

Unaffected mosaic *C9orf72* case

RNA foci, dipeptide proteins, but upregulated *C9orf72* expression

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Abstract

Objective

Suggested *C9orf72* disease mechanisms for amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration include *C9orf72* haploinsufficiency, G₄C₂/C₄G₂ RNA foci, and dipeptide repeat (DPR) proteins translated from the G₄C₂ expansion; however, the role of small expansions (e.g., 30–90 repeats) is unknown and was investigated here.

Methods

We conducted a molecular and pathology study of a family in which the father (unaffected at age 90) carried a 70-repeat allele in blood DNA that expanded to ≈1,750 repeats in his children, causing ALS.

Results

Southern blotting revealed different degrees of mosaicism of small and large expansions in the father's tissues from the CNS. Surprisingly, in each mosaic tissue, *C9orf72* mRNA levels were significantly increased compared to an ALS-affected daughter with a large expansion. Increased expression correlated with higher levels of the 70-repeat allele (the upregulation was also evident at the protein level). Remarkably, RNA foci and DPR burdens were similar or even significantly increased (in cerebellum) in the unaffected father compared to the daughter with ALS. However, the father did not display TDP-43 pathology and signs of neurodegeneration.

Conclusion

The presence of RNA foci and DPR pathology was insufficient for disease manifestation and TDP-43 pathology in the mosaic *C9orf72* carrier with upregulated *C9orf72* expression. It is important to conduct an investigation of similar cases, which could be found among unaffected parents of sporadic *C9orf72* patients (e.g., 21% among Finnish patients with ALS). Caution should be taken when consulting carriers of small expansions because disease manifestation could be dependent on the extent of the somatic instability in disease-relevant tissues.

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Glossary

ALS = amyotrophic lateral sclerosis; DPR = dipeptide repeat; FTLN = frontotemporal lobar degeneration.

A G₄C₂-repeat expansion in *C9orf72* is the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD)¹⁻³ with 100% penetrance of ALS in men by 86 years of age.⁴ The cascade leading to TDP-43 pathology in degenerating neurons of *C9orf72* carriers is unknown.⁵ Three suggested pathomechanisms arise from the mutation⁶: downregulation of *C9orf72* expression that could lead to *C9orf72* haploinsufficiency, nuclear sense (G₄C₂) and antisense (C₄G₂) RNA foci, and dipeptide repeat (DPR) inclusions (poly-GA, -GP, -GR, -PA, -PR).

Some factors could modify the *C9orf72* phenotype. Intermediate *ATXN2* alleles (27–33 CAG repeats) render susceptibility to ALS,⁷ and the *TMEM106B* A allele of rs1990622 was associated with a later onset,⁸ while homozygosity for the G allele of rs3173615 protects against FTLN.⁹ Size of the expansions may also affect disease severity.¹⁰ Initially, >30 repeats were suggested to be pathogenic^{1,2}; however, several 30- to 70-repeat carriers have been free from disease.¹¹⁻¹³ Small expansions (<90 repeats) are unmethylated, while methylation of the large expansions is unequivocal¹⁴ and associated with ≈50% downregulation of *C9orf72* expression.^{1,2,14-16} In contrast, a 70-repeat expansion increases *C9orf72* expression (by 3-fold vs normal control¹¹). This 70-repeat allele (on the typical ≈200-kb risk haplotype) jumped to pathogenic length (≈1,750 repeats) in the next generation,¹¹ indicating that the expansions have occurred on multiple occasions.^{17,18}

We conducted a comprehensive study of a neurologically healthy carrier of a 70-repeat allele in blood¹¹ who recently came to autopsy at age 90. We investigated *C9orf72* at the DNA, RNA, and protein levels and assessed RNA foci, DPRs, and TDP-43 pathology.

Methods

Standard protocol approvals, registrations, and patient consents

The CNS and peripheral tissues from Canadian PED25 family members (figure 1) were collected in accordance with the ethics review board. We received written informed consent for all study participants.

Human samples

The proband (9686) carried a 70-repeat *C9orf72* expansion in blood DNA,¹¹ was free from symptoms of ALS and FTLN up to age 90 on the basis of an assessment by neurologists (L.Z. and R.S.), and died of chronic obstructive pulmonary disease. Autopsy results for 9686 were compared to his daughter (9548), diagnosed with ALS at age 57, who had

a large expansion (≈1,750 repeats) in all investigated tissues.¹¹

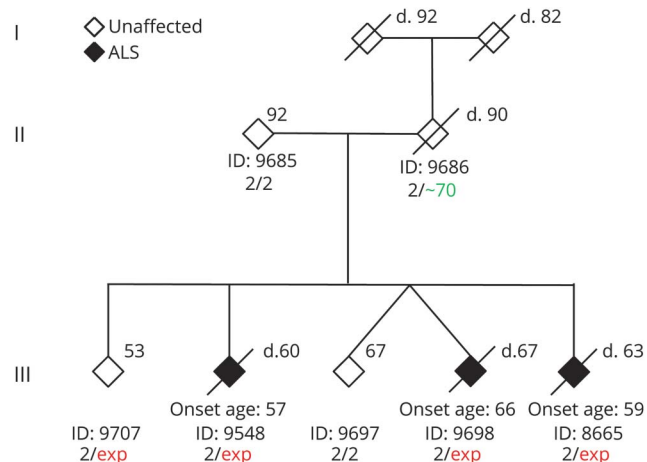
Genetic and epigenetic analyses

C9orf72 was genotyped by repeat-primed PCR as described previously.² Southern blot for 9686 was conducted as reported¹¹ to estimate the size of the expansion in heart, liver, kidney, lung, tongue, testicles, epididymis, thalamus, cervical and thoracic spinal cord, brainstem, cerebellum, and frontal, motor, temporal, and occipital cortices. To study the DNA methylation of *C9orf72*, we used an aliquot from the same DNA preparation as for Southern blot. DNA methylation level at the 5' and 3' regions flanking the G₄C₂ repeat was analyzed by direct bisulfite sequencing.¹⁶ Methylation status of the repeat region was investigated by a (G₄C₂)_n methylation assay.¹⁴ *ATXN2* and *TMEM106B* were genotyped as described previously.¹⁹

