

The Friedreich ataxia GAA repeat expansion mutation induces comparable epigenetic changes in human and transgenic mouse brain and heart tissues

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3 **The Friedreich ataxia GAA repeat expansion mutation induces comparable**
4 **epigenetic changes in human and transgenic mouse brain and heart**
5 **tissues**
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ABSTRACT

Friedreich ataxia (FRDA) is caused by a homozygous GAA repeat expansion mutation within intron 1 of the *FXN* gene, leading to reduced expression of frataxin protein. Evidence suggests that the mutation may induce epigenetic changes and heterochromatin formation, thereby impeding gene transcription. In particular, studies using FRDA patient blood and lymphoblastoid cell lines have detected increased DNA methylation of specific CpG sites upstream of the GAA repeat and histone modifications in regions flanking the GAA repeat. In this report we show that such epigenetic changes are also present in FRDA patient brain and heart tissues, the primary affected systems of the disorder. Bisulfite sequence analysis of the *FXN* flanking GAA regions reveals a shift in the FRDA DNA methylation profile, with upstream CpG sites becoming consistently hypermethylated and downstream CpG sites becoming consistently hypomethylated. We also identify differential DNA methylation at three specific CpG sites within the *FXN* promoter and one CpG site within exon 1. Furthermore, we show by chromatin immunoprecipitation (ChIP) analysis that there is overall decreased histone H3 and H4 acetylation together with increased H3K9 methylation of FRDA brain tissue. Further studies of brain and heart tissues from our GAA repeat expansion-containing FRDA YAC transgenic mice reveal comparable epigenetic changes to those detected in FRDA patient tissue. We have thus developed a mouse model that will be a valuable resource for future therapeutic studies targeting epigenetic modifications of the *FXN* gene to increase frataxin expression.

INTRODUCTION

FRDA is an autosomal recessive neurodegenerative disorder that is predominantly caused by a homozygous GAA repeat expansion mutation within intron 1 of the *FXN* gene (1). Normal individuals have 5 to 30 GAA repeat sequences, whereas affected individuals have from approximately 70 to more than 1,000 GAA triplets (2). The GAA repeat shows somatic instability, with progressive expansion throughout life, particularly in the cerebellum and dorsal root ganglia (DRG) (3-5). The effect of the GAA expansion mutation is to reduce the expression of frataxin (6), a mitochondrial protein that acts as an iron chaperone in iron-sulphur cluster and heme biosynthesis (7-9). Frataxin insufficiency leads to oxidative stress, mitochondrial iron accumulation and resultant cell death, with the primary site of pathology being in the large sensory neurons of the DRG and the dentate nucleus of the cerebellum (10). The outcome is progressive spinocerebellar neurodegeneration, causing symptoms of ataxia, dysarthria, muscle weakness, and sensory loss, together with cardiomyopathy, and diabetes. At present there is no effective treatment for FRDA, and affected individuals generally die in early adulthood from the associated heart disease.

Preclinical and clinical trials using antioxidants and iron chelators have demonstrated some limited success in alleviating FRDA heart pathology (11-16). However, a more effective overall therapeutic strategy may be to target the immediate effects of the GAA repeat expansion mutation to restore normal levels of frataxin expression. The exact mechanism by which the GAA repeat

1
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3 expansion leads to decreased frataxin expression is unknown, but several
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5 models have been put forward. Firstly, it has been suggested that the GAA
6
7 repeat expansion may adopt abnormal DNA or DNA/RNA hybrid structures that
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9 interfere with *FXN* gene transcription (17-20). Secondly, there is evidence that
10
11 GAA repeat expansions produce a heterochromatin-mediated gene silencing
12
13 effect (21). Epigenetic mechanisms, such as DNA methylation and the
14
15 associated deacetylation and methylation of histones are known to affect gene
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17 expression by chromatin remodelling (22), and these epigenetic changes are
18
19 likely to underpin any GAA repeat-induced heterochromatin-mediated gene
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21 silencing effects. In support of this hypothesis, research has recently shown
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23 increased DNA methylation of three specific CpG sites immediately upstream of
24
25 the expanded GAA repeat sequence in FRDA patient lymphoblastoid cell lines
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27 and primary lymphocytes, and one of the three CpG sites was identified as an
28
29 important enhancer of frataxin expression (23). Other studies have identified
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31 specific histone modifications that are associated with gene silencing within the
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33 GAA repeat expansion-flanking regions of the *FXN* intron 1 sequence in FRDA
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35 lymphoblastoid cell lines and primary lymphocytes (23, 24). These changes
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37 include deacetylation of histone H3 and H4 lysine residues and increased di- and
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39 trimethylation of H3K9. Based on the hypothesis that the acetylation state of the
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41 core histones is responsible for gene silencing, novel histone deacetylase
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43 (HDAC) inhibitor compounds have been developed and have been shown to
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45 increase *FXN* transcription in FRDA lymphoblastoid cells and primary
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47 lymphocytes (24).
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These previous epigenetic studies have provided valuable insights into the possible mechanism of GAA-induced transcription inhibition, but they do not address the issue of whether such epigenetic changes are actually present in the most clinically relevant FRDA tissues. Therefore, we decided to investigate epigenetic profiles of the *FXN* gene in FRDA patient autopsy brain and heart tissue. By bisulfite sequencing and ChIP analysis we now report changes in DNA methylation and histone modifications that are consistent with inhibition of *FXN* transcription. With a view to future epigenetic-based FRDA therapies, we also investigated the *FXN* epigenetic profiles within brain and heart tissue from our Y47, YG8 and YG22 FRDA YAC transgenic mouse models (25-27). We find that the GAA repeat expansion-containing FRDA mouse models (YG8 and YG22) exhibit comparable epigenetic changes to those detected in FRDA patient tissue. Therefore, these are excellent FRDA mouse models in which to investigate the therapeutic effects of epigenetically-acting compounds, such as novel HDAC inhibitors or DNA methylation inhibitors.

RESULTS

***FXN* gene DNA methylation profiles are distinctly altered in human FRDA brain and heart tissues**

A previous investigation of the *FXN* gene in FRDA patient lymphoblastoid cell lines and blood samples has detected hypermethylation at three specific CpG sites immediately upstream of the expanded GAA repeat sequence. One of the three CpG sites was further identified as an important enhancer element for

