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# Hypermethylation of repeat expanded *C9orf72* is a clinical and molecular disease modifier

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## Abstract

*C9orf72* promoter hypermethylation inhibits the accumulation of pathologies which have been postulated to be neurotoxic. We tested here whether *C9orf72* hypermethylation is associated with prolonged disease in *C9orf72* mutation carriers. *C9orf72* methylation was quantified from brain or blood using methylation-sensitive restriction enzyme digest-qPCR in a cross-sectional cohort of 118 *C9orf72* repeat expansion carriers and 19 non-carrier family members. Multivariate regression models were used to determine whether *C9orf72* hypermethylation was associated with age at onset, disease duration, age at death, or hexanucleotide repeat expansion size. Permutation analysis was performed to determine whether *C9orf72* methylation is heritable. We observed a high correlation between *C9orf72* methylation across tissues including cerebellum, frontal cortex, spinal cord and peripheral blood. While *C9orf72* methylation was not significantly different between ALS and FTD and did not predict age at onset, brain and blood *C9orf72* hypermethylation was associated with later age at death in FTD (brain: $\beta = 0.18$, $p = 0.006$; blood: $\beta = 0.15$, $p < 0.001$), and blood *C9orf72* hypermethylation was associated with longer disease duration in FTD ($\beta = 0.03$, $p = 0.007$). Furthermore, *C9orf72* hypermethylation was associated with smaller hexanucleotide repeat length ($\beta = -16.69$, $p = 0.033$). Finally, analysis of pedigrees with multiple mutation carriers demonstrated a significant association between *C9orf72* methylation and family relatedness ($p < 0.0001$). *C9orf72* hypermethylation is associated with

prolonged disease in *C9orf72* repeat expansion carriers with FTD. The attenuated clinical phenotype associated with *C9orf72* hypermethylation suggests that slower clinical progression in FTD is associated with reduced expression of mutant *C9orf72*. These results support the hypothesis that expression of the hexanucleotide repeat expansion is associated with a toxic gain of function.

### Keywords

Neurodegeneration; Frontotemporal lobar degeneration; Frontotemporal dementia; Amyotrophic lateral sclerosis; Epigenetics

## Introduction

Hexanucleotide repeat expansions in *C9orf72* are the most frequent genetic cause of autosomal dominant amyotrophic lateral sclerosis (ALS) and frontotemporal degeneration (FTD) [15, 59]. The *C9orf72* mutation is associated with highly variable clinical phenotypes including ALS, FTD, Alzheimer's disease and others, and with a highly variable clinical course ranging from rapidly fatal motor neuron disease to a "slowly progressive" form of FTD [2, 6–8, 10, 14, 28, 29, 34–36, 38, 40, 45, 49, 56, 57, 61, 62]. Several studies have implicated *C9orf72* repeat expansion size [2, 19, 20, 37, 65] and single-nucleotide polymorphisms in *TMEM106B* as disease modifiers in *C9orf72* mutation carriers [26, 66]. However, the basis for much of the clinical heterogeneity amongst *C9orf72* mutation carriers remains unknown.

Studies of other repeat expansion diseases indicate that DNA hypermethylation adjacent to trinucleotide repeat mutations is an epigenetic disease modifier, most notably in cases of Fragile X syndrome and Friedreich's ataxia [9, 22, 33, 51, 52]. We and others have recently shown that the *C9orf72* repeat expansion is associated with *C9orf72* promoter hypermethylation and histone trimethylation which contribute to transcriptional silencing of mutant *C9orf72* [4, 48, 68]. Moreover, we found that epigenetic silencing of mutant *C9orf72* is associated with a decreased accumulation of neuropathologic inclusions associated with the *C9orf72* mutation, namely RNA foci and dipeptide repeat (DPR) protein aggregates, raising the possibility that *C9orf72* promoter hypermethylation mitigates disease pathogenesis [48].

We hypothesized that epigenetic silencing of mutant *C9orf72* is associated with prolonged survival amongst *C9orf72* hexanucleotide repeat expansion carriers. To test this hypothesis, we studied the relationship between *C9orf72* promoter hypermethylation and clinical phenotype, disease onset, disease duration, age of death, and *C9orf72* hexanucleotide repeat length in a cross-sectional cohort of *C9orf72* repeat expansion carriers.

## Materials and methods

### Study cohort

Subjects were evaluated at the Penn Frontotemporal Degeneration Center, the ALS Center at Pennsylvania Hospital or the Penn Alzheimer's Disease Center, or underwent autopsy at the

Center for Neurodegenerative Disease Research [64]. The clinical diagnosis of ALS was made using the El Escorial criteria and FTD was diagnosed using established clinical criteria [30, 58]. In this study, patients presenting with ALS were defined as our ALS cohort and included subjects with mild cognitive impairment [63] and subjects who subsequently met criteria for FTD. Patients presenting with FTD were defined as our FTD cohort and included subjects who subsequently met criteria for ALS. Additional analyses in which the cohort was subdivided into ALS, ALS with mild cognitive impairment (ALS-MCI), ALS with FTD (ALS-FTD) and FTD are included in the Fig. 1e and supplementary materials. Detailed clinical characteristics were obtained from an integrated clinical and autopsy database [70] and by retrospective chart review of clinical visits. Age of onset data was unavailable for four subjects (1 ALS, 3 FTD). All clinical protocols were approved by the University of Pennsylvania Institutional Review Board.

### *C9orf72* genotyping and Southern blotting

Genomic DNA from blood was extracted with the Quick-Gene-610L kit (AutoGen, Holliston, MA, USA), while genomic DNA from brain and other tissues was extracted with the QIAamp DNA mini kit or the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA). *C9orf72* genotyping with repeat-primed PCR was performed as described previously [7]. Southern blot hybridization of *Alu*I- and *Dde*I-digested genomic DNA was performed to determine the hexanucleotide repeat length of *C9orf72* using a digoxigenin-labeled (GGGGCC)5 oligonucleotide probe as previously described [2]. Southern blot images were analyzed in ImageJ to determine the modal hexanucleotide repeat size defined as the point of highest density.

### Bisulfite conversion-based mapping of CpG methylation

Genomic DNA was extracted from two *C9orf72* patient-derived lymphoblastoid cell lines (ND10966 and ND14442, Coriell NINDS Repository, Camden, NJ, USA), five cerebella from *C9orf72* expansion carriers and four cerebella from control cases. DNA was digested for 6 h with 10 U of *Hha*I (New England Biolabs, Ipswich, MA, USA) and purified by phenol:chloroform:isoamyl alcohol extraction. Purified DNA was quantified by NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA) and 100 ng of DNA was bisulfite converted using the EpiTect Bisulfite Kit (Qiagen). Bisulfite-converted DNA was subjected to PCR to amplify the marked *C9orf72* promoter region (Fig. 1a), and the PCR product was cloned into pGEM as described previously [48]. Six to sixteen individual clones from each case were Sanger sequenced using a T7 promoter sequencing primer.

### Bisulfite cloning

Bisulfite cloning was performed on cerebellar genomic DNA from four *C9orf72* repeat expansion cases as described previously [48]. Five clones per case were analyzed, in addition to the five or six individual clones per case previously published in Liu et al. [48] to yield a total of 9–11 clones per case.