Analyses of *C9orf72* expression

Reverse transcription PCR^{11,16} and Western blotting²⁰ were performed as reported (details in appendix e-1, links.lww.com/WNL/A75). Relative quantification of reverse transcription PCR was calculated with the ddCt method after normalization to the reference genes. Gene expression levels were normalized to the level of *C9orf72* expression in

Figure 1 Updated pedigree of Canadian family of British origin (PED25)



C9orf72 genotype is shown beneath the corresponding diamond. Age at the time of examination is shown in the top right corner. Age at death is indicated by the prefix d (the postmortem interval was within 12 hours). Onset age is indicated for individuals with disease above the identification number. The 70-repeat allele carrier (9686) died at age 90. Four of his 5 offspring had large *C9orf72* repeat expansions, including 3 with amyotrophic lateral sclerosis (ALS).

corresponding tissues of 9548. *C9orf72* expression in blood was assessed for family members (9686, 9548, 9697) and normalized to expression in the wild-type allele carrier (9697). Two protein isoforms are produced from *C9orf72* transcript variants: C9-L (54 kDa) and C9-S (23 kDa), with C9-L found predominantly in the urea-soluble fraction and C9-S present in the low- and high-salt Triton X-100 fractions.²⁰ Thus, we extracted protein using the high-salt Triton X-100 buffer (to quantify C9-S), followed by high salt-sucrose buffer, and solubilized the remaining pellet in urea buffer (to quantify C9-L). For Western blotting, we used anti-C9-L or anti-C9-S rabbit polyclonal in-house primary antibodies (densitometric values normalized to β -actin).

RNA foci and DPR analyses

Details for RNA fluorescence in situ hybridization and DPR assessment are given in appendix e-1, links.lww.com/WNL/A75. For quantification, investigators were blinded, and random images were taken from each region. We used 563' TYE-conjugated probes recognizing either the sense or antisense RNA foci. The number of nuclei positive for RNA foci was expressed as a percentage of total nuclei (all cell types). The number of RNA foci per nuclei was also quantified. Primary antibodies for each DPR were mouse anti-GA (MABN889, Millipore, Billerica, MA), rabbit anti-GP (NBP2-25018, Novus, St. Louis, MO), rat anti-GR (MABN778, Millipore), and rabbit anti-PA (Rb8604) and anti-PR (Rb8736) from Dr. Petrucelli's laboratory.

Statistics

A nonparametric Mann-Whitney *U* test was used to compare intensities of the Southern blot bands (70-repeat vs 2-repeat alleles) between CNS and non-CNS tissues. Linear regression was used to evaluate the relationship between the ratio of Southern blot band intensity and the ratio of *C9orf72* expression (9686 vs 9548) in multiple tissues (data meet normal distribution). Statistical analysis was conducted with SPSS (IBM, Armonk, NY), and $p < 0.05$ was accepted as statistically significant. For Western blot, an unpaired 2-tailed *t* test was used (GraphPad Software Inc, La Jolla, CA). Two-tailed *t* tests (GraphPad) were used to determine differences between 9686 and 9548 for burden of RNA foci or DPRs ($p < 0.0125$ and $p < 0.01$ after Bonferroni correction were deemed significant, respectively).

Results

The 70-repeat allele is somatically unstable

Repeat-primed PCR detected an allele with >50 repeats in all examined tissues of 9686 (figure e-1, links.lww.com/WNL/A73). Southern blot of CNS tissues revealed the presence of the 2-repeat and 70-repeat alleles together with a large expansion (most evident in the cerebellum) of a size similar to that of an unrelated *C9orf72*-ALS case, indicative of pathologic length (figure 2, A and B). In non-CNS tissues (figure 2B), a weak signal for large expansions was detected in liver

but not in kidney, heart, lung, tongue, testicles, or epididymis (similar to blood DNA¹¹).

To assess the degree of instability of the small expansion, we quantified the band intensity ratio between the 70-repeat and 2-repeat alleles (a lower ratio indicates a greater number of cells without the 70-repeat allele because of its expansion). CNS tissues had a lower level of the 70-repeat allele than peripheral tissues ($p = 0.001$), especially in cerebellum with only $\approx 2\%$ of the 70-repeat expansion remaining (figure 2C). In contrast, the 70-repeat allele remained stable in most non-CNS tissues with the exception of liver and kidney, which had an estimated loss of the 70-repeat allele by $\approx 74\%$ and $\approx 34\%$, respectively (figure 2C). Hence, 9686 is a mosaic carrier of small and large *C9orf72* expansions, with varying degrees of somatic instability between tissues.

Some reports suggested that ALS/FTLD could be caused²¹ or modified²² by inheritance of variants in several genes. Previously, we excluded additional pathogenic mutations in *SOD1*, *FUS*, and *GRN* in 9548.¹¹ Here, we analyzed *ATXN2* and *TMEM106B* (table e-1, links.lww.com/WNL/A74), which did not explain the phenotypic difference between 9686 and 9548 (e.g., we did not detect intermediate *ATXN2* alleles or homozygosity for the rs3173615 G allele in *TMEM106B*).