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3 frataxin expression (23). This same study also reported a lack of any DNA
4 methylation in the promoter region of either FRDA or unaffected cells.
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6 However, cultured cells are known to often develop non-physiological DNA
7 methylation profiles. Furthermore, FRDA is a systemic disorder that is known to
8 have differentially affected tissues and cell types. Therefore, we chose to
9 investigate the DNA methylation status in two of the primary affected tissues in
10 FRDA, namely brain and heart. We obtained brain and heart autopsy tissues
11 from an FRDA patient (GAA repeat sizes of 750 and 650) and an unaffected
12 individual, and we firstly determined the *FXN* transcription levels of the samples
13 by quantitative RT-PCR. The FRDA brain and heart samples showed 12% and
14 67% *FXN* expression, respectively, compared with the unaffected samples (Fig.
15 1). We then analysed the DNA methylation status of the samples by performing
16 bisulfite sequence analysis of three regions of the *FXN* gene: (i) a 475bp
17 sequence that encompasses part of the *FXN* promoter, exon 1 and start of intron
18 1, containing 59 CpG sites; (ii) a 286bp sequence upstream of the GAA repeat,
19 containing 8 CpG sites, and (iii) a 275bp sequence downstream of the GAA
20 repeat, containing 12 CpG sites (Fig. 2). A comparison of the bisulfite sequences
21 from the FRDA patient and control brain and heart tissues reveals a certain
22 degree of DNA methylation in all 8 of the upstream GAA CpG sites (Fig. 3C and
23 D) and all 12 of the downstream GAA CpG sites (Fig. 3E and F). However, the
24 data show a consistent shift in the DNA methylation pattern around the GAA
25 repeat in both tissue types. The FRDA upstream GAA CpG sites are
26 comparatively hypermethylated, whereas the FRDA downstream GAA CpG sites
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3 are comparatively hypomethylated (Fig. 3C-F). The greatest increase in DNA
4 methylation within the upstream GAA region is seen at CpG site 4. Significantly
5 increased DNA methylation is also seen at CpG sites 5 and 6, the latter of which
6 corresponds to the previously described E-box enhancer element (23). We
7 observed 100% methylation at CpG site 6 in FRDA brain tissue (*FXN* mRNA
8 level of 12%, Fig.1) compared with 80% methylation in heart tissue (*FXN* mRNA
9 level of 67%, Fig.1). Thus, the upstream GAA DNA methylation changes in both
10 FRDA brain and heart are consistent with their proposed roles in inhibition of
11 *FXN* transcription. However, the finding of decreased DNA methylation in the
12 downstream GAA region (Fig. 3E and F) is somewhat unexpected, since all of
13 the 12 CpG sites fall within an Alu repeat sequence and such sequences are
14 usually repressed by heavy DNA methylation.

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32 Another particularly interesting finding was the identification of differential DNA
33 methylation at three specific CpG sites within the *FXN* promoter (sites 5, 7 and 8)
34 and one CpG site within exon 1 (site 23) (Fig. 3A and B). All of the other 55 CpG
35 sites in the total of 59 CpG sites analysed show complete lack of DNA
36 methylation, as to be expected for a CpG island that is situated at the start of a
37 gene. CpG sites 5, 7 and 8 show incomplete methylation in the unaffected heart,
38 but complete methylation in the FRDA heart (Fig. 3B). Therefore, these CpG
39 sites may be involved in reducing initiation of *FXN* gene transcription in FRDA
40 heart. However, the DNA methylation pattern is different in brain tissue. Here we
41 identified approximately 20% DNA methylation at all of the four CpG sites in the
42 unaffected tissue, but very little change, and even a decrease, in DNA
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3 methylation in FRDA tissue (Fig. 3A). Furthermore, the fact that we have
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5 identified some degree of DNA methylation at all in this region contrasts with the
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7 previous report that DNA methylation is absent in the *FXN* promoter region of
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9 both FRDA and unaffected lymphoblastoid cells (23). Therefore, we have shown
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11 that the influence of DNA methylation on *FXN* gene expression is likely to be
12
13 complex, with some similarities (CpG site usage) but also some distinct
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15 differences (degree of CpG methylation) identified between different somatic
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17 tissues.
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***FXN* gene histone modifications are altered in human FRDA brain tissue**

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25 Previous studies of the promoter, upstream GAA and downstream GAA regions
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27 of the *FXN* gene have identified specific histone modifications that are associated
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29 with gene silencing within the GAA repeat expansion-flanking regions of the *FXN*
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31 intron 1 sequence in FRDA lymphoblastoid cell lines and primary lymphocytes
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33 (23, 24). We have now investigated acetylated histone H3 and H4 and
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35 methylated histone H3K9 modifications by ChIP analysis of the *FXN* promoter,
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37 upstream GAA and downstream GAA regions (Fig. 2) in autopsy brain tissues
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39 from an FRDA patient and an unaffected individual. Our results show overall
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41 decreased histone H3 and H4 acetylation of FRDA brain tissue, particularly in the
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43 downstream GAA region (Fig.4). All of the 6 acetylated histone residues that we
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45 have examined show a GAA-induced gradient of comparative acetylation that is
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47 highest in the *FXN* promoter and lowest in the downstream GAA region. The
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49 single most altered histone residue is H3K9, which exhibits progressive
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3 decreases in acetylation to comparative levels of 63%, 35% and 9% in the *FXN*
4 promoter, upstream GAA and downstream GAA regions, respectively. There is
5
6 also a consistently increased H3K9 di- and tri-methylation of FRDA brain tissue
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8 in all three of the *FXN* gene regions (Fig. 4). These changes concur with the
9
10 previous findings of increased H3K9 di- and tri-methylation in the upstream GAA
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12 region of other cell types (23, 24). However, we have now extended these
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14 studies to show that in FRDA brain the H3K9 di- and tri-methylation spreads to
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16 both *FXN* promoter and downstream GAA regions.
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25 **DNA methylation profiles of *FXN* transgenic mouse brain and heart tissues** 26 **resemble the profiles of human tissue**

