN mRNA levels in cells with 800 GAA triplets. However, we did never observe such an effect for the FRDA cell line with the longest repeats (Fig 5D and data not shown). Thus, treatment with HMT inhibitors such as BIX-01294 might be beneficial for patients with shorter repeats, although the increase is only very modest. Nevertheless, as an aberrant non-B-form DNA conformation adopted by the long GAA repeats appears to constitute the main obstacle for RNAPII, we believe that strategic chemical design and high-throughput screening to identify compounds with the potential to promote transcription elongation through expanded GAA repeat tracts should become a major focus in the quest for novel therapeutic strategies to treat FRDA. Combined with HMT inhibitors or the benzamide-based HDACs, such molecules may have synergistic effects on FXN gene expression.

CONCLUSIONS

To address the molecular mechanisms of FXN silencing we have done an analysis of epigenetic changes on the FXN gene that occur upon expansion of GAA repeat tracts. Our results demonstrate that long GAA repeats constitute an obstacle to the transcription machinery in FRDA patient-derived cells irrespective of the H3K9 methylation status of the FXN gene. Our data urge the FRDA community to find novel therapeutic strategies to overcome this elongation problem, which may develop into promising new treatments for this devastating neurodegenerative disease that has thus far remained incurable.

MATERIALS AND METHODS

Cell culture propagation and drug treatments

Transformed B-lymphocytes from FRDA-affected (GM15850, GM04079 and GM16209) and unaffected individuals (GM15851) were obtained from the Coriell Cell Repository (http://ccr.coriell.org/). Cells were propagated in RPMI1640 medium supplemented with 15% FCS and 2 mM L-glutamine at 37°C in 5% CO_2. Confluent cells (1×10^6 cells/ml) were treated with 115 μM of DRB (Sigma, A9415) for 5 h or with 4 μM of BIX-01294 (Sigma, B9311) for 72 h. BIX-01294 treated cells were diluted to 1×10^6 cells/ml with fresh cell media supplemented with BIX-01294 after each 24 h treatment.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described (Greene et al, 2007) with some modifications. Briefly, the cells were crosslinked in phosphate-buffered saline (PBS) at room temperature for 5 min. Thereafter, the cells were washed in PBS and lysed in a buffer containing 0.5% SDS, 10 mM EDTA (2,2′,2″,2‴-(ethane-1,2-diyldinitrilo)tetraacetic acid) and 50 mM Tris–HCl pH 8.0. The chromatin from lysed cells was sheared by sonication to 200 and 500 bp fragments using Bioruptor (Diagenode). For each immunoprecipitation experiments, the amount of lysate corresponding to 3×10^6 cells was used in combination with 5 μg of the respective antibody. The antibodies H3K9me2 (ab1220), H3K9me3 (ab8898), H3K4me2 (ab7766) and H3K36me3 (ab9050) were purchased from Abcam. A Combination of IgM (M8644, Sigma) and H14 (MMS-134R, Covance) antibodies was used for S5P

immunoprecipitation. Immunocomplexes were recovered using Protein G-coupled magnetic beads (Invitrogen), washed extensively and eluted. The amount of recovered DNA was determined using quantitative real-time PCR and relative fold enrichments were calculated as described elsewhere (http://www.sabiosciences.com/manuals/chipqpcranalysis.xls). Primer sequences and detailed locations are available in the Supporting Information Table 1.

4-Thiouridine labelling

4-thiouridine (4sU) labelling was done as described previously (Dolken et al, 2008). Briefly, exponentially growing cells (0.8×10^6 cells/ml) were incubated with 200 μM of 4sU (Sigma, T4509). Total RNA was extracted with Trizol reagent at different time points and 4sU labelled RNA was biotinylated (Biotin-HPDP, Pierce). A total RNA sample was saved and from the rest biotinylated RNA was affinity purified by using the Macs Streptavidin Kit (Miltenyi). Unbound RNA was also collected. The purified and total RNA samples were converted to cDNA and analysed by qPCR.

qRT-PCR and Northern blot

For qRT-PCR experiments, total RNA was extracted from cell lines with the Absolutely RNA Miniprep Kit (Statagene). One microgram of total RNA was reverse transcribed with AffinityScript enzyme (Stratagene) using random primers according to the manufacturer's instructions. Taqman Gene Expression assays (Applied Biosystems) were used to measure FXN (Hs00175940_m1), c-myc (Hs00153408_m1), GAPDH (P/N 402869) and 18S rRNA (P/N 4308329) expressions. The relative changes in FXN and c-Myc expressions were determined after normalization to 18S rRNA or to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). For Northern blot experiments, RNA was extracted with Trizol reagent (Invitrogen). Approximately 20 μg of total RNA was separated on 1.2% agarose gel and transferred to a positively charged Nylon membrane (Roche). The membrane was hybridized with a ^{32}P-dCTP labelled human FXN cDNA probe.