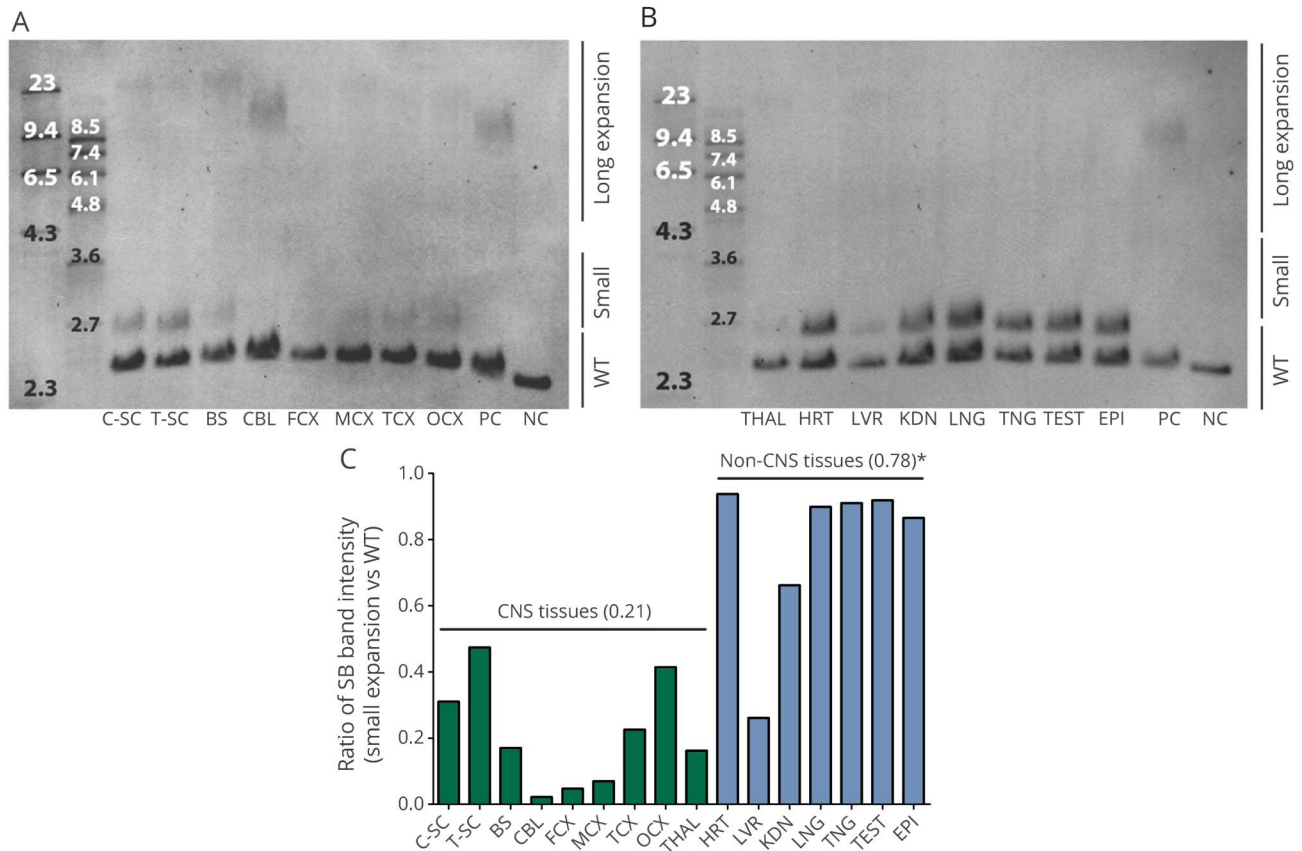
Large expansions trigger DNA methylation of the G₄C₂ repeat

No methylation was detected at CpG islands adjacent to the G₄C₂ repeat for 9686 or 9548 (tables e-2 and e-3, links.lww.com/WNL/A74). In contrast, the G₄C₂ repeat was methylated in all 10 CNS tissues, liver, and kidney of 9686 (figure 3, A and B), where large expansions were identified by Southern blot (figure 2, A and B) or partial loss of the 70-repeat allele. Hence, the (G₄C₂)_n methylation test revealed the presence of a large expansion even in a small fraction of the cells (it could detect methylation in a mixture of DNA standards containing >5% of highly methylated DNA¹⁴).

Small expansion upregulates C9orf72 at the RNA and protein levels

Total *C9orf72* transcript levels from heart (2/70 alleles) and 5 mosaic tissues of 9686 were compared to the same tissues from 9548 (2/1,750 genotype). The mosaic carrier had higher *C9orf72* expression compared to 9548 ($p < 0.05$ for frontal cortex and $p < 0.01$ for other tissues) by 51% in cerebellum, 45% in frontal cortex, 325% in cervical spinal cord, 106% in thoracic spinal cord, 79% in liver, and 440% in heart (figure 4A). Consistent with a previous study,¹¹ 9686 had much higher *C9orf72* expression in blood than 9548 or a noncarrier sibling (9697 with 2/2 genotype) by 530% and 440%, respectively (figure e-2, links.lww.com/WNL/A73). The ratio of *C9orf72* expression (9686 vs 9548) and the ratio of Southern blot band intensity (70-repeat vs 2-repeat alleles) in the investigated tissues fit a linear regression model ($p = 0.046$, $R^2 = 0.67$) (figure 4B), indicating that the small