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28 Having determined the epigenetic profiles around the human *FXN* gene, we then
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30 investigated the epigenetic profiles of the *FXN* transgene in brain and heart
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32 tissue isolated from YG8 and YG22 GAA repeat expansion-containing *FXN* YAC
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34 transgenic mice (26) compared with Y47 normal-sized GAA repeat-containing
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36 *FXN* YAC transgenic mice (27). Initial determination of *FXN* transgene
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38 expression showed YG8 (90+190 GAA repeats) and YG22 (190 GAA repeats) to
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40 have decreased mRNA levels of approximately 26% and 35% in brain and 57%
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42 and 56% in heart compared with Y47 (Fig. 5). Thus, inhibition of *FXN* expression
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44 in transgenic mouse brain was not as severe as the 12% observed in the
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46 750/650 GAA repeat-containing human FRDA brain (Fig. 1), whereas levels of
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48 *FXN* expression in heart were more comparable. DNA methylation analysis was
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50 then performed on the single GAA repeat expansion-containing YG22 GAA
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3 repeat transgenic mouse compared to the Y47 non-GAA repeat control. As the
4 mouse transgenes consist of entire human *FXN* gene sequence, we were able to
5 investigate the DNA methylation profiles of exactly the same three regions of the
6 *FXN* gene that we had previously analysed in human tissue (Fig. 2). Our data
7 show that the DNA methylation profiles of upstream GAA regions of both YG22
8 transgenic mouse brain and heart tissues closely resemble those found in human
9 tissues (Fig. 6C and D). Namely, there is a consistent hypermethylation of the
10 upstream GAA region induced by the GAA repeat expansion, with the most
11 prominent hypermethylation at CpG sites 4, 5 and 6. However, the degree of
12 DNA methylation at CpG sites 4 and 6 in YG22 transgenic mouse brain tissue is
13 less than that observed in FRDA human brain tissue, which is consistent with the
14 comparative *FXN* expression levels of 35% and 12%, respectively (Figs. 1 and
15 5). The downstream GAA region differs from the human situation in that there is
16 hypermethylation at all CpG sites, which is retained upon introduction of the GAA
17 repeat expansion (Fig. 6E and F). Thus, there is no GAA-induced decrease in
18 DNA methylation as detected in the human tissues. The promoter/exon 1 regions
19 of the *FXN* transgenes in both mouse brain and heart tissues show a similarity to
20 the human tissues in that DNA methylation is found at only four specific CpG
21 sites: 5, 7, 8 and 23 (Fig. 6A and B). However, the changes in the DNA profiles of
22 these four CpG sites upon introduction of the GAA repeat expansion differ
23 markedly from those found in the human tissues. This time, the brain tissue
24 shows an overall increase in DNA methylation, whereas the heart tissue shows
25 an overall decrease in DNA methylation. Assessment of the entire mouse DNA
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3 methylation data indicates a similar overall DNA methylation profile around the
4 start of the *FXN* gene that is consistent with inhibition of *FXN* transcription.
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8 However, there are also some specific differences, which may result from
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10 epigenetic-control or transcriptional-control variations between the human and
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12 the mouse that will require further investigation.
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18 **Histone modifications of *FXN* transgenic mouse brain tissue are**
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20 **comparable to histone modifications of human tissue**
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22 Acetylated histone H3 and H4 and di- and tri-methylated histone H3K9
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24 modifications were detected by ChIP analysis of the three regions of the *FXN*
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26 transgene (Fig. 2) in brain tissue isolated from both YG8 and YG22 GAA repeat
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28 expansion-containing *FXN* YAC transgenic mice (26) and Y47 normal-sized GAA
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30 repeat-containing *FXN* YAC transgenic mice (27). Our results show overall GAA
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32 repeat-induced decreases in histone H3 and H4 acetylation and increases in
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34 H3K9 methylation for both YG8 and YG22 transgenic mice (Fig. 7), as we
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36 previously identified in human FRDA tissue (Fig. 4). However, the level of
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38 deacetylation in the transgenic mouse tissue was not as great as that seen in the
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40 human tissue, possibly as a consequence of the smaller transgenic GAA repeat
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42 expansion sizes (190+90 for YG8 and 190 for YG22, compared with 750/650 for
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44 FRDA patient). Also, H4K16 acetylation is actually increased in all three *FXN*
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46 transgene regions of both YG8 and YG22 compared with Y47, which is different
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48 to the finding in human tissues. The greatest consistent histone residue changes
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50 that we found between the non-GAA (Y47) and both of the GAA (YG8 and YG22)
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3 transgenic brain tissue samples were decreases in acetylated H3K9 and
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5 increases in di- and tri-methylated H3K9. The H4K12 residue also showed a
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7 significant degree of deacetylation, but only in the YG8 transgenic tissue. All of
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9 these major histone residue changes in mouse brain tissue reflect the GAA
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11 repeat-induced histone residue changes that we detected in human tissue.
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13 Furthermore, as with the human samples, we similarly identified a GAA repeat-
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15 induced gradient of decreased H3K9 acetylation in both YG8 and YG22
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17 transgenic mouse tissues, with the highest comparative levels of acetylation in
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19 the *FXN* promoter and the lowest comparative levels in the downstream GAA
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21 region. The increases in H3K9 di- and tri-methylation were consistent throughout
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23 all of the three *FXN* gene regions in both YG8 and YG22 transgenic mice, once
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25 again agreeing with our findings in human FRDA tissue.
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34 **DISCUSSION**