### *C9orf72* promoter methylation assay

For quantitative assessment of methylation levels, we used methylation-sensitive restriction enzyme DNA digestion coupled with quantitative PCR (Fig. 1) as described previously [48]. Briefly, 100 ng of genomic DNA was digested for 6 h with 2 units of *Hha*I (New England Biolabs, Ipswich, MA, USA) and 2 units of *Hae*III (NEB) or only with 2 units of *Hae*III followed by heat inactivation. qPCR was done using 12.5 ng of digested DNA per reaction with 2× FastStart SYBR Green Master mix (Roche Applied Science, Indianapolis, IN, USA) using primers amplifying the differentially methylated *C9orf72* promoter region (5′-CAGTGTGAAAATCATGCTTGAGAGA-3′ and 5′-TTTGTGCTTGGTAGGCAGTG-3′). The difference in the number of cycles to threshold amplification between double versus single digested DNA was used as measure of CpG methylation. *Hae*III was added to the DNA digestion reactions to digest the hexanucleotide repeat as we found empirically that the presence of the hexanucleotide repeat expansion mutation resulted in a slight reduction in PCR efficiency.

### Statistical analysis

We applied Wilcoxon rank-sum tests and Kruskal–Wallis non-parametric ANOVA to compare *C9orf72* hypermethylation according to *C9orf72* mutation status, clinical diagnosis, site of motor neuron disease onset, and FTD clinical phenotypes. Multivariate linear regression analysis was performed to determine the relationship between *C9orf72* promoter hypermethylation and age at onset, age at death, disease duration, or hexanucleotide repeat length (mode). Because disease duration values were not normally distributed, disease duration was natural log (ln) transformed when used as dependent variable in linear regression analyses. Spearman's test of correlation or univariate linear regressions were used to assess the association between *C9orf72* methylation and age at collection as well as to compare *C9orf72* methylation in cerebellum and peripheral blood, frontal cortex or spinal cord. One-way ANOVA was used to compare linear regression models. Statistical analysis of the association of repeat size and promoter methylation within families was done with a linear mixed-effects model where the fixed effects were age at sample collection and *C9orf72* promoter methylation, and the random effect was family group [43]. For both multiple linear regression models and linear mixed-effects models, we report the β value for each predictor, which represents the change in outcome (in years for age at onset and age at death, in natural log years for disease duration and number of repeats for repeat length) associated with a unit change in the predictor (e.g., methylation increases by 1 %). A permutation test was used to analyze *C9orf72* promoter hypermethylation within families as described below. Unadjusted *p* values are reported as given hypotheses were made a priori. Notably, for analyses where multiple comparisons are made (pairwise comparisons of methylation in cerebellum versus blood, frontal cortex and spinal cord; linear regression models involving age at onset, age at death and disease duration), *p* values less than 0.0167 would be considered significant after using a Bonferroni correction, and so the reported unadjusted *p* values would remain significant. The R Statistical package (v3.0.1) was used for statistical analyses and GraphPad Prism Software (v5.02, GraphPad, San Diego, CA, USA) was used for data visualization.

## Results

### Study cohort characteristics

*C9orf72* promoter methylation was analyzed in *C9orf72* hexanucleotide repeat expansion carriers and non-expanded family members. Genomic DNA was extracted from cerebellum ($n$ = 22), peripheral blood ($n$ = 99) or both ($n$ = 16). The cohort included 118 individuals with the *C9orf72* repeat expansion as identified by repeat-primed PCR and Southern blotting. 60 mutation carriers presented with motor neuron disease, while 45 individuals presented with FTD. 13 repeat expansion carriers had no clinical phenotype (i.e., prodromal cases). The median age at onset, age at death and disease duration was higher in FTD relative to ALS (Table 1).

Cerebellar DNA was available for 38 mutation carriers including 22 presenting with ALS and 16 presenting with FTD. Blood DNA was available for 96 mutation carriers including 50 with ALS and 33 with FTD. Among the subjects for whom we had blood DNA, 70 were unrelated while 26 were members of one of 9 families. In addition to the repeat expansion carriers, we also measured *C9orf72* methylation in 19 non-expanded, unaffected family members as controls.

### Validation of *Hha*I-qPCR methylation assay for *C9orf72* promoter region

*C9orf72* promoter methylation was quantified using methylation-sensitive restriction enzyme digestion with qPCR [48]. *Hha*I is a restriction enzyme which is able to cut the *C9orf72* promoter only in the absence of CpG methylation, and so qPCR was used to quantify the percent DNA resistant to *Hha*I digestion as a measure of *C9orf72* methylation (Fig. 1a–c). To validate this restriction enzyme-based assay, we sought to determine whether CpG methylation at the *Hha*I cleavage site was associated with methylation of adjacent CpG nucleotides.

Adjacent to the *Hha*I cut site within the qPCR amplicon, there is also an *Hpa*II cut site (Fig. 1a). *Hpa*II also specifically cuts unmethylated CpGs. In a subset of 28 peripheral blood DNA samples, we quantified the resistance against *Hha*I or *Hpa*II digest compared to mock digested DNA using qPCR. Both enzymes gave highly comparable results (Fig. 1d; $R^2$ = 0.984, $p < 0.0001$), suggesting that CpG methylation at these two sites is highly concordant.

To further determine if other CpG sites within the *C9orf72* promoter region are also methylated when the *Hha*I digest site is methylated, genomic DNA from *C9orf72* repeat expansion carriers was *Hha*I digested ($n$ = two *C9orf72* patient-derived lymphoblastoid cell lines and five cerebella of *C9orf72* expansion carriers) followed by bisulfite cloning to assess CpG methylation downstream of the *Hha*I cut site. The cloned DNA included 25 CpG sites with the second CpG corresponding to the *Hha*I cut site assessed by our digest-qPCR assay (Fig. 1a). This procedure demonstrated that *Hha*I resistant DNA is densely methylated across the entire *C9orf72* promoter (Fig. 1e and S1), indicating that *Hha*I resistance is a proxy for dense promoter hypermethylation. Notably, CpG sites 12, 23 and 24 remained hypomethylated (Fig. 1e). Interestingly, there are exactly 146 bp of DNA between CpG sites 12 and 24 which corresponds to the length of DNA within a single nucleosome. Therefore, these sites are likely protected from CpG methylation due to nucleosomal positioning which

is known to be present around transcription start sites and is known to hinder accessibility of DNA methyltransferases [25].

A similar analysis was performed on genomic DNA from non-expanded controls (*n* = four cerebella). Although amplification was low due to the low level of *C9orf72* promoter methylation in controls, *Hha*I resistant DNA from non-expanded controls also demonstrated dense promoter hypermethylation (Fig. 1e and S1). This indicates that the low level of *Hha*I resistance in non-expanded genomic DNA is also associated with dense promoter hypermethylation.

Finally, *Hha*I-based methylation measurements were directly compared to the gold standard bisulfite cloning. Bisulfite cloning (without predigestion with *Hha*I) was performed on cerebellar DNA from four *C9orf72* repeat expansion carriers to determine the percentage of hypermethylated clones. Both bisulfite cloning and digest-qPCR assays produced highly comparable results, further validating our restriction enzyme-based qPCR quantification assay (Fig. 1f and S2).

## *C9orf72* promoter hypermethylation in brain is associated with later age at death in FTD patients

We have recently found that *C9orf72* promoter hypermethylation is associated with a reduction in pathologic RNA foci and DPR aggregates, suggesting that epigenetic silencing of mutant *C9orf72* may protect against disease pathogenesis [48]. Based on these findings, we hypothesized that *C9orf72* hypermethylation is associated with longer survival. To test this hypothesis, we first analyzed brain DNA from individuals harboring the *C9orf72* mutation as a discovery cohort to determine whether *C9orf72* methylation affects clinical disease phenotype, age at death, disease duration, or age at onset.