Figure 2 Southern blots of different tissues from individual 9686



(A) Southern blot of CNS tissues including spinal cord and brain tissues. (B) Southern blot of thalamus and non-CNS tissues. (C) Bar figure showing the ratio of Southern blot band intensity between small expansion and wild-type alleles in different tissues. CNS tissues have a significantly smaller ratio than non-CNS tissues (* $p < 0.05$), suggesting that CNS tissues have a higher instability than non-CNS tissues. BS = brainstem; CBL = cerebellum; C-SC = cervical spinal cord; EPI = epididymis; FCX = frontal cortex; HRT = heart; KDN = kidney; LNG = lung; LVR = liver; MCX = motor cortex; NC = negative control; OCX = occipital cortex; PC = positive control; TCX = temporal cortex; TEST = testicle; THAL = thalamus; TNG = tongue; T-SC = thoracic spinal cord; WT = wild-type.

expansion drives *C9orf72* upregulation. Next, we compared the protein levels of C9-L and C9-S in frontal cortex and cerebellar tissue of 9686 and 9548. C9-L was higher in frontal cortex ($p = 0.04$) and cerebellum ($p = 0.04$) of 9686 vs 9548 (figure 4, C–E); however, no difference was evident in C9-S levels (figure 4, D–F).

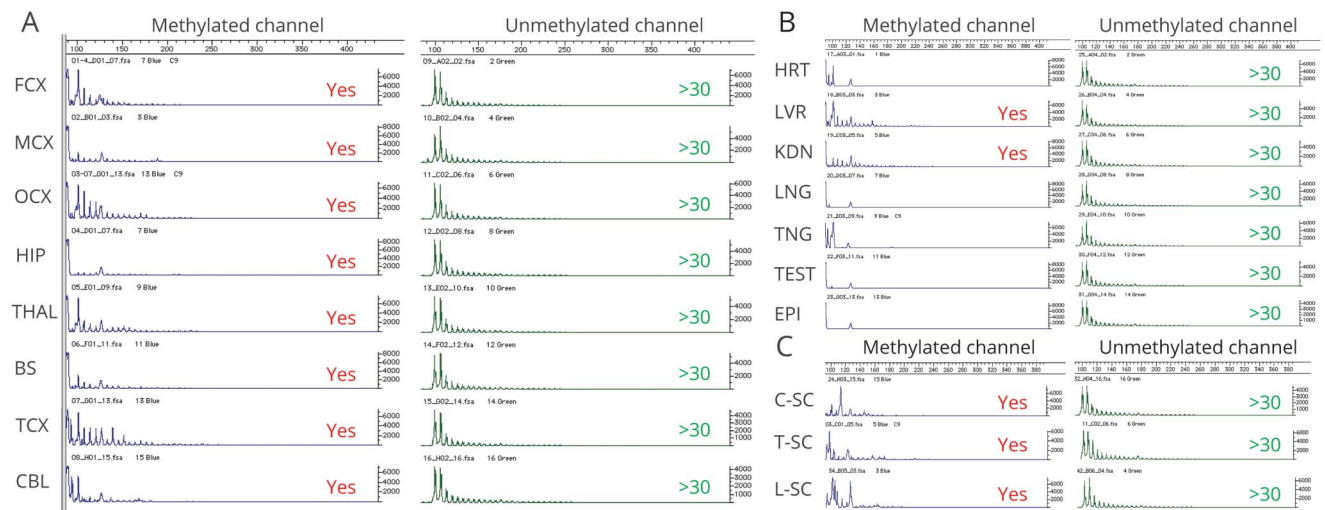
Brain pathology in PED25 family

We quantified the burden of RNA foci in brain regions (figure 5, A–Q) and spinal cord of 9686 and 9548. There was no difference between 9686 and 9548 in the number of nuclei positive for sense RNA foci in frontal cortex ($6.63 \pm 2.24\%$ vs $6.64 \pm 0.42\%$), motor cortex ($10.28 \pm 0.39\%$ vs $9.27 \pm 1.26\%$), or hippocampus ($21.55 \pm 2.06\%$ vs $20.13 \pm 1.07\%$). Remarkably, in cerebellum of 9686, there were twice as many nuclei positive for sense RNA foci compared to ALS-affected 9548 ($21.20 \pm 1.42\%$ vs $11.38 \pm 0.76\%$; $p = 0.0037$) (figure 5I). The burden of antisense RNA foci was similar between 9686 and 9548 (figure 5R). In addition, we assessed the number of RNA foci per nucleus, which suggested similar dynamics of RNA foci production between 9686 and 9548 (figure e-3,

links.lww.com/WNL/A73). Quantification of sense or antisense RNA foci in motor neurons of the cervical and lumbar spinal cord did not reveal any differences between 9686 and 9548 (figure e-4).

All 5 types of DPR inclusions were detectable in brains of 9686 and 9548 (figure e-5, links.lww.com/WNL/A73). Our result is consistent with the reported relative abundance of DPRs in *C9orf72* cases (GA > GP > GR > PA/PR), with PA and PR inclusions being very rare.²³ To determine whether DPR burden is altered in 9686 compared to 9548, we scored the average number of DPR inclusions per field from different brain regions (figure e-5L). In agreement with a prior report,²³ DPR inclusions in the spinal cord were too scarce for quantification. Strikingly, in granule cells of the cerebellum, DPR burden was increased (up to 7 times) in neurologically normal 9686 compared to 9548: GA (205.80 ± 11.62 vs 30.33 ± 4.13 , $p < 0.001$), GP (49.60 ± 6.64 vs 30.50 ± 2.40 , $p < 0.01$), and GR (7.48 ± 0.66 vs 1.00 ± 0.22 , $p < 0.001$, respectively) (figure e-5L). There was no difference in DPR burden in frontal cortex (figure e-5M) or hippocampus