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36 For the consideration of future FRDA therapy, it is first essential to understand
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38 the mechanism of GAA-induced inhibition of *FXN* gene transcription. Previous
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40 studies of FRDA have implicated epigenetic changes, including the detection of
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42 increased DNA methylation of specific CpG sites upstream of the GAA repeat
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44 and histone modifications in regions flanking the GAA repeat that are both
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46 consistent with transcription inhibition (23, 24). However, no DNA or histone
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48 methylation changes have previously been identified in the *FXN* promoter or
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50 downstream GAA regions, and clinically relevant FRDA brain and heart tissues
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52 have not previously been investigated. Different trinucleotide repeat expansion
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3 mutations have been shown to induce *cis*-acting epigenetic changes in several
4 other human disorders (28, 29). Thus, DNA methylation of the CGG repeat
5 upstream of the *FMR1* gene has been identified as a main epigenetic switch in
6 Fragile X syndrome, with histone acetylation playing an ancillary role (30).
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8 Decreased Sp1 interaction associated with DNA hypermethylation upstream of
9 the CTG repeat in the *DMPK* gene has also been reported for congenital
10 myotonic dystrophy type 1 (29). Furthermore, both CTG and GAA repeat
11 expansions have been shown to induce similar heterochromatin formation by
12 position effect variegation studies of transgenic mice (21). However, it is still
13 uncertain if different trinucleotide repeat sequences produce similar overall
14 epigenetic effects or not.
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29 Our investigations of the *FXN* gene in both FRDA human and transgenic
30 mouse brain and heart tissues have now confirmed the presence of previously
31 described DNA methylation changes (23) in the upstream GAA region of these
32 clinically important tissues. Furthermore, our data have revealed an overall shift
33 in the DNA methylation profile, moving from hypomethylation in the downstream
34 GAA region towards hypermethylation in the upstream GAA region. This shift in
35 DNA methylation profile could be explained by the known position of the GAA
36 repeat within an Alu sequence, since Alu sequences have been shown to act as
37 methylation centres leading to bi-directional spread of DNA methylation (31).
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39 Thus, the hypermethylation detected in the FRDA upstream GAA region may be
40 due to the GAA repeat mutation enhancing the effect of a putative methylation
41 centre at the 5' end of the Alu sequence. At the same time, the addition of the
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3 GAA repeat sequence would put extra distance between the methylation centre
4 at the 5' end of the Alu sequence and the downstream GAA region. This may
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6 impede the spread of methylation to the downstream region when the distance is
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8 large enough (2.25kb for 750 human GAA repeats), but not when the distance is
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10 smaller (600bp for 190 transgenic mouse GAA repeats).
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15 We have additionally identified differential DNA methylation at four specific
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17 CpG sites within the *FXN* promoter and exon 1 regions that have not previously
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19 been reported. The three CpG sites within the promoter region (sites 5, 7 and 8)
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21 are immediately upstream of the ATG translation start site, at nucleotide
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23 positions -27, -18 and -11, respectively. CpG sites 5 and 7 are also contained
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25 within Sp1 transcription factor binding sites (32). Interestingly, the region
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27 between -64 and the start of translation has previously been suggested to
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29 contain sequences important for positive regulation of frataxin production,
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31 although no candidate sequences were identified (33). Therefore, the 3
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33 differentially methylated CpG sites that we have now uncovered in the *FXN*
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35 promoter, and in particular the two Sp1 recognition sites, are likely to represent
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37 these important regulatory sequences.
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44 By comparison with other instances of trinucleotide repeat-induced DNA
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46 methylation changes that inhibit transcription (28, 29), one would have predicted
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48 general hypermethylation to be associated with the FRDA GAA repeat expansion
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50 mutation. However, we actually identified three occurrences in human tissues
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52 (promoter and downstream GAA regions in brain, and downstream GAA region in
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54 heart) and one occurrence in mouse tissues (promoter region in heart) where
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3 there was in fact GAA repeat expansion-induced decrease in DNA methylation.
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5 This suggests the possible occurrence of demethylation and resultant active *FXN*
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7 gene expression, at least for some cells within the tissue. DNA demethylation
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9 has previously been shown to occur both passively due to DNA replication upon
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11 cell division (34) and actively in a process that may involve RNA (35), although
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13 the DNA demethylating activity has yet to be identified. DNA demethylation has
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15 also previously been associated with processes of DNA damage and repair. The
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17 formation of 8-OH-dG by oxidative DNA damage has been shown to affect the
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19 activity of human DNA methyltransferase and inhibit CpG methylation (36), and
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21 DNA demethylation has also been shown to occur as a result of homologous
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23 recombination repair of DNA damaged by double-strand breaks (37). However,
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25 DNA demethylation has not previously been considered for FRDA. A close
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27 inspection of our data reveals potential DNA demethylation in the *FXN* promoter
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29 region only when CpG site 6 of the upstream GAA region is 100% methylated.
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31 Therefore, we now propose that the shutdown of transcription due to major
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33 epigenetic changes at the upstream GAA region may result in attempts to
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35 upregulate *FXN* transcription by Sp1 binding and subsequent DNA demethylation
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37 in the promoter region. In support of this proposal, Sp1 binding is known to occur
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39 independent of CpG methylation status (32), but at the same time has been
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41 shown to inhibit CpG methylation (38). Furthermore, DNA demethylation has
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43 previously been shown to occur when there are few methylated CpG sites within
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45 a CpG island, but not when all of the CpG sites are methylated (39), which is
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47 exactly the situation that we find for the *FXN* promoter region. However, the GAA
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3 repeat expansion-induced decreases in DNA methylation at the *FXN* promoter
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5 are not consistent throughout all human and mouse brain and heart tissues,
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7 suggesting the involvement of other factors. Such factors may include differential
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9 susceptibility of the brain and heart tissues to DNA damage and/or GAA repeat
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11 instability. Indeed, FRDA is a disorder that is known to involve both oxidative
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13 DNA damage (40) and somatic instability of GAA repeats (3-5). Therefore, cells
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15 that are initially methylated at the *FXN* promoter region may lose this methylation
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17 as part of the GAA repeat instability process, wherein demethylation subsequent
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19 to DNA damage repair (37) may be selected for due to beneficial effect of *FXN*
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21 expression and hence cell viability. The GAA repeat expansion-induced
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23 decreases in DNA methylation that we have observed in the downstream GAA
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25 region of human tissues, but not transgenic mouse tissues, are more likely due to
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27 differently sized GAA repeats within the Alu sequence, as we have previously
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29 discussed. However, potential DNA demethylation in this downstream GAA
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31 region could also indirectly lead to an increase in *FXN* transcription due to the
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33 removal of inhibitory effects on RNA polymerase II elongation.
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41 Our investigations of histone modifications within *FXN* gene in both FRDA
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43 human and transgenic mouse brain tissues have now confirmed the changes
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45 previously reported for H3 and H4 deacetylation in the *FXN* promoter, upstream
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47 GAA and downstream GAA regions and H3K9 methylation in the upstream GAA
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49 region (23, 24). Furthermore, we have extended the H3K9 methylation analysis
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51 to include the *FXN* promoter and downstream GAA regions that to our knowledge
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53 have not previously been reported for any FRDA tissue. Our findings from both
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3 human and transgenic mouse tissues indicate significant H3K9 deacetylation,
4 which becomes more severe upon progression from the *FXN* promoter, through
5 the upstream GAA region to the downstream GAA region. This correlates well
6 with the results for both di- and tri-methylation of H3K9, which show a generally
7 similar gradient of progressive increase from the *FXN* promoter, through the
8 upstream GAA region, to the downstream GAA region. The only exception is the
9 very high level of di-methylated H3K9 in FRDA brain tissue, which is higher than
10 that in the downstream GAA region. All of the H3K9 changes correlate well with
11 the DNA methylation changes in both human and transgenic mouse brain
12 tissues. Thus, the patterns of progressively increasing H3K9 deacetylation and
13 increasing H3K9 di- and tri-methylation in transgenic mouse brain correspond
14 exactly to the pattern of increasing DNA methylation. Similarly, the patterns of
15 progressively increasing H3K9 deacetylation and increasing H3K9 tri-methylation
16 in human FRDA brain with a peak of H3K9 di-methylation in the upstream GAA
17 region equate very well to the corresponding DNA methylation profiles.
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19 Therefore, our combined data thus far indicate major roles for DNA methylation,
20 histone H3K9 deacetylation and histone H3K9 methylation in the inhibition of
21 *FXN* transcription in brain and heart tissues, with a less prominent role for
22 deacetylation of other histone residues. The more severe epigenetic changes
23 within the *FXN* intron 1 region compared with the promoter region support a
24 hypothesis of transcription inhibition due to interference with elongation rather
25 than initiation. Further work will be required to determine the exact relationships
26 between DNA methylation, histone acetylation and methylation, heterochromatin
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3 formation and transcription inhibition. However, our results are consistent with
4 the generally described pathway for gene inactivation wherein initial histone
5 H3K9 deacetylation leads to H3K9 methylation, recruitment of HP1, histone
6 deacetylases, DNA methyltransferases and eventual long-term shut down of
7 transcription by DNA methylation (41). However, this situation is not likely to be
8 universal for all trinucleotide repeat disorders, as highlighted by research on the
9 *FMR1* gene which has shown both histone deacetylation and H3K9 methylation
10 in the absence of DNA methylation without interfering in active gene transcription
11 (42).
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24 For now, the exact mechanism by which the GAA repeat mutation inhibits
25 frataxin expression remains elusive. However, accumulating evidence, including
26 the findings of this report, now highlights the importance of epigenetic changes
27 that lead to heterochromatin formation. The epigenetic changes that we and
28 others have now identified in FRDA do not in any way negate the importance of
29 any abnormal DNA or DNA/RNA hybrid structures in the inhibition of frataxin
30 expression, but rather suggest the involvement of several combined
31 mechanisms. Indeed the existence of abnormal DNA structures may help to
32 explain why the GAA repeat mutation induces epigenetic changes in the first
33 place. Thus, there are reports that non-B DNA structures such as hairpins may
34 induce DNA methylation (43, 44), and GAA repeats have been shown to form
35 hairpins (45). Alternatively, small double-stranded RNA (dsRNA) has also been
36 shown to induce transcriptional gene silencing through a mechanism that
37 involves DNA methylation (46, 47). However, dsRNA has failed to induce DNA
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3 methylation in a study of mouse oocytes (48) and dsRNA targeted to the *HD*
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5 gene does not induce DNA methylation at the target huntingtin genomic locus in
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7 human cells (49). Thus, further studies are still required to identify any possible
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9 involvement of non-B DNA structures (such as GAA hairpins or triplex
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11 structures), DNA/RNA hybrids or dsRNA in the establishment of epigenetic
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13 changes and heterochromatin formation in FRDA.
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18 In light of the epigenetic changes that we and others have identified in
19
20 FRDA tissues and cells, several novel epigenetic-based therapeutic approaches
21
22 can now be considered for FRDA. Firstly, histone deacetylase (HDAC) inhibitors
23
24 can be used, and indeed these have already shown considerable promise by
25
26 decreasing acetylation of histones and thereby increasing *FXN* transcription in
27
28 FRDA cells (24). Secondly, pharmacological approaches could be taken to
29
30 decrease H3K9 methylation, as have recently been described for the combined
31
32 use of mithramycin and cystamine in Huntington disease mice (50). Thirdly,
33
34 therapies to decrease DNA methylation should now be considered for FRDA, as
35
36 have previously been tried for other trinucleotide repeat disorders. In particular,
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38 5-azadeoxycytidine (5-azadC) has been shown to remove DNA methylation of
39
40 the CCG repeat expansion, increase H3 and H4 acetylation, decrease H3K9
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42 methylation, increase H3K4 methylation and reactivate the *FMR1* gene (30, 51).
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44 Combined HDAC inhibitor and 5-azadC treatment has also been shown to
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46 synergistically increase *FMR1* gene activity (52). Finally, short dsRNA molecules
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48 complementary to promoter sequences have recently been shown to induce
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50 gene activation (53, 54), and such approaches may also prove effective in
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3 increasing *FXN* transcription. Our identification of a transgenic FRDA mouse
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5 model that shows comparable epigenetic changes to those seen in FRDA
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7 patients will now provide a valuable resource in the study of all such epigenetic-
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9 based FRDA therapies.
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17 **MATERIALS AND METHODS**