Western blot

Total cell lysates were prepared as previously described (Punga et al, 2006) and analysed on 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Anti-FXN monoclonal antibody (SKU#456300, Invitrogen) was used for immunodetection.

Author contributions

T.P. planned and performed experiments and evaluated the data. M.B. obtained funding, oversaw the study, and assisted in data evaluation and interpretation. T.P. and M.B. wrote the paper.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

For more information

Online Mendelian Inheritance in Man (OMIM)
FRDA
http://www.ncbi.nlm.nih.gov/entrez/dispomim. cgi?id=229300
FXN
http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id= 606829

Ensemble (human frataxin gene)
http://www.ensembl.org/Homo_sapiens/Location/View?db=core;g=ENSG00000165060;r=9:71650175-71689129;t=ENST00000377270

Coriell Cell Repositories (FRDA cell line collection)
http://ccr.coriell.org/sections/collections/NIGMS/frda.aspx?PgId=236

Friedreich's Ataxia Research Alliance (FARA)
http://www.curefa.org/

Homepage of Marc Bühler's laboratory
http://www.fmi.ch/research/groupleader/?group=122

References

Al-Mahdawi S, Pinto RM, Ismail O, Varshney D, Lymperi S, Sandi C, Trabzuni D, 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

Bannister AJ, Schneider R, Myers FA, Thorne AW, Crane-Robinson C, Kouzarides T (2005) Spatial distribution of di- and tri-methyl lysine 36 of histone H3 at active genes. J Biol Chem 280: 17732-17736

Baralle M, Pastor T, Bussani E, Pagani F (2008) Influence of Friedreich ataxia GAA noncoding repeat expansions on pre-mRNA processing. Am J Hum Genet 83: 77-88

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K (2007) High-resolution profiling of histone methylations in the human genome. Cell 129: 823-837

Bell O, Wirbelauer C, Hild M, Scharf AN, Schwaiger M, MacAlpine DM, Zilbermann F, van Leeuwen F, Bell SP, Imhof A, *et al* (2007) Localized H3K36 methylation states define histone H4K16 acetylation during transcriptional elongation in Drosophila. EMBO J 26: 4974-4984

Bidichandani SI, Ashizawa T, Patel PI (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

Buhler M (2009) RNA turnover and chromatin-dependent gene silencing. Chromosoma 118: 141-151

Buhler M, Moazed D (2007) Transcription and RNAi in heterochromatic gene silencing. Nat Struct Mol Biol 14: 1041-1048

Buhler M, Haas W, Gygi SP, Moazed D (2007) RNAi-dependent and -independent RNA turnover mechanisms contribute to heterochromatic gene silencing. Cell 129: 707-721

Buratowski S (2009) Progression through the RNA polymerase II CTD cycle. Mol Cell 36: 541-546

Burnett R, Melander C, Puckett JW, Son LS, Wells RD, Dervan PB, Gottesfeld JM (2006) DNA sequence-specific polyamides alleviate transcription inhibition associated with long GAA.TTC repeats in Friedreich's ataxia. Proc Natl Acad Sci USA 103: 11497-11502

Campuzano V, Montermini L, Molto MD, Pianese L, Cossee M, Cavalcanti F, Monros E, Rodius F, Duclos F, Monticelli A, *et al* (1996) Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. Science 271: 1423-1427

Campuzano V, Montermini L, Lutz Y, Cova L, Hindelang C, Jiralerspong S, Trottier Y, Kish SJ, Faucheux B, Trouillas P, *et al* (1997) Frataxin is reduced in

Friedreich ataxia patients and is associated with mitochondrial membranes. Hum Mol Genet 6: 1771-1780

Carrozza MJ, Li B, Florens L, Suganuma T, Swanson SK, Lee KK, Shia WJ, Anderson S, Yates J, Washburn MP, *et al* (2005) Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell 123: 581-592

Chou CJ, Herman D, Gottesfeld JM (2008) Pimelic diphenylamide 106 is a slow, tight-binding inhibitor of class I histone deacetylases. J Biol Chem 283: 35402-35409

Dion V, Wilson JH (2009) Instability and chromatin structure of expanded trinucleotide repeats. Trends Genet 25: 288-297

Dolken L, Ruzsics Z, Radle B, Friedel CC, Zimmer R, Mages J, Hoffmann R, Dickinson P, Forster T, Ghazal P, *et al* (2008) High-resolution gene expression profiling for simultaneous kinetic parameter analysis of RNA synthesis and decay. RNA 14: 1959-1972