There was no difference in *C9orf72* methylation in individuals who presented with ALS (*n* = 22; Mdn = 6.09) versus FTD (*n* = 16; Mdn = 6.41; $r = -0.08$, $p = 0.6049$, Wilcoxon rank-sum test). We also detected no difference in *C9orf72* promoter methylation in the cerebellum when comparing ALS patients with different sites of onset (bulbar, cervical or lumbosacral; $\chi^2(2) = 0.25$, $p = 0.8842$, Kruskal–Wallis ANOVA).

A multivariate linear regression model correcting for gender and age at onset (*n* = 37 unrelated *C9orf72* mutation carriers after removing one autopsy case for which complete clinical data were not available) showed a highly significant interaction between *C9orf72* methylation and disease diagnosis such that increased *C9orf72* promoter hypermethylation in individuals with FTD was associated with a later age at death ($\beta = 0.18$, $p = 0.006$; Table 2). This corresponds to a 1.8-year later age at death for every 10 % increase in *C9orf72* promoter methylation. To demonstrate that *C9orf72* promoter hypermethylation contributed significantly to this statistical model, an alternate multivariate linear regression model was generated in which *C9orf72* methylation was omitted. This alternate model did significantly worse at predicting age at death (ANOVA $F(2,31) = 6.97$, $p = 0.0032$). Additional multivariate regression analyses failed to reveal a significant relationship between cerebellar *C9orf72* hypermethylation and either disease duration or age at onset (Table 2).

### *C9orf72* promoter hypermethylation in peripheral blood

*C9orf72* methylation was then measured in peripheral blood DNA of 96 expansion carriers (Mdn = 6.04), and 19 related, non-expanded unaffected controls (Mdn = 0.97, IQR = 0.69–1.63). *C9orf72* promoter methylation levels were significantly higher in repeat expansion carriers compared to non-expanded controls ($p < 0.001$, Wilcoxon rank-sum test; Fig. 2a). Overall, 33.3 % ($n = 32$) of the *C9orf72* repeat expansion carriers exhibited *C9orf72* promoter methylation values greater than 10 %, in contrast with the non-expanded control group in which *C9orf72* promoter methylation values were all less than 10 %. Similar to what was observed using cerebellar DNA, peripheral blood *C9orf72* promoter methylation did not differ between patients who presented with ALS ($n = 50$; Mdn = 6.062) versus FTD ($n = 33$; Mdn = 6.41, $r = -0.106$, $p = 0.3155$, Wilcoxon rank-sum test). Similar results were observed when further subdividing the cohort into four disease subcategories (ALS, ALS-MCI, ALS-FTD and FTD, Table S1, $\chi^2(3) = 2.65$, $p = 0.4493$, Kruskal–Wallis non-parametric one-way ANOVA). Furthermore, no difference in blood *C9orf72* promoter methylation was observed when subdividing individuals with motor neuron disease based on site of onset (bulbar, cervical and lumbosacral; $\chi^2(2) = 1.139$, $p = 0.5659$, Kruskal–Wallis ANOVA) or when subdividing individuals with cognitive impairment into individuals with behavioral variant FTD versus primary progressive aphasia ($p = 0.9422$, Wilcoxon rank-sum test). Overall these results suggest that *C9orf72* methylation was similar between different clinical phenotypes.

### Promoter methylation is stable across time and tissue types

*C9orf72* methylation in repeat expansion carriers was not associated with age at peripheral blood collection ($r_s = -0.166$, $p = 0.1206$, Spearman rank correlation) suggesting that *C9orf72* methylation is stable over time. This is in contrast with *C9orf72* repeat length which has been reported as being dependent on age of collection [65]. Additionally, we identified 16 repeat expansion carriers for which we had DNA available from both cerebellum and peripheral blood, allowing for a direct comparison of *C9orf72* promoter methylation in peripheral blood and brain. *C9orf72* promoter methylation between peripheral blood and cerebellum was highly consistent ($R^2 = 0.621$, $p = 0.0003$; Fig. 2b). The time between blood and brain collection ranged between 0.4 and 9.5 years. There was no correlation between differences in *C9orf72* methylation between blood and cerebellum and the collection time interval ($r_s = 0.14$, $p = 0.6174$, Spearman rank correlation). This further supports that *C9orf72* promoter methylation is stable across time.

Genomic DNA was available from both cerebellum and frontal cortex from 23 *C9orf72* repeat expansion carriers, and from both cerebellum and spinal cord for 13 patients. *C9orf72* promoter methylation in frontal cortex and cerebellum (Fig. 2c) as well as in spinal cord and cerebellum (Fig. 2d) was highly concordant (frontal cortex: $R^2 = 0.659$, $p < 0.0001$; spinal cord: $R^2 = 0.853$, $p < 0.0001$). Tissues from three additional autopsies were available from which genomic DNA was extracted from multiple central and peripheral tissues. *C9orf72* promoter methylation was measured in 8 different brain regions, in 3–4 spinal cord sections and in up to 8 different peripheral tissues (Fig. 2e). One of the three cases was hypermethylated (Case 2: Mdn = 41.69), while the other two cases were hypomethylated (Case 1: Mdn = 5.20; Case 3: Mdn = 3.39). While there was some variability between

tissues, *C9orf72* promoter methylation was overall highly consistent across all analyzed tissues types in these three cases (Case 1: IQR = 4.18–5.67; Case 2: IQR = 35.98–45.56; Case 3: IQR = 2.40–4.14). These results indicate that *C9orf72* promoter methylation is stable across a wide variety of tissues.

### *C9orf72* promoter hypermethylation in FTD patients is associated with longer survival

Given the strong correlation between peripheral blood and brain *C9orf72* methylation, we sought to replicate our autopsy cohort results by determining whether *C9orf72* hypermethylation in peripheral blood of repeat expansion carriers is associated with age at death. Complete clinical information including age at death and disease duration was available for 47 unrelated *C9orf72* mutation carriers. A multivariate linear regression model correcting for gender, age at onset and clinical diagnosis revealed a significant interaction such that increased peripheral blood *C9orf72* methylation in individuals with FTD was associated with a later age at death ($\beta = 0.15$, $p < 0.001$, Table 3). This corresponds to a 1.5-year later age at death for every 10 % increase in methylation. These findings based on peripheral blood DNA were highly similar to what was observed using cerebellar DNA. To demonstrate that *C9orf72* promoter methylation contributed significantly to this statistical model, an alternate linear regression model was generated in which *C9orf72* methylation was omitted. This alternate model did significantly worse at predicting age at death (ANOVA $F(2,41) = 10.23$, $p = 0.0003$).

In addition to replicating the association between *C9orf72* hypermethylation and age at death in FTD, multivariate linear regression analysis correcting for gender, age at onset and diagnosis also revealed that increased blood *C9orf72* methylation in FTD was associated with longer disease duration ($\beta = 0.03$, $p = 0.007$, Table 3). Because disease duration was ln transformed, an increase of 1 % in *C9orf72* promoter methylation would result in a 3 % increase of disease duration. Once again, a regression model in which *C9orf72* methylation was omitted resulted in a model which performed significantly worse relative to the linear regression model containing *C9orf72* methylation (ANOVA $F(2,41) = 5.03$, $p = 0.0111$). Age of onset data was available for 77 unrelated *C9orf72* mutation carriers from which no association was observed between peripheral blood *C9orf72* hypermethylation and age at onset (Table 3).