Figure 3 Chromatograms of the (G₄C₂)_n methylation assay in multiple tissues of individual 9686



Methylation and nonmethylation channels detect methylated (blue) or unmethylated (green) repeat alleles. The red “yes” indicates that a methylated repeat expansion was detected. (A) (G₄C₂)_n methylation results in brain DNA. (B) (G₄C₂)_n methylation results in non-CNS DNA. (C) (G₄C₂)_n methylation results in spinal cord DNA. BS = brainstem; CBL = cerebellum; C-SC = cervical spinal cord; EPI = epididymis; FCX = frontal cortex; HIP = hippocampus; HRT = heart; KDN = kidney; LNG = lung; L-SC = lumbar spinal cord; LVR = liver; MCX = motor cortex; OCX = occipital cortex; TCX = temporal cortex; TEST = testicle; THAL = thalamus; TNG = tongue; T-SC = thoracic spinal cord.

(figure e-5N), and although the number of GA inclusions was lower in the motor cortex of 9686 compared to 9548 (3.70 ± 1.77 vs 5.53 ± 2.36 , $p < 0.001$) (figure e-5O), other DPRs did not show differences in this region. Our findings do not rule out DPRs as mediators of toxicity; rather, we consider that alone they are insufficient for neurodegeneration.

The neuropathologic diagnosis of the daughter (9548) was ALS with findings typical for *C9orf72* carriers. This included gliosis and loss of motor neurons from the anterior horn, TDP-43 and p62 inclusions in remaining motor neurons, and degeneration of corticospinal tracts. Neuronal p62 inclusions, some with a starburst morphology, were scattered throughout all cortical regions, the Ammon horn, the entorhinal cortex, and the cerebellar granular layer. There was a normal population of Betz cells, with some containing p62- and TDP-43–positive inclusions. In contrast, the father (9686) did not have any evidence of upper or lower motor neuron degeneration, and there was no TDP-43 pathology in the motor pathway. There was no evidence of FTLTDP-43; the frontal and temporal cortices were not atrophic and did not contain TDP-43 inclusions. The most significant finding was the typical starburst-shaped p62 inclusions encountered in *C9orf72* carriers known to reflect DPRs. These inclusions were observed in the cerebellar granular layer, pyramidal neurons of CA1, and all sampled areas of the cerebral cortex. Other minor coexisting neurodegenerative phenomena included Alzheimer-type neuropathologic changes and scant TDP-43 pathology restricted to the mesial temporal regions (short neurites, neuronal cytoplasmic inclusions in

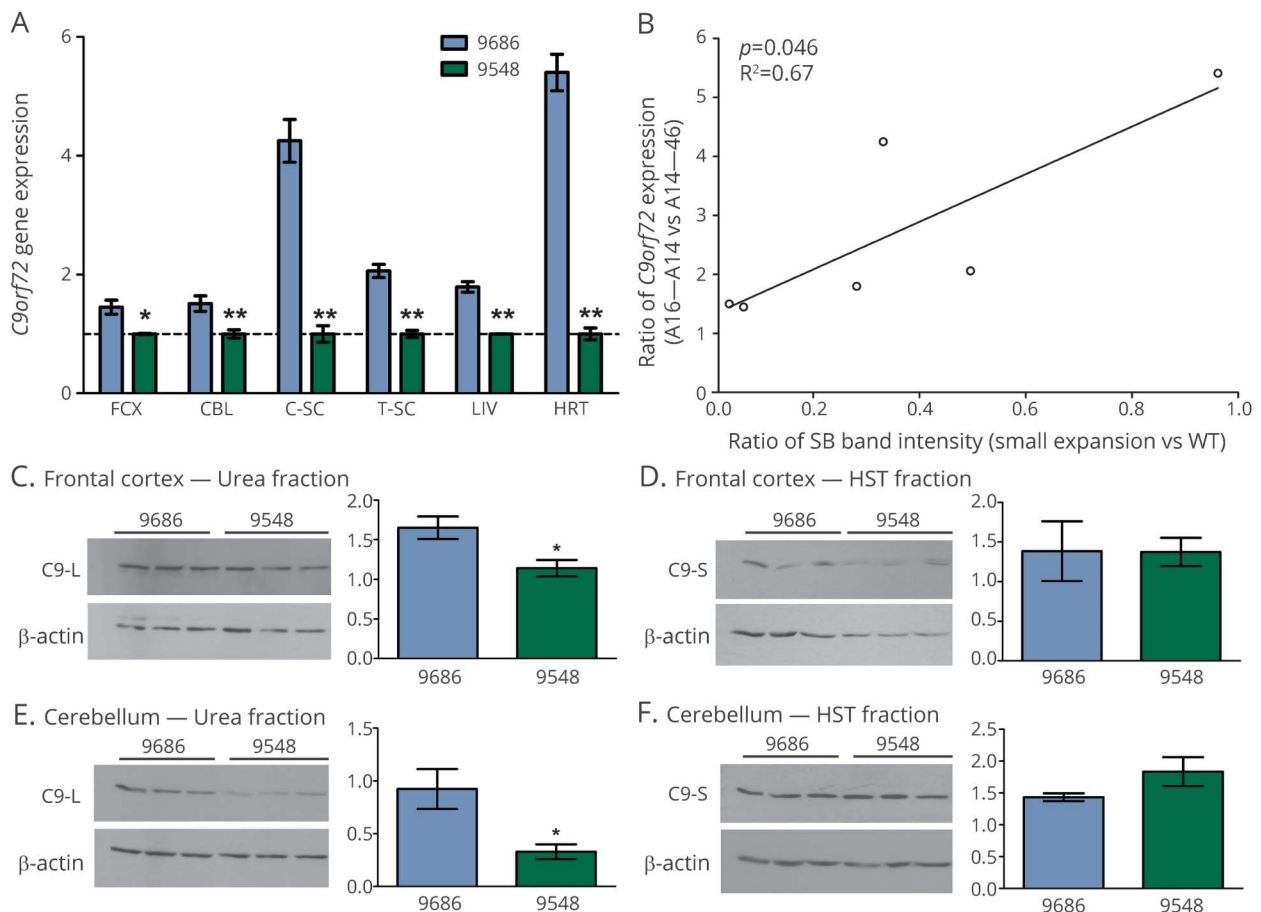
the superficial layers of the entorhinal cortex, and dot-like inclusions of the dentate granular layer).