Festenstein R (2006) Breaking the silence in Friedreich's ataxia. Nat Chem Biol 2: 512-513

Greene E, Mahishi L, Entezam A, Kumari D, 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

Guenther MG, Levine SS, Boyer LA, Jaenisch R, Young RA (2007) A chromatin landmark and transcription initiation at most promoters in human cells. Cell 130: 77-88

Herman D, Jenssen K, Burnett R, Soragni E, Perlman SL, Gottesfeld JM (2006) Histone deacetylase inhibitors reverse gene silencing in Friedreich's ataxia. Nat Chem Biol 2: 551-558

Jenuwein T (2006) The epigenetic magic of histone lysine methylation. FEBS J 273: 3121-3135

Komarnitsky P, Cho EJ, Buratowski S (2000) Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev 14: 2452-2460

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

Krogan NJ, Kim M, Tong A, Golshani A, Cagney G, Canadien V, Richards DP, Beattie BK, Emili A, Boone C, *et al* (2003) Methylation of histone H3 by Set2 in Saccharomyces cerevisiae is linked to transcriptional elongation by RNA polymerase II. Mol Cell Biol 23: 4207-4218

Kubicek S, O'Sullivan RJ, August EM, Hickey ER, Zhang Q, Teodoro ML, Rea S, Mechtler K, Kowalski JA, Homon CA, *et al* (2007) Reversal of H3K9me2 by a small-molecule inhibitor for the G9a histone methyltransferase. Mol Cell 25: 473-481

Kumari D, Usdin K (2009) Chromatin remodeling in the noncoding repeat expansion diseases. J Biol Chem 284: 7413-7417

McGarvey KM, Fahrner JA, Greene E, Martens J, Jenuwein T, Baylin SB (2006) Silenced tumor suppressor genes reactivated by DNA demethylation do not return to a fully euchromatic chromatin state. Cancer Res 66: 3541-3549

Mirkin SM (2007) Expandable DNA repeats and human disease. Nature 447: 932-940

Murakami H, Goto DB, Toda T, Chen ES, Grewal SI, Martienssen RA, Yanagida M (2007) Ribonuclease activity of Dis3 is required for mitotic progression and provides a possible link between heterochromatin and kinetochore function. PLoS ONE 2: e317

Ohshima K, Montermini L, Wells RD, 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

Pandolfo M (2009) Friedreich ataxia: the clinical picture. J Neurol 256: 3-8

Phatnani HP, Greenleaf AL (2006) Phosphorylation and functions of the RNA polymerase II CTD. Genes Dev 20: 2922-2936

Pokholok DK, Harbison CT, Levine S, Cole M, Hannett NM, Lee TI, Bell GW, Walker K, Rolfe PA, Herbolsheimer E, *et al* (2005) Genome-wide map of nucleosome acetylation and methylation in yeast. Cell 122: 517-527

Punga T, Bengoechea-Alonso MT, Ericsson J (2006) Phosphorylation and ubiquitination of the transcription factor sterol regulatory element-binding protein-1 in response to DNA binding. J Biol Chem 281: 25278-25286

Rai M, Soragni E, Jenssen K, Burnett R, Herman D, Coppola G, Geschwind DH, Gottesfeld JM, Pandolfo M (2008) HDAC inhibitors correct frataxin deficiency in a Friedreich ataxia mouse model. PLoS ONE 3: e1958

Sakamoto N, Chastain PD, Parniewski P, Ohshima K, Pandolfo M, Griffith JD, Wells RD (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

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

Schubeler D, MacAlpine DM, Scalzo D, Wirbelauer C, Kooperberg C, van Leeuwen F, Gottschling DE, O'Neill LP, Turner BM, Delrow J, *et al* (2004) The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. Genes Dev 18: 1263-1271

Schulz JB, Boesch S, Burk K, Durr A, Giunti P, Mariotti C, Pousset F, Schols L, Vankan P, Pandolfo M (2009) Diagnosis and treatment of Friedreich ataxia: a European perspective. Nat Rev Neurol 5: 222-234

Sobell HM (1985) Actinomycin and DNA transcription. Proc Natl Acad Sci USA 82: 5328-5331

Vakoc CR, Mandat SA, Olenchock BA, Blobel GA (2005) Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. Mol Cell 19: 381-391

Xu C, Soragni E, Chou CJ, Herman D, Plasterer HL, Rusche JR, Gottesfeld JM (2009) Chemical probes identify a role for histone deacetylase 3 in Friedreich's ataxia gene silencing. Chem Biol 16: 980-989

Yamaguchi Y, Wada T, Handa H (1998) Interplay between positive and negative elongation factors: drawing a new view of DRB. Genes Cells 3: 9-15