Similar results were obtained upon subdividing the cohort into four disease subtypes (ALS, ALS-MCI, ALS-FTD, FTD). Again no association was observed between peripheral blood *C9orf72* hypermethylation and age at onset. However, there was a significant association between *C9orf72* hypermethylation and both age at death and disease duration in FTD and/or ALS-FTD (Table S2).

### Promoter methylation levels are heritable

The mechanisms regulating differential *C9orf72* promoter methylation in repeat expansion carriers are not known. However, prior studies have suggested that *C9orf72* methylation levels are similar amongst closely related family members [68]. We analyzed the nine families in our study for which peripheral blood *C9orf72* promoter hypermethylation measurements were available from at least two repeat expansion carrier family members.

Figure 3a highlights six families in which all individuals within these families exhibited low *C9orf72* hypermethylation ranging from 1.1 to 10.0 %. In contrast, there were three families, including one family with five first-degree relatives harboring the hexanucleotide repeat expansion, where individuals exhibited high levels of *C9orf72* methylation ranging from 20.2 to 41.9 %, suggesting that *C9orf72* hypermethylation is heritable (Fig. 3b). Overall, it appeared that *C9orf72* methylation was stable both within generations and across different generations. Non-expanded, unaffected family members are not shown because *C9orf72* hypermethylation is only observed in repeat expansion carriers, and to better preserve anonymity of the research participants. Many repeat expansion carriers in the nine pedigrees were clinically unaffected at sample collection. Four pedigrees had multiple affected individuals. Of those four pedigrees, individuals within two of the families exhibited concordant clinical phenotypes (all motor neuron disease, or all FTD), while individuals within the other two families exhibited discordant clinical phenotypes (mixtures of both motor neuron disease and FTD). We observed no relationship between *C9orf72* methylation and concordance of clinical diagnosis in these families.

To more rigorously test whether there is a significant relationship between *C9orf72* methylation and family relatedness, we used a permutation test to determine if affiliation within a certain family is associated with *C9orf72* methylation. First, a univariate linear regression model was generated relating different families to *C9orf72* methylation. Then, each of the 26 studied family members was randomly assigned to one of the nine families, while the number of family members within each family was kept constant; thereby an alternate linear regression model was generated in which the relationship between the dependent and independent variables was broken. This process was repeated 10,000 times to generate a random set of linear regression models. The true linear regression model revealed an $F$ statistic of 3.41, while the 10,000 random linear regression models were associated with $F$ statistics ranging from 0.049 to 2.711 (Fig. 3c). Thus, maintaining the true relationship between family membership and *C9orf72* methylation predicted *C9orf72* methylation significantly better than when the relationship between family membership and *C9orf72* methylation was randomly broken ($p < 0.0001$). These results suggest that *C9orf72* methylation is heritable and raises the possibility that there may be a latent polymorphism which promotes *C9orf72* methylation in a subset of *C9orf72* repeat expansion carriers.

### *C9orf72* promoter hypermethylation is associated with a shorter repeat size

Finally, DNA methylation has been shown to affect repeat expansion size in several repeat expansion-associated diseases [12, 17, 18, 47]. To determine whether *C9orf72* promoter methylation affects hexanucleotide repeat expansion size, hexanucleotide repeat expansion size was measured by Southern blot of peripheral blood of 93 repeat expansion carriers. A multivariate regression analysis demonstrated that both age at collection and *C9orf72* promoter methylation influenced repeat expansion size ($n = 76$ unrelated repeat expansion carriers, Fig. 4a and Table 4). As has been previously noted by others [65], later age of sample collection was associated with longer repeat sizes ($\beta = 38.48$, $p = 0.001$). This corresponds to an increase of 38.5 repeats per 1 year increase in age at collection. In contrast, increasing *C9orf72* promoter methylation levels are associated with smaller repeat size ($\beta = -16.69$, $p = 0.033$), i.e., an increase of 10 % in *C9orf72* promoter methylation

results in a decrease of repeat size by 167 repeats. To further explore the relationship between repeat size and promoter methylation, 24 individuals were identified within eight families for which both peripheral blood hexanucleotide repeat expansion size and *C9orf72* promoter methylation values were available from at least two family members. A representative Southern blot of DNA from five individuals from a single family is shown in Fig. 4b. We observed that within families, increased *C9orf72* methylation was associated with reduced hexanucleotide repeat expansion size. To verify this observation, a linear mixed-effects model was generated to control for family relatedness which again revealed that *C9orf72* promoter methylation was associated with reduced hexanucleotide repeat expansion size ($\beta = -51.44$, $p = 0.010$, Table 4). This corresponds to a decrease in repeat size by 51.4 repeats with every 1 % increase of *C9orf72* promoter methylation within family members. We also analyzed the relationship between repeat size, methylation and age at collection in cerebellum ($n = 38$, Fig. 4c). There was no significant effect of methylation or age of collection on repeat size in cerebellum (Table 4), perhaps due to the smaller sample size compared to the peripheral blood dataset, or due to the fact that the *C9orf72* repeat expansion may be more stable in cerebellum relative to other tissues [20, 65].

## Discussion

The hexanucleotide repeat expansion in *C9orf72* is associated with a variable clinical phenotype including ALS, ALS with FTD, FTD, Alzheimer's disease and others [2, 6–8, 10, 14, 28, 29, 34–36, 38, 40, 45, 49, 56, 57, 61, 62]. Additionally, both ALS and FTD are characterized by clinical heterogeneity in terms of age at onset and disease duration [2, 6–8, 10, 14, 28, 29, 34–36, 38, 40, 45, 49, 56, 57, 61, 62]. The clinical heterogeneity associated with the *C9orf72* mutation indicates that there are likely several environmental or genetic disease modifiers which remain to be discovered.

We hypothesized that epigenetic silencing of mutant *C9orf72* associated with DNA methylation prolongs survival. We found that *C9orf72* methylation appears to be stable across a diverse set of central and peripheral tissues as well as over time. Additionally, *C9orf72* hypermethylation in both cerebellum and peripheral blood is associated with later age at death in *C9orf72* mutation carriers with FTD. Moreover, *C9orf72* hypermethylation in blood is associated with longer disease duration and shorter *C9orf72* hexanucleotide repeat length. These results provide evidence that *C9orf72* hypermethylation is associated with prolonged survival in *C9orf72* repeat expansion carriers with FTD, supporting the hypothesis that epigenetic silencing of mutant *C9orf72* may be protective.

We did not see an effect of *C9orf72* promoter hypermethylation on disease duration or age at death in ALS patients. The pyramidal motor system may have less functional reserve compared to the CNS networks which regulate executive or language function, resulting in more rapid progression to death in ALS such that *C9orf72* hypermethylation may not lead to a significant modifying effect (see Table 1). Indeed, ALS is known to exhibit a rapid clinical course compared to FTD as seen in the cohort reported here (Table 1), compounded by the fact that the *C9orf72* mutation is associated with even more rapid clinical decline relative to cases without the *C9orf72* mutation [38].

A previous study examined *C9orf72* promoter methylation in repeat expanded ALS patients, non-expanded ALS patients and normal controls [68]. Similar to us, they found that *C9orf72* promoter methylation in blood is significantly higher in *C9orf72* repeat expansion carriers than in non-expanded controls, and that there is no correlation between *C9orf72* methylation and age at onset or age at sample collection [68]. These results were recently confirmed in a cohort of FTD patients [69]. We and others have observed faster disease progression in cases with the *C9orf72* mutation relative to non-expanded cases [6, 7, 10, 14, 36, 38, 49, 61]. Therefore, there is an overall inverse relationship between *C9orf72* methylation and disease duration due to the fact that *C9orf72* hypermethylation is seen only in individuals with the hexanucleotide repeat expansion [48, 68, 69]. Rather than studying *C9orf72* hypermethylation in a cohort of both repeat expanded and non-expanded cases, our goal here was to determine whether epigenetic silencing of mutant *C9orf72* is a disease modifier within *C9orf72* carriers. In this context, we found that increased *C9orf72* methylation levels in FTD patients are associated with prolonged survival.