Discussion

We have conducted a study of a neurologically normal individual (9686) at age 90 carrying a 70-repeat *C9orf72* allele in blood that expanded to a pathogenic range in 4 offspring.¹¹ We were unable to collect a sperm sample from 9686, but the 70-repeat allele likely expanded to $\approx 1,750$ repeats in the germ cells of 9686, rather than arising 4 times separately in each child. Therefore, on the background of the permissive risk haplotype, small expansions (premutations) may jump to pathogenic lengths in the parental generation, which could explain the high frequency of sporadic *C9orf72* patients (e.g., 21% among Finnish patients with ALS^{1,2}). Such a frequency is unusually high for an autosomal dominant disorder, even if family history in some sporadic patients is masked by the early death of parents or complexity of *C9orf72* subphenotypes (e.g., neuropsychiatric symptoms).

Southern blot revealed the mosaicism of small and large expansions in 9686 with a weak signal for large expansions in spinal cord and frontal, temporal, and motor cortices, whereas cerebellum showed mainly large expansion. Somatic instability was described for other small expansions (e.g., 61–92 repeats^{17,18}). However, detailed investigation of *C9orf72* expression and brain pathology (RNA foci, DPRs, TDP-43) in these cases was unreported (table e-4, links.lww.com/WNL/A74). A study of a 30-repeat carrier, unaffected at age 84, did not show repeat instability in the investigated frontal cortex and cerebellum. However, it is unclear if he carried the risk

Figure 4 Relative quantification of *C9orf72* at the RNA and protein levels



(A) We analyzed *C9orf72* expression levels in brain tissues (frontal cortex [FCX] and cerebellum [CBL]), cervical (C-SC) and thoracic spinal cord (T-SC), and non-CNS tissues (liver [LIV] and heart [HRT]) from 9686 (*C9orf72* genotype: 2/70/expansion) and 9548 (*C9orf72* genotype: 2/expansion). Tissues carrying the small expansion had higher *C9orf72* expression than tissues with large expansions ($*p < 0.05$, $**p < 0.01$). The relative quantification of each sample of 9686 was adjusted to that of 9548. Error bars represent the standard error of the triplicates. (B) A correlation analysis to understand the relationship between repeat expansion size and *C9orf72* expression. The ratio of Southern blot (SB) band intensity (70-repeat allele vs wildtype [WT]) in 9686 is significantly correlated with the ratio of *C9orf72* expression (9686 vs 9548) ($p = 0.046$). (C) C9-L levels were examined in the urea fraction of frontal cortex tissue of 9686 and 9548. Three separate samples of tissue were used from each case. C9-L levels were normalized to β -actin and revealed to be significantly higher in 9686 ($*p = 0.044$). (D) C9-S levels were examined from the high-salt Triton X-100 (HST) fraction. No difference was seen between 9686 and 9548. (E) C9-L was significantly increased in the urea fraction of cerebellum of 9686 compared to 9548 ($*p = 0.042$). C9-L was normalized to β -actin, and 3 separate samples were used. (F) C9-S levels in the HST fraction of cerebellum did not differ between 9686 and 9548. Data are mean \pm SEM.