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20 **Tissues.** Human brain and heart tissue samples were obtained from autopsies of
21
22 an FRDA patient (750/650 GAA repeats) and a non-FRDA individual, in
23
24 accordance with UK Human Tissue Authority ethical guidelines. Mouse brain and
25
26 heart tissues were dissected from our previously reported *FXN* YAC transgenic
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28 mouse models: Y47 (2 copies of 9 GAA repeats); YG8 (2 copies of 90 and 190
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30 GAA repeats), and YG22 (1 copy of 190 GAA repeats) (25, 27).
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34 **mRNA expression analysis.** Total RNA was isolated from frozen tissues by
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36 homogenization with Trizol (Invitrogen) and cDNA was then prepared by using
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38 AMV Reverse transcriptase (Invitrogen) with oligo-dT primers. Levels of human
39
40 or mouse transgenic *FXN* mRNA expression were assessed by quantitative RT-
41
42 PCR using an ABI7400 sequencer and SYBR[®] Green (Applied Biosystems) with
43
44 the following primers: FxnRTF 5'-CAGAGGAAACGCTGGACTCT-3'
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46 and FxnRTR 5'-AGCCAGATTTGCTTGTTTGGC-3'
47
48 (24). Human *GAPDH* or mouse *Gapdh* RT-PCR primers were used as control
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50 standards: human: GapdhhF 5'-GAAGGTGAAGGTCGGAGT-3' and
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52 GapdhhR 5'-GAAGATGGTGATGGGATTTTC-3'
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3 mouse GapdhmF 5'-ACCCAGAAGACTGTGGATGG-3' and GapdhmR 5'-
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5 GGATGCAGGGATGATGTTCT-3'
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8 **Bisulfite sequencing.** Genomic DNA was isolated from frozen tissue by
9
10 standard phenol/chloroform extraction and ethanol precipitation. 2ug of genomic
11
12 DNA was digested with *EcoRI* prior to bisulfite treatment using the CpGenome kit
13
14 (Calbiochem). Nested PCR was carried out on bisulfite-treated DNA to amplify
15
16 three regions of the *FXN* gene using the following primers: Pro 1st primer pair:
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18 SL1F1 5'-TAGTTTTTAAGTTTTTTTTGTTTAG-3' and SL1R1 5'-
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20 CAAAACAAAATATCCCCTTTTC-3'; Pro 2nd primer pair: SL1F2 5'-
21
22 GTTTTTTATAGAAGAGTGTGTTG-3' and SL1R2 5'-
23
24 CAAAACCAATATAAATACAACC-3'; Up 1st primer pair: F1G 5'-
25
26 GAGGGATTTGTTTGGGTAAAG-3' and R1G 5'-
27
28 ATACTAAATTCACCATATTAACC-3'; Up 2nd primer pair: F2G 5'-
29
30 GATTTGTTTGGGTAAAGGTTAG-3' and R2G 5'-
31
32 CTCCCAAATACTAAAATTATAAAC-3'; Down 1st primer pair: NH1F 5'-
33
34 AAGAAGAAGAAGAAAATAAAGAAAAG-3' and SLGR2 5'-
35
36 TCCTAAAAAAATCTAAAACCATC-3'; Down 2nd primer pair: NH2F 5'-
37
38 AGAAGAAGAAAATAAAGAAAAGTTAG-3' and SLGR1 5'-
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40 AAAACCATCATAACCACACTTAC-3'. PCR products were then resolved on
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42 agarose gels, purified with GeneClean (BIO101) and cloned into pCR4.0
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44 (Invitrogen) prior to DNA sequencing. A minimum of 8 clones were sequenced for
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46 each tissue sample.
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3 **ChIP analysis.** Histone modifications at the three *FXN* gene regions were
4 detected by ChIP analysis of FRDA human and mouse tissues. This procedure
5 involved initial cross-linking of DNA and protein by formaldehyde treatment of
6 homogenised frozen tissue samples. DNA was then sheared by sonication,
7 followed by immunoprecipitation with commercially available anti-histone and
8 anti-acetylated histone H3 and H4 antibodies: H3K9ac, H3K14ac, H4K5ac,
9 H4K8ac, H4K12ac, H4K16ac and H3K9me2 (Upstate), and H3K9me3
10 (Diagenode). For each experiment, normal rabbit serum (SIGMA) was used as a
11 minus antibody immunoprecipitation control. After reversal of cross-linking,
12 quantitative RT-PCR amplification of the resultant co-immunoprecipitated DNA
13 was carried out with SYBR[®] Green in an ABI7400 sequencer (Applied
14 Biosystems) using three sets of *FXN* primers (Pro, Up and Down) and human
15 *GAPDH* control for the human samples as previously described (24). For the
16 analysis of transgenic mouse samples, the same three sets of *FXN* primers were
17 used together with the following mouse *Gapdh* control primers: GapdhMF, 5'-
18 TGACAAGAGGGCGAGCG-3' and GapdhMR, 5'-GGAAGCCGAAGTCAGGAAC-
19 3'. Each tissue sample was subjected to two independent ChIP procedures,
20 followed by triplicate quantitative PCR analysis.
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49
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52 Faisal Specialist Hospital and Research Center, KSA.
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CONFLICT OF INTEREST

There is none declared.

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LEGENDS TO FIGURES

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Figure 1. Quantitative RT-PCR analysis of *FXN* mRNA isolated from brain and heart autopsy samples of an FRDA patient (750 and 650 GAA repeats) and an unaffected individual. Data are normalised to the *FXN* mRNA level found in the unaffected individual. Two individual cDNA samples were analysed for each tissue and each reaction was carried out in triplicate. The means and SEMs of these values are shown.