We also observed that *C9orf72* methylation is stable within and across generations. We demonstrated statistically a significant relationship between *C9orf72* methylation and family relatedness. These results are consistent with a previous report that suggested that *C9orf72* methylation is heritable [68]. Genetic polymorphisms are known to influence DNA methylation [3, 27, 39]. Our results raise the possibility that there may be a latent polymorphism which promotes *C9orf72* hypermethylation in *C9orf72* repeat expansion carriers. *TMEM106B* polymorphisms have been reported to be genetic modifiers in FTD individuals with the *C9orf72* repeat expansion [26, 66]. Importantly, we found no relationship between *TMEM106B* genotype and *C9orf72* promoter methylation (data not shown).

The factors which regulate *C9orf72* methylation are not known. Interestingly, several repeat expansion disease loci are associated with local heterochromatin formation including *FMR1* and *FXN* [23]. *FMR1* silencing occurs very early during embryogenesis and has been linked to the formation of RNA–DNA duplexes within the *FMR1* promoter [13, 16]. Whether a similar mechanism causes silencing of mutant *C9orf72* remains to be seen. Although the molecular association between repeat expansions and local epigenetic silencing is remarkably consistent across several diseases, the clinical consequences of epigenetic silencing are divergent. Thus, in contrast to what we observe for *C9orf72*, DNA hypermethylation in Friedreich's ataxia and Fragile X syndrome is associated with faster disease progression or more severe disease phenotype [9, 22, 33, 51, 52]. Both Friedreich's ataxia and Fragile X syndrome are caused by a loss of gene function. Our results imply that the *C9orf72* mutation does not cause disease through a loss of function.

*C9orf72* repeat expansion length has also been studied as a potential disease modifier. One study found that a threshold *C9orf72* repeat length from cerebellar DNA is associated with longer survival in individuals with FTD [65]. Three studies reported a positive correlation between repeat size and age at onset [2, 37, 65]. Two other studies failed to observe an association between *C9orf72* repeat length and age at onset [19, 20]. However, these associations are confounded by the fact that the *C9orf72* hexanucleotide repeat exhibits somatic instability such that repeat length in peripheral blood does not correlate with repeat

length in brain [65]. Moreover, *C9orf72* repeat length changes according to age at collection, confounding potential associations between repeat length and clinical disease [65]. In contrast, *C9orf72* methylation did not vary according to age at sample collection and thereby may represent a more stable disease biomarker.

*C9orf72* hexanucleotide repeat expansions characteristically exhibit a high degree of somatic instability [2, 37, 65]. The molecular mechanisms that lead to GGGGCC repeat instability are not known. However, DNA methylation, by virtue of altering DNA transcription or replication dynamics, has been shown in most cases to stabilize microsatellite repeats [12, 17, 18, 31, 47]. The significant inverse correlation between *C9orf72* promoter methylation and hexanucleotide repeat expansion length is thus another example of epigenetic regulation of microsatellite instability.

The *C9orf72* hexanucleotide repeat expansion has been postulated to cause disease due to a gain of toxic function linked to the accumulation of RNA foci [15]. RNA foci are predominantly intranuclear accumulations of hexanucleotide repeat containing RNA which have been reported to bind to several RNA-binding proteins including Pur α, hnRNPA3, hnRNP-H, ADARB2 and nucleolin [21, 32, 44, 54, 60, 71]. The sequestration of RNA-binding proteins within RNA foci has been postulated to lead to transcriptomic abnormalities which lead to neurodegeneration [21, 32, 42, 44, 54, 60, 71]. Additionally, repeat expanded RNA can be translated into DPR proteins which aggregate within neurons and may be toxic [1, 41, 50, 53, 55].

Alternatively, the *C9orf72* mutation has been suggested to result in haploinsufficiency linked to epigenetic silencing of mutant *C9orf72*. The repeat expansion mutation is associated with reduced *C9orf72* mRNA and protein [4, 48, 67, 68]. *C9orf72* protein regulates endosomal trafficking, consistent with bioinformatics-based analysis which indicated that *C9orf72* is structurally related to the DENN family of proteins which regulate organelle trafficking [24, 46, 72].

Epigenetic silencing of mutant *C9orf72* leads to reduced expression of *C9orf72* mRNA [4, 5, 48, 68, 69]. Moreover, knockdown of the *C9orf72* homolog in zebrafish results in motor neuron axonal degeneration [11]. However, we have found that *C9orf72* hypermethylation is associated with a protective phenotype in *C9orf72* repeat expanded lymphoblast cells, and that at time of autopsy, brain tissue from individuals within the *C9orf72* mutation carrier cohort reported here demonstrates that *C9orf72* hypermethylation is associated with reduced RNA foci and DPR aggregate burden [48]. Thus, it was unclear whether epigenetic silencing of mutant *C9orf72* is deleterious or protective against disease. The association between *C9orf72* hypermethylation and prolonged survival and smaller repeat length that we report here supports the toxic gain of function hypothesis, and indicates that transcriptional silencing of mutant *C9orf72* may be protective, at least in terms of disease progression after onset in FTD cases. Importantly, we did not observe a significant relationship between *C9orf72* hypermethylation and age at onset or clinical phenotype (ALS versus FTD, site of onset, FTD phenotype). The factors that influence disease onset and clinical phenotypes remain unknown.

Our results have implications in terms of developing therapies for *C9orf72* mutation carriers. While haploinsufficiency is not entirely excluded as a disease mechanism, therapies to increase *C9orf72* gene expression may be detrimental, particularly if these therapies increase expression of the mutant allele. Conversely, enhancing epigenetic silencing of mutant *C9orf72* or other methods to suppress mutant *C9orf72* may be beneficial, including recent efforts to develop antisense oligonucleotides to target mutant *C9orf72* RNA for degradation [21, 42, 60].