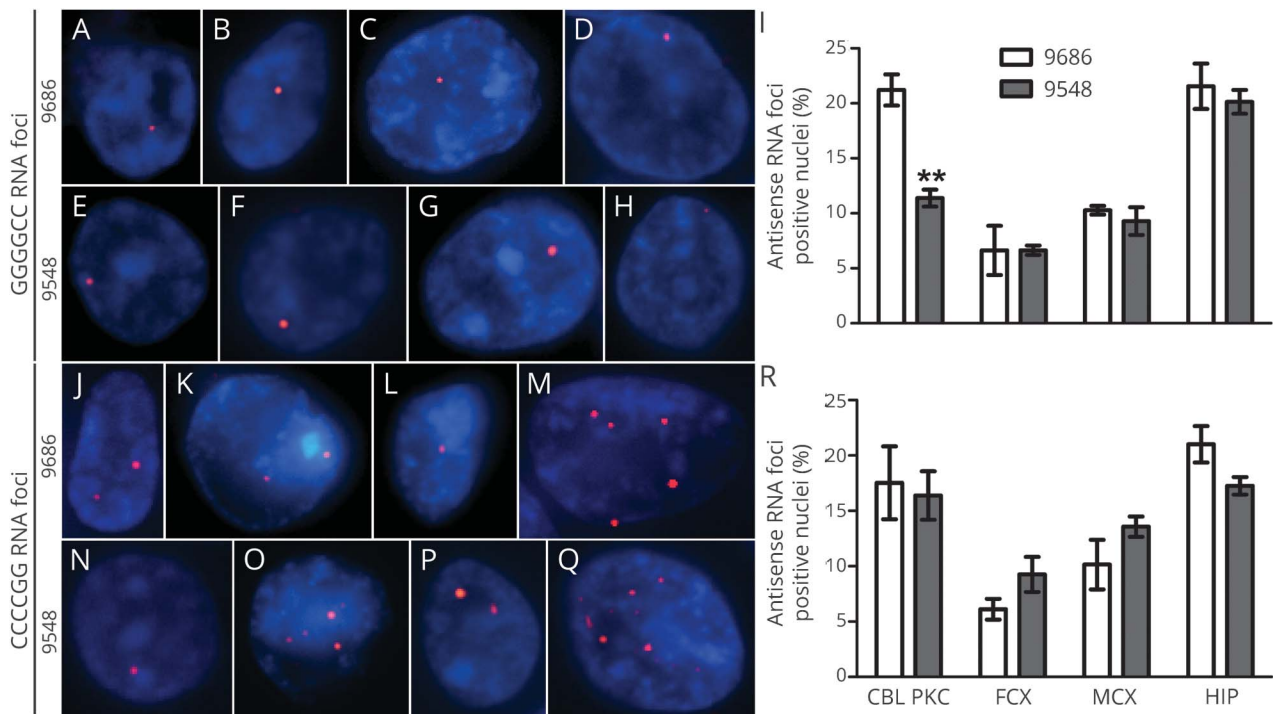
haplotype.¹² Like 9686, this individual had RNA foci and DPRs but lacked TDP-43 pathology. Similar to 9686, non-CNS tissues of reported mosaic cases rarely have large expansions, which may reflect differences in efficiency of DNA repair and cell turnover rate between CNS (lifetime) and non-CNS (days) cells. In contrast to 9686, some mosaic *C9orf72* cases had strong signals for large expansions in ALS/FTLD-relevant regions, leading to a typical disease course.^{10,17,18,24} Thus, the degree of mosaicism in disease-relevant regions may be a key determinant for clinical manifestation.

Toxic effects of RNA foci and DPRs have been demonstrated in model systems; however, the neurodegeneration in *C9orf72* cases does not correlate with the distribution of RNA foci²⁵ or DPRs^{23,26} (only 1 report suggests an association

between cerebellar GP inclusions and cognitive score in a small FTLD cohort²⁷). Similarly, downregulation of *C9orf72* orthologs caused mobility defects in *Caenorhabditis elegans*²⁸ and motor neuron degeneration in zebrafish²⁹; however, knockout mice lack signs of neurodegeneration and show only immune system dysfunction,³⁰ suggesting that *C9orf72* downregulation alone is insufficient to cause disease. However, a recent report of a patient with ALS with a loss-of-function *C9orf72* mutation (p.I201fsX235) supports a role of *C9orf72* haploinsufficiency in ALS.³¹

We have previously shown that the 70-repeat expansion in blood DNA of 9686 is associated with upregulation of *C9orf72*.¹¹ A similar phenomenon was reported for *FMR1* in which unaffected carriers of a premutation (55–200 CGG repeats) have upregulation of *FMR1*, whereas larger

Figure 5 RNA foci analyses in brains of individuals 9686 and 9548



RNA fluorescence in situ hybridization with a probe complementary to the sense-direction sequence (GGGGCC) was used to detect RNA foci (red) in nuclei (blue) of the cerebellum (CBL), frontal cortex (FCX), motor cortex (MCX), and hippocampus (HIP) of 9686 (A–D, respectively) and 9548 (E–H, respectively). Quantification of the total number of nuclei positive for sense RNA foci (I) revealed more sense RNA foci-positive cells in the cerebellum of 9686 compared to 9548 ($n = 3$, $**p < 0.001$); however, there were no differences in other regions. Antisense RNA foci (red, J–Q), detected with a probe complementary to the antisense sequence (CCCCGG), were also seen in the cerebellum, frontal cortex, motor cortex, and hippocampus of 9686 (J–M) and 9548 (N–Q). Quantification of the number of antisense RNA foci-positive nuclei did not reveal any significant differences between 9686 and 9548 (R). $n = 3$ slides per region. Data are mean \pm SEM. PKC = Purkinje and molecular cell layer.

expansions cause fragile X mental retardation syndrome by silencing *FMR1* through hypermethylation.³² Consistent with prior findings,¹¹ tissues from 9686 revealed higher *C9orf72* expression at RNA and protein levels compared to tissues from the ALS-affected daughter (9548). The extent of *C9orf72* upregulation correlated with the level of the 70-repeat allele, suggesting that small expansions drive upregulation, which may offset haploinsufficiency caused by large expansions. Because *C9orf72* plays important roles in nucleocytoplasmic transport, autophagy, intercellular trafficking, and TDP-43 aggregation,^{20,33–38} modulating *C9orf72* expression might be a therapeutic option. Indeed, a recent report found that increased expression of *C9orf72* variant 1 conferred a survival advantage in expansion carriers.³⁹ However, it is also possible that unknown genetic or environmental factors protected 9686 from disease. For instance, DNA methylation analysis of the 353 age-linked CpGs from the genome-wide 450k BeadChip revealed that acceleration of the epigenetic clock in *C9orf72* carriers is significantly correlated with a shorter disease duration and earlier age at onset.¹⁹ Notably, 9686 was aging more slowly than other PED25 family members (his epigenetic age was ≈ 9 years younger than his chronologic age).¹⁹ However, it currently is not clear whether the epigenetic clock reacts to aging or causes aging.