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Figure 2. Schematic representation of 2.2kb at the 5' end of the *FXN* gene, indicating the promoter / exon 1 (Pro), upstream GAA (Up) and downstream GAA (Down) regions that were analysed by ChIP (black boxes) and bisulfite sequencing (hatched boxes). Numbers above indicate the position of CpG sites

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3 within the promoter and upstream GAA regions. The positions of the ATG
4 translation start codon, exon 1 open reading frame and GAA repeat sequence
5 within the Alu repeat sequence are shown. Numbers below indicate the
6 chromosome 9 base pair numbering according to the 2006 build of the UCSC
7 human DNA sequence database.
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18 Figure 3. DNA methylation analysis of the *FXN* promoter (A, B), upstream GAA
19 (C, D) and downstream GAA (E, F) regions of human brain and heart tissues. In
20 each case the percentage of methylated CpG sites is shown as determined from
21 the analysis of 8 to 12 independent cloned DNA sequences. Only eleven CpG
22 sites are represented for the promoter region (A, B), as sites 11-22 and 24-59 did
23 not show any methylation in either FRDA or unaffected samples in brain or heart.
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34 Figure 4. Analysis of histone modifications in human brain tissue. ChIP
35 quantitative PCR results for the *FXN* promoter/exon1 (Pro), upstream GAA (Up)
36 and downstream GAA (Down) amplified regions are represented as the relative
37 amount of immunoprecipitated DNA compared with input DNA, having taken
38 negligible –Ab control values into account. *FXN* values were normalised with
39 human *GAPDH* and all values have been adjusted so that all of the Upstream
40 GAA values from the unaffected individual are 100%. In each case two individual
41 ChIP samples were analysed in triplicate. The means and SEMs of these values
42 are shown.
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3 Figure 5. Quantitative RT-PCR analysis of transgenic *FXN* mRNA isolated from
4 Y47, YG8 and YG22 mouse brain and heart tissues. Data are normalised to the
5 *FXN* mRNA level found in the non-GAA transgenic mouse. Two individual cDNA
6 samples were analysed for each tissue and each reaction was carried out in
7 triplicate. The means and SEMs of these values are shown.
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18 Figure 6. DNA methylation analysis of the *FXN* promoter (A, B), upstream GAA
19 (C, D) and downstream GAA (E, F) regions of YG22 (GAA) and Y47 (non-GAA)
20 transgenic mouse brain and heart tissues. In each case the percentage of
21 methylated CpG sites is shown as determined from the analysis of 8 to 25
22 independent cloned DNA sequences. Only eleven CpG sites are represented for
23 the promoter region, as sites 11-22 and 24-59 did not show any methylation in
24 either FRDA or unaffected samples in brain or heart.
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36 Figure 7. Analysis of histone modifications in transgenic mouse brain tissue.
37 ChIP quantitative PCR results for the transgenic *FXN* promoter/exon1 (Pro),
38 upstream GAA (Up) and downstream GAA (Down) amplified regions are
39 represented as the relative amount of immunoprecipitated DNA compared with
40 input DNA, having taken negligible –Ab control values into account.. *FXN* values
41 were normalised with mouse *GAPDH* and all values have been adjusted so that
42 all of the upstream GAA values from the non-GAA transgenic mouse (Y47) are
43 100%. In each case two individual ChIP samples were analysed in triplicate. The
44 means and SEMs of these values are shown.
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Fig.1