The strengths of this study include a well-characterized cohort of *C9orf72* mutation carriers, the use of DNA from multiple different sources, and the use of a highly quantitative methylation assay. Replication of our results with independent *C9orf72* mutation carrier cohorts would be desirable. Moreover, because of our retrospective study design, prospective longitudinal studies are necessary to validate the predictive power of *C9orf72* promoter methylation in terms of clinical disease progression. Furthermore, a more thorough analysis of *C9orf72* methylation over the entire *C9orf72* promoter from multiple DNA sources including multiple brain regions is needed to further elucidate potential tissue-specific differences in *C9orf72* methylation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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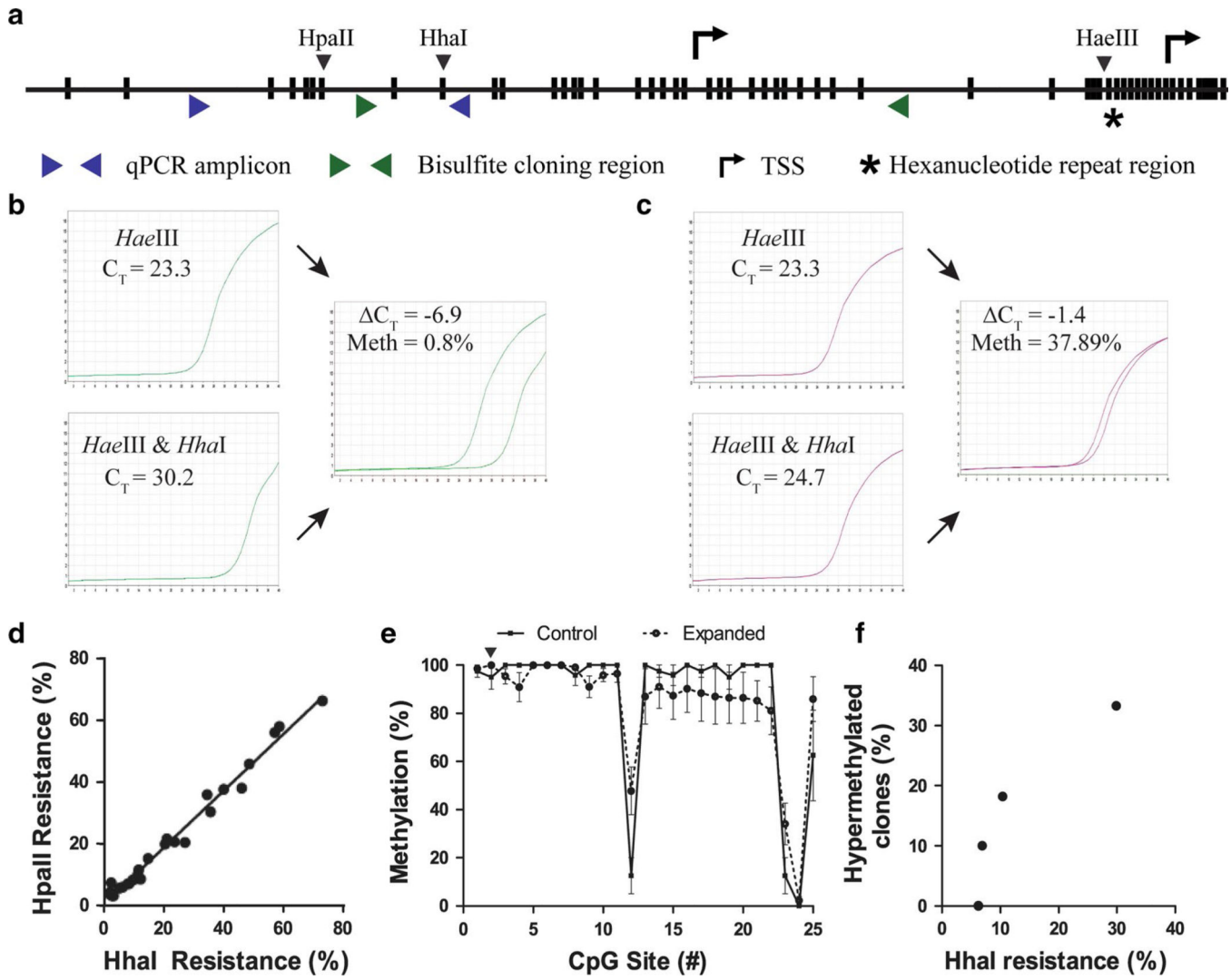


**Fig. 1.**

*C9orf72* promoter methylation assay. **a** Schematic representation of the 5′ end of the *C9orf72* gene in which individual CpG dinucleotides are represented by *vertical bars*, the transcriptional start sites (TSS) are designated by *arrows*, the hexanucleotide repeat region is designated by a *star*, the binding sites of the qPCR primers used in the *C9orf72* promoter methylation assay are represented as *blue triangles*, and the binding sites of the primers used in bisulfite cloning are represented as *green triangles*. The *Hha*I and the *Hpa*II restriction enzyme recognition sites within the qPCR amplicon as well as the first *Hae*III restriction enzyme recognition site are shown. Representative qPCR amplification curves for *Hae*III only versus *Hae*III–*Hha*I double digested DNA for **b** a hypomethylated (0.8 %) and **c** a hypermethylated (37.89 %) case. The resulting $\Delta C_T$ values are used to calculate the percent DNA resistance to *Hha*I digestion ($2^{\Delta C_T} \times 100$) as a measure of DNA methylation. **d** Scatterplot and linear regression of *Hpa*II resistance versus *Hha*I resistance as measured by restriction enzyme digest-qPCR ($n = 28$). **e** Bisulfite cloning of *Hha*I resistant DNA. The percent methylation and standard error for each CpG site are plotted for repeat expanded cases (*dashed line*) and non-expanded controls (*solid line*). The *Hha*I site which is assessed

by the digest-qPCR assay is marked with an *inverted triangle*. **f** Direct comparison of *Hha*I-qPCR and bisulfite cloning. The percentage of bisulfite-treated DNA clones exhibiting hypermethylation (methylation of at least 10 of 25 CpG sites) is plotted as a function of *Hha*I resistance ($n = 4$)