The striking difference between the mosaic case (9686) and the large expansion carrier (9548) is the absence of ALS/FTLD symptoms and TDP-43 pathology in 9686. Burdens of RNA foci and DPRs were similar between 9686 and 9548, except for the cerebellum, which surprisingly had more DPR inclusions and sense RNA foci in the neurologically normal father compared to the daughter with ALS. Our results suggest that RNA foci and DPRs alone are insufficient to drive neurodegeneration or require additional stressors such as downregulation of *C9orf72* expression (figure e-6, links.lww.com/WNL/A73). Although a recent study reported that small and large DPRs have differential effects in vitro,⁴⁰ because DPRs are not associated with regions of neurodegeneration in carriers of large expansions,^{23,26} it is possible that the absence of disease in 9686 is due to upregulated *C9orf72* expression rather than fewer long DPRs. This is in agreement with the observation that the highest *C9orf72* expression among CNS tissues of unaffected individuals was detected in the cerebellum,¹ a region with high abundance of DPRs in *C9orf72* cases but no degeneration.^{23,26}

Currently, there are only limited data on the properties of small expansions, yet they may provide important knowledge on *C9orf72*-related disease: of the 3 potential pathogenic

mechanisms, only downregulation of *C9orf72* expression was absent in the unaffected father. This individual lacked signs of ALS or FTLT, but the burden of RNA foci and DPRs was similar to that of an ALS-affected daughter with large expansion. Because the current study is limited to a single family, it is important to investigate other small expansion carriers who could be identified among unaffected parents of sporadic *C9orf72* cases, especially frequent in the Finnish population.^{1,2} We suggest that caution should be taken when consulting carriers of small *C9orf72* expansions in blood because disease manifestation may be dependent on the extent of the somatic instability in disease-relevant CNS tissues.

Author contributions

Dr. McGoldrick was responsible for the tissue collection and processing, Western blotting, immunohistochemistry and RNA fluorescence in situ hybridization experiments, and subsequent analysis. Dr. Zhang was responsible for DNA methylation and RNA quantification of *C9orf72* and Southern blot analysis. Dr. van Blitterswijk conducted Southern blotting. Ms Sato and Ms Moreno participated in DNA/RNA preparation. Ms Zhang assisted in immunohistochemistry, and Dr. Xiao assisted in Western blotting and *C9orf72* antibody generation. Mr McKeever, Dr. Weichert, and Dr. Schneider participated in tissue collection and processing. Dr. Keith carried out pathologic analysis. Dr. Petrucelli provided reagents. Dr. Zinman was responsible for obtaining clinical findings and editing the manuscript. Dr. Rademakers contributed to Southern blotting experiments and editing the manuscript. Dr. Robertson and Dr. Rogaeva supervised the project and wrote the manuscript with Dr. McGoldrick and Dr. Zhang.

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Unaffected mosaic *C9orf72* case

RNA foci, dipeptide proteins, but upregulated *C9orf72* expression

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Study question

What roles do small *C9orf72* expansions have in amyotrophic lateral sclerosis (ALS)?

Summary answer

Small expansions in *C9orf72* cause RNA foci and dipeptide repeat protein (DPR) pathology, but in contrast to large expansions, there is higher *C9orf72* RNA and protein expression, and this may act to preclude ALS manifestation.

What is known and what this paper adds

Large repeat expansions in *C9orf72* are the most commonly known cause of ALS, but the possible pathogenic role of small expansions (30–70 repeats) is unclear. This study of an individual carrying a 70-repeat allele elucidates the neuropathologic effects of such repeats.

Participants and setting

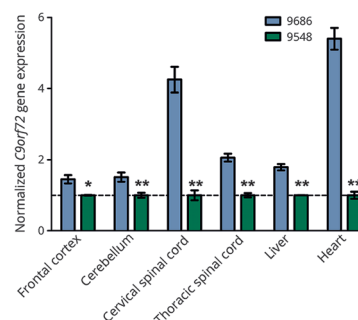
The proband was a neurologically normal white individual, deceased at 90 years of age, who carried a 70-repeat *C9orf72* expansion in blood DNA. The proband was compared at autopsy to family members, including an offspring who carried an ≈1,750-repeat expansion and had ALS.

Design, size, and duration

The study used repeat-primed PCR to investigate *C9orf72* genotypes and Southern blotting to evaluate organ-specific expansion sizes in the proband. Reverse transcription PCR was used to quantify tissue-specific *C9orf72* expression, Western blotting to determine the expression of *C9orf72* protein isoform, in situ hybridization to study the presence of RNA foci, and immunohistochemistry to study DPRs.

Main results and the role of chance

C9orf72 alleles with >50 repeats were found in all of the proband's tissues. Southern blotting revealed large *C9orf72* expansions that were similar in size to those observed in the ALS-affected offspring. It also revealed 2- and 70-repeat alleles, but the ratio significantly differed between the CNS and peripheral tissues ($p = 0.001$), with larger expansions being more prevalent in the CNS. The proband's *C9orf72* expression levels were higher than the offspring's levels ($p < 0.05$ for various tissues). The proband also had higher CNS



expression levels of the *C9orf72* protein than the offspring ($p = 0.04$). Compared to the offspring, the proband had similar numbers of sense and antisense RNA foci-positive nuclei in most brain regions but had twice as many sense RNA foci in the cerebellum ($p = 0.0037$). The burden of DPR pathology was broadly similar across all brain regions.

Bias, confounding, and other reasons for caution

It is unclear what factors might have been protecting this individual from developing ALS, so the therapeutic implications of these results are unclear.

Generalizability to other populations

This study focused on a single individual and his family, so the results may not be generalizable. However, it highlights the need to further characterize autopsy tissues from unaffected parents carrying *C9orf72* small or mosaic expansions, whose offspring carrying large expansions are disease affected, to uncover the roles of RNA foci, DPRs, and *C9orf72* expression in contributing to disease manifestation.

Study funding/potential competing interests

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