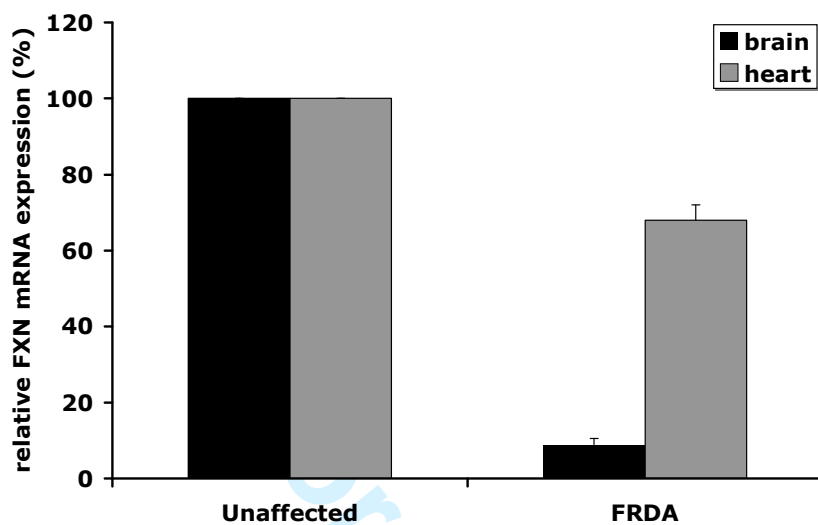


Fig.2

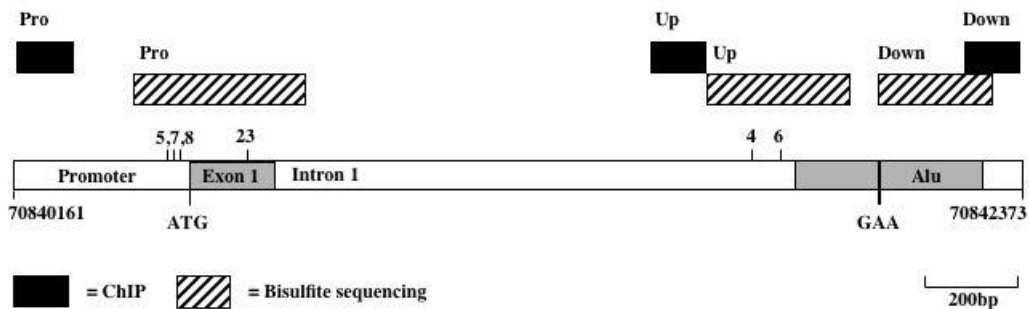


Fig. 3

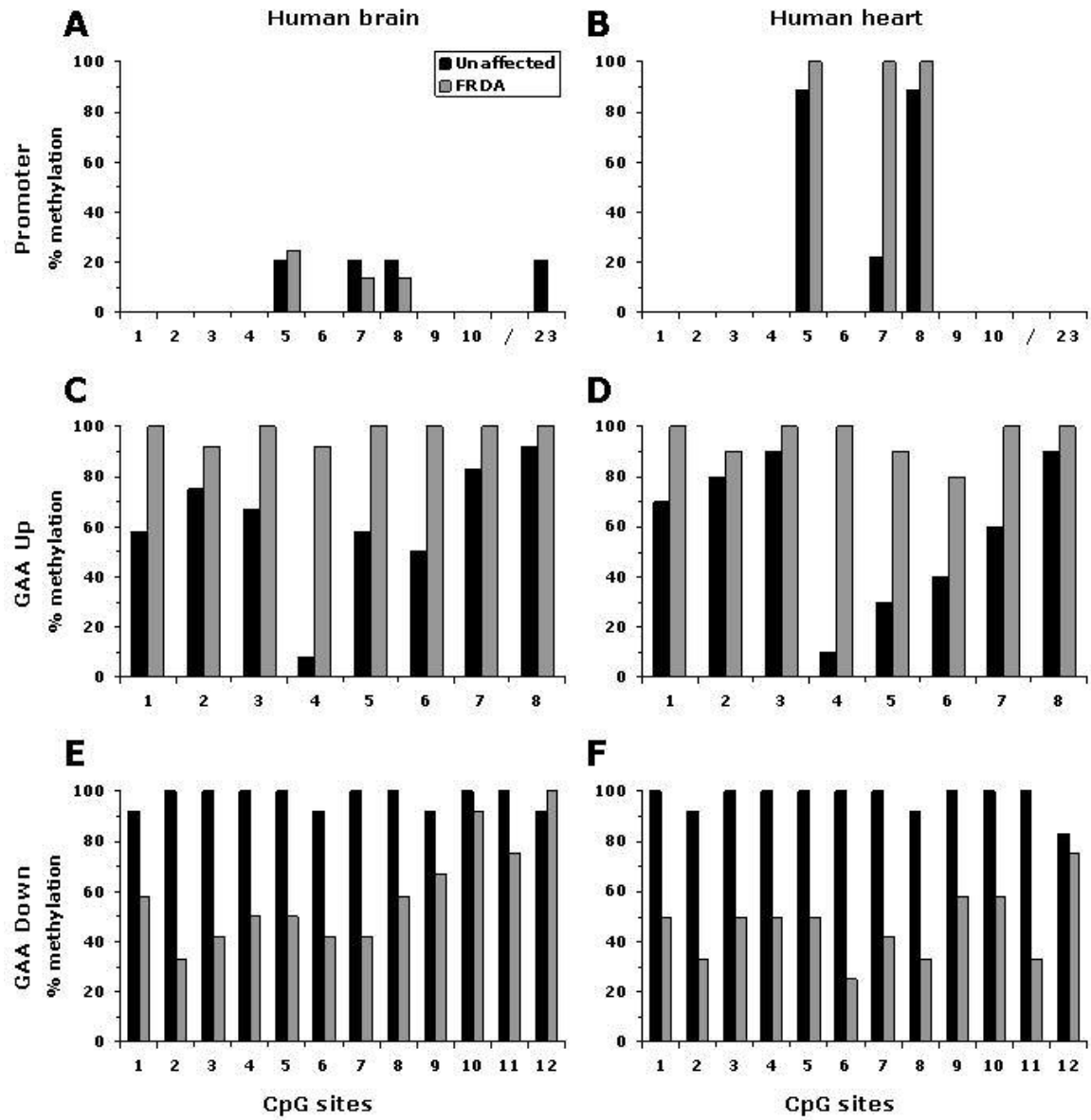


Fig. 4

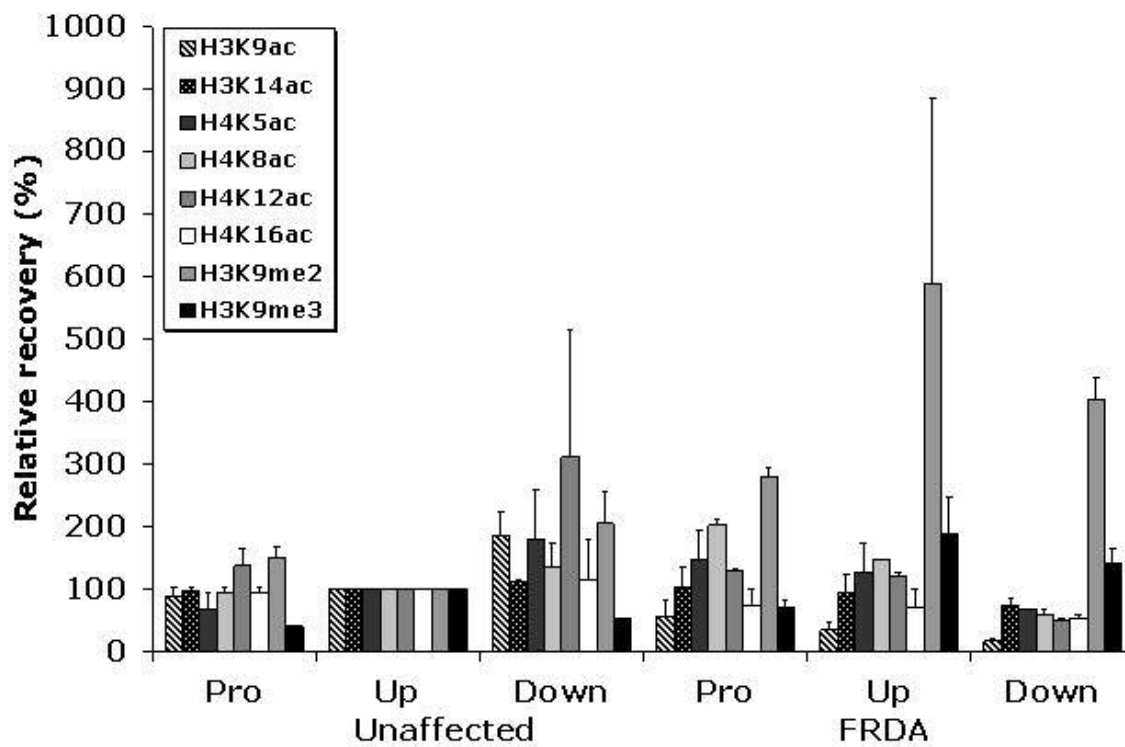


Fig. 5

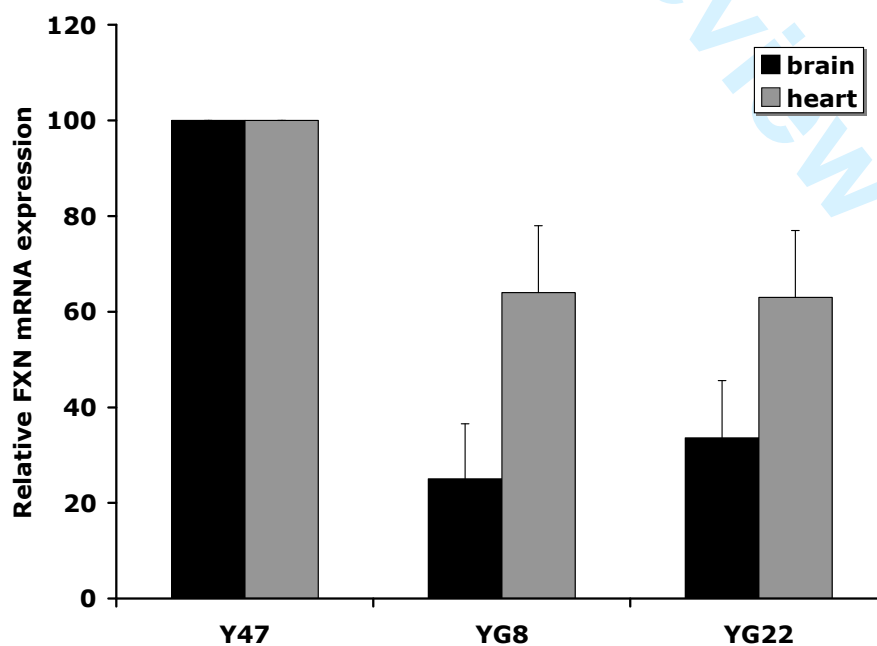


Fig. 6

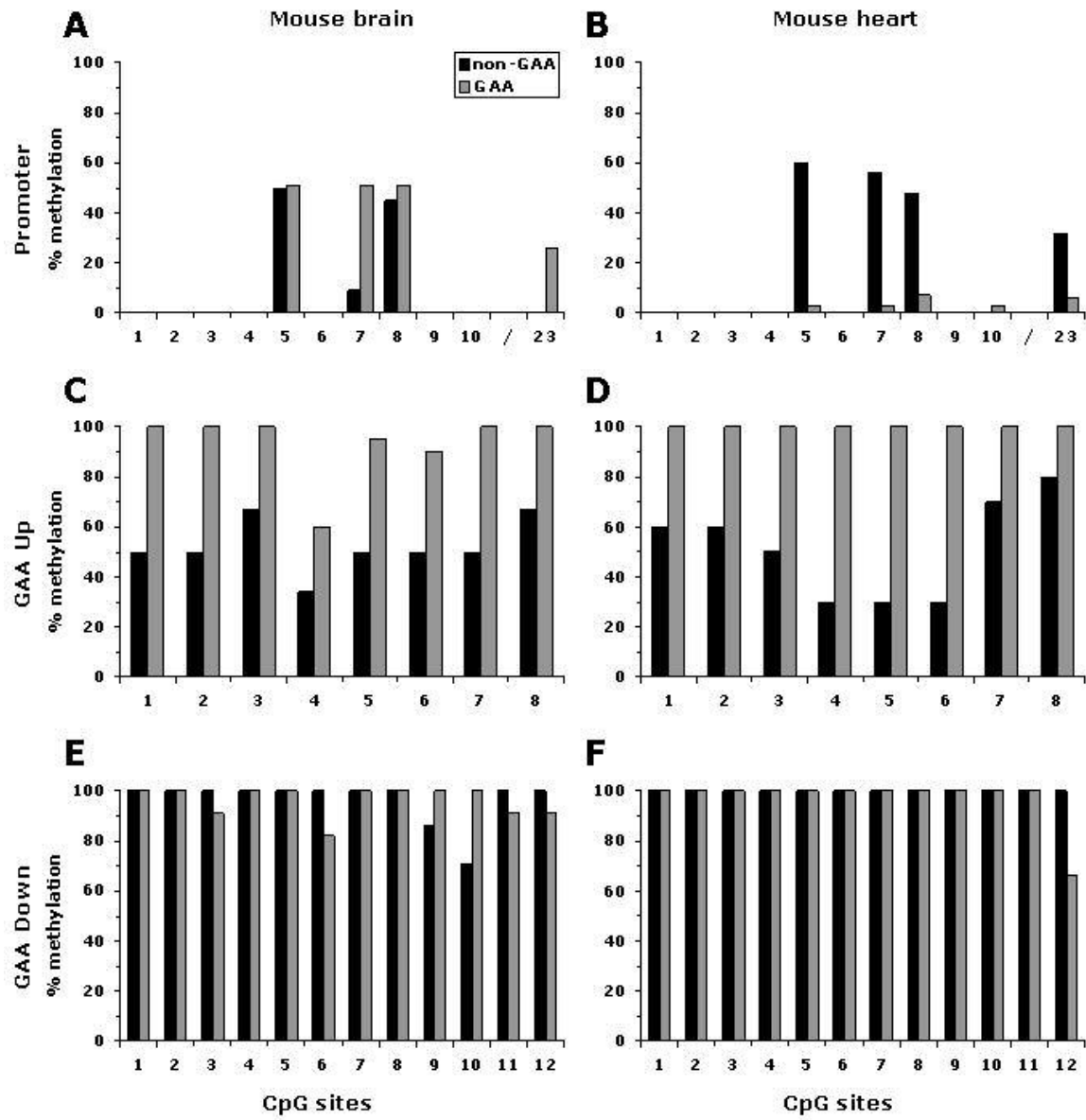
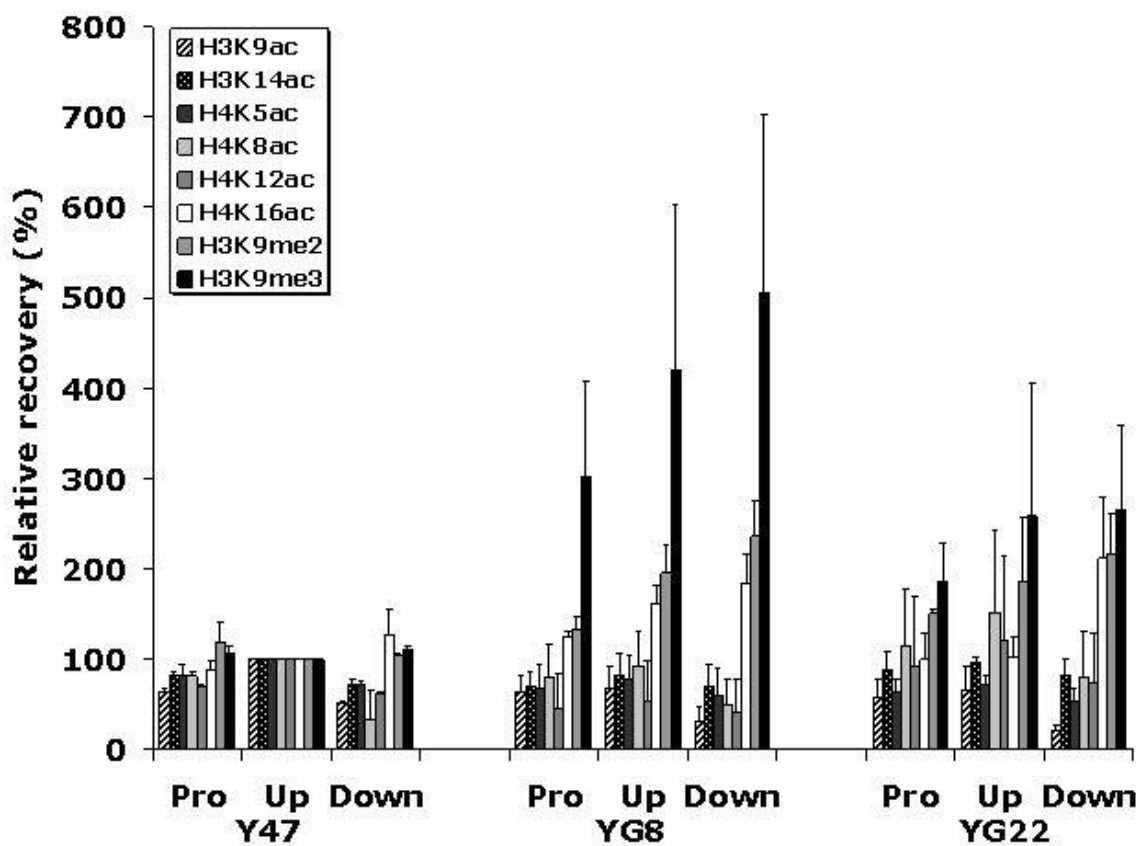


Fig.7



ABBREVIATIONS

5-azadC: 5-azadeoxycytidine; 8-OH-dG: 8-hydroxyl-2'-deoxyguanosine; Ab: antibody; ChIP: chromatin immunoprecipitation; DRG: dorsal root ganglia; FRDA: Friedreich ataxia; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; H3K9: histone 3 lysine 9 residue; HDAC: histone deacetylase; HP1: heterochromatin protein 1; RT-PCR: reverse transcriptase polymerase chain reaction; SEM: standard error of the mean; Sp1: specificity protein 1.

For Peer Review