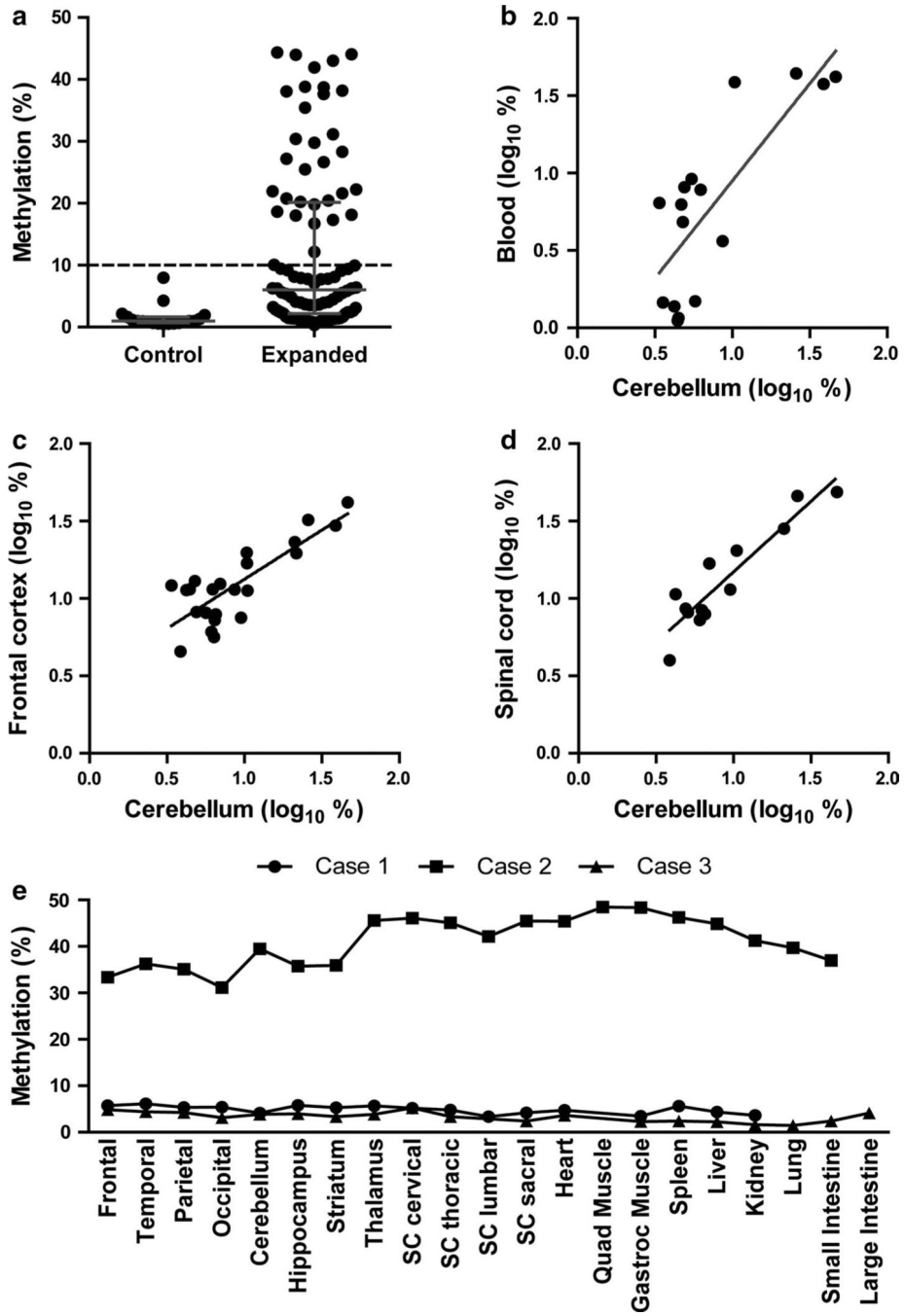


**Fig. 2.**

*C9orf72* promoter methylation in peripheral tissue and cerebellum. **a** *C9orf72* methylation as measured by restriction enzyme digestqPCR of peripheral blood from controls ($n$ = 19) or repeat expansion cases ($n$ = 96) are shown. *Horizontal gray line* represents median and *error bars* show interquartile range. The *dashed line* represents 10 % methylation. Wilcoxon rank-sum test: $p < 0.001$. **b** Relationship between $\log_{10}$-transformed *C9orf72* methylation from peripheral blood versus cerebellum ($n$ = 16 repeat expansion carriers, $R^2 = 0.621$, $p$ = 0.0003). **c** Relationship between $\log_{10}$-transformed *C9orf72* methylation from frontal cortex

versus cerebellum ($n$ = 23 repeat expansion carriers, $R^2$ = 0.659, $p$ < 0.0001). **d** Relationship between $\log_{10}$-transformed *C9orf72* methylation from spinal cord versus cerebellum ($n$ = 13 repeat expansion carriers, $R^2$ = 0.853, $p$ < 0.0001). **e** *C9orf72* promoter methylation across central and peripheral tissues. Percent methylation for each available brain, spinal cord (SC) or peripheral tissue is shown for three *C9orf72* repeat expansion cases

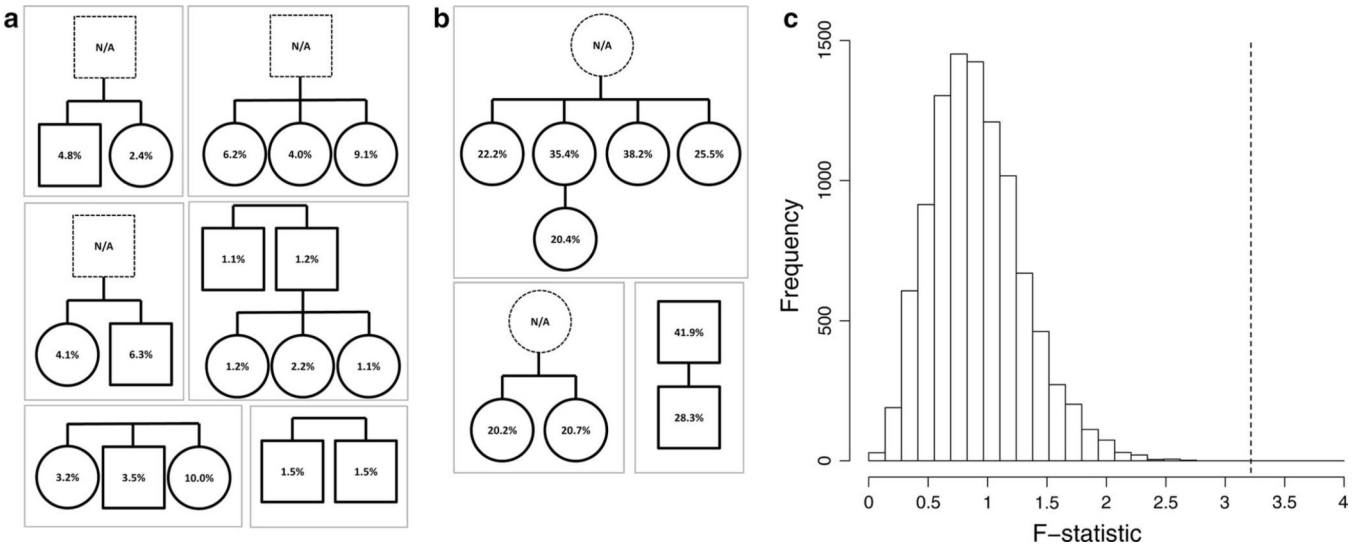


**Fig. 3.**

*C9orf72* promoter methylation within family pedigrees. *C9orf72* promoter methylation values are shown for individuals from families with (**a**) low or (**b**) high peripheral blood *C9orf72* methylation. Male family members are represented as *squares* and females as *circles*. *Dotted squares* or *circles* represent inferred repeat expansion carriers for which no DNA was available. Non-expanded family members are not shown. **c** Frequency histogram of *F* statistics from 10,000 randomly permutated linear models. *Dotted line* represents the *F* statistic obtained with the true linear model (permutation test $p < 0.0001$)

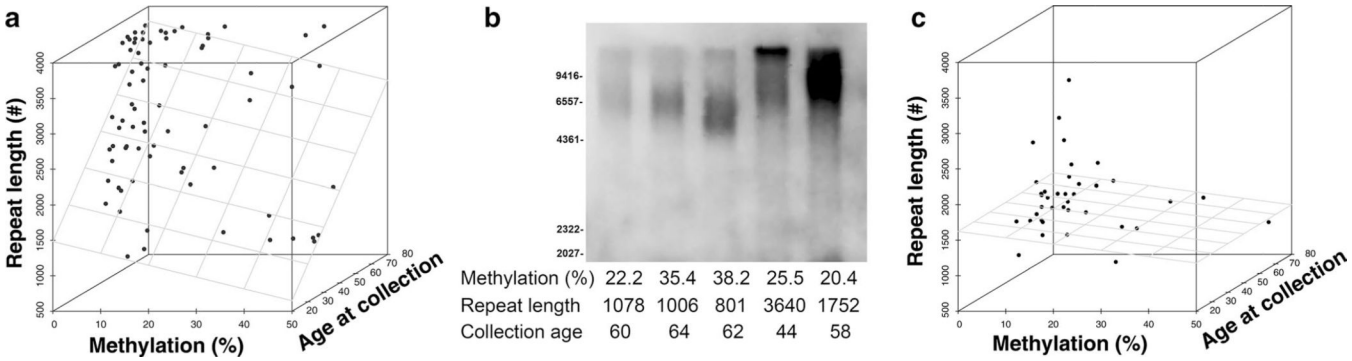


**Fig. 4.**

*C9orf72* promoter hypermethylation is associated with shorter repeat length. **a** Scatterplot of the relationship between repeat length, promoter methylation and age at sample collection of peripheral blood DNA (*n* = 77). The results of the multivariate regression model (Table 4) are overlaid as a *gray-colored plane*. **b** Representative Southern blot for the *C9orf72* hexanucleotide repeat expansion from peripheral blood DNA of five repeat expansion carriers from a single family. Values for *C9orf72* promoter methylation, hexanucleotide repeat length, and age at DNA collection are shown. **c** Scatterplot of the relationship between repeat length, promoter methylation and age at sample collection of cerebellar DNA (*n* = 38). The results of the multivariate regression model (Table 4) are overlaid as a *gray-colored plane*

## Table 1

Clinical characteristics of *C9orf72* repeat expansion carriers

| | Overall | ALS | FTD |
|---|---|---|---|
| Cerebellum, *n* | 38 | 22 | 16 |
| Gender, no. male (%) | 23 (60.53) | 15 (68.18) | 8 (50) |
| Age at onset, year (IQR) | 55.89 (52–64.63) | 54.85 (52–63.25) | 61 (51–66) |
| Age at death, year (IQR) | 59.98 (55–70.66) | 57.81 (54.92–65.67) | 68.22 (56.82–74.7) |
| Disease duration, year (IQR) | 2.87 (2.09–7.11) | 2.36 (1.61–2.92) | 7.23[a] (5.53–10.11) |
| Methylation, pct (IQR) | 6.19 (4.64–10.37) | 6.09 (4.76–13.18) | 6.41 (4.33–9.94) |
| Blood, *n* | 96 | 50 | 33 |
| Deaths | 48 | 37 | 11 |
| Gender, no. male (%) | 52 (54.17) | 30 (60.0) | 21 (63.64) |
| Age at onset, year (IQR) | 55.95 (51.15–62.15) | 55.12 (51.30–63.5) | 58.0 (50.73–62.0) |
| Age at death, year (IQR) | 61.69 (55.16–67.42) | 59.89 (55.05–67.71) | 62.75 (55.56–67.48) |
| Disease duration, year (IQR) | 2.68 (2.03–4.08) | 2.53 (1.95–3.31) | 5.29[b] (2.87–9.98) |
| Methylation, pct (IQR) | 6.04 (2.17–20.13) | 5.74 (1.71–10.5) | 6.22 (1.82–29.35) |

Data are shown as median and interquartile range (IQR) unless otherwise stated

[a] $p < 0.001$,

[b] $p = 0.0012$ compared to ALS, Wilcoxon rank-sum test

**Table 2**

Cerebellar *C9orf72* promoter methylation and age at onset, age at death and disease duration

| | **Age at onset (*n* = 37)** | | | **Age at death (*n* = 37)** | | | **Disease duration (ln transformed, *n* = 37)** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **β (SE)** | **95 % CI** | ***p* value** | **β (SE)** | **95 % CI** | ***p* value** | **β (SE)** | **95 % CI** | ***p* value** |
| Intercept | 61.07 (3.21) | 54.43 to 67.61 | <0.001 | 0.13 (2.53) | −5.02 to 5.28 | 0.960 | 0.27 (0.66) | −1.08 to 1.61 | 0.690 |
| Diagnosis (FTD) | −0.99 (4.03) | −9.20 to 7.24 | 0.806 | 3.39 (0.90) | 1.55 to 5.24 | <0.001 | 0.87 (0.24) | 0.39 to 1.35 | <0.001 |
| Gender (male) | −5.61 (2.90) | −11.51 to 0.29 | 0.062 | −0.02 (0.69) | −1.42 to 1.38 | 0.980 | −0.03 (0.18) | −0.40 to 0.33 | 0.865 |
| Age at onset | n.a. | n.a. | n.a. | 1.04 (0.04) | 0.96 to 1.12 | <0.001 | 0.01 (0.01) | −0.01 to 0.03 | 0.341 |
| Methylation | −0.03 (0.19) | −0.41 to 0.35 | 0.884 | −0.01 (0.04) | −0.10 to 0.07 | 0.759 | −0.01 (0.01) | −0.03 to 0.02 | 0.604 |
| Methylation × diagnosis (FTD) | 0.11 (0.27) | −0.44 to 0.67 | 0.684 | 0.18 (0.06) | 0.06 to 0.31 | 0.006 | 0.03 (0.02) | −0.01 to 0.06 | 0.097 |
| $R^2$ | 0.116 | | | 0.967 | | | 0.644 | | |

*SE* standard error, *CI* confidence interval, *ln* natural log, *n.a.* not applicable

**Table 3**

Peripheral blood *C9orf72* promoter methylation and age at onset, age at death and disease duration (two disease subgroups)

| | **Age at onset (*n* = 77)** | | | **Age at death (*n* = 47)** | | | **Disease duration (ln transformed, *n* = 47)** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **β (SE)** | **95 % CI** | ***p* value** | **β (SE)** | **95 % CI** | ***p* value** | **β (SE)** | **95 % CI** | ***p* value** |
| Intercept | 56.57 (1.89) | 52.81 to 60.34 | <0.001 | 2.86 (1.85) | −0.86 to 6.59 | 0.129 | 0.93 (0.50) | 0.03 to 2.04 | 0.071 |
| Diagnosis (FTD) | −0.70 (2.61) | −5.90 to 4.51 | 0.791 | 0.53 (0.88) | −1.25 to 2.31 | 0.553 | 0.19 (0.24) | −0.32 to 0.65 | 0.440 |
| Gender (male) | 2.79 (1.89) | −0.97 to 6.55 | 0.144 | −0.07 (0.51) | −1.10 to 0.97 | 0.899 | −0.10 (0.14) | −0.41 to 0.15 | 0.460 |
| Age at onset | n.a. | n.a. | n.a. | 1.00 (0.03) | 0.94 to 1.06 | <0.001 | 0.00 (0.01) | −0.02 to 0.02 | 0.889 |
| Methylation | −0.16 (0.11) | −0.38 to 0.06 | 0.151 | −0.01 (0.03) | −0.06 to 0.04 | 0.661 | −0.01 (0.01) | −0.02 to 0.01 | 0.411 |
| Methylation × diagnosis (FTD) | 0.13 (0.15) | −0.16 to 0.42 | 0.379 | 0.15 (0.04) | 0.07 to 0.22 | <0.001 | 0.03 (0.01) | 0.01 to 0.05 | 0.007 |
| $R^2$ | 0.054 | | | 0.968 | | | 0.453 | | |

*SE* standard error, *CI* confidence interval, *ln* natural log, *n.a.* not applicable

**Table 4**

*C9orf72* promoter methylation and hexanucleotide repeat expansion length

| | **Repeat length, blood[a] (*n* = 76 unrelated individuals)** | | **Repeat length, blood[b] (*n* = 24 individuals in eight families)** | | **Repeat length, brain[c] (*n* = 38 unrelated individuals)** | |
|---|---|---|---|---|---|---|
| | **β (SE)** | ***p* value** | **β (SE)** | ***p* value** | **β (SE)** | ***p* value** |
| Intercept | 713.24 (701.41) | 0.313 | 2,408.56 (1,080.22) | 0.043 | 1,615.56 (514.98) | 0.004 |
| Age at collection | 38.48 (11.62) | 0.001 | 15.15 (20.26) | 0.467 | 0.38 (8.11) | 0.963 |
| *C9orf72* methylation | −16.69 (7.68) | 0.033 | −51.44 (17.32) | 0.010 | −8.89 (7.83) | 0.264 |

*SE* standard error

[a] Linear multivariate regression model ($R^2$ = 0.189)

[b] Linear mixed-effects model using age at sample collection and methylation as fixed effects and family group as random effect

[c] Linear multivariate regression model ($R^2$ = 0.036)