Jump from Pre-mutation to Pathologic Expansion in *C9orf72*

Zhengrui Xi,[1,9] Marka van Blitterswijk,[2,9] Ming Zhang,[1,9] Philip McGoldrick,[1] Jesse R. McLean,[1] Yana Yunusova,[3,4] Erin Knock,[1] Danielle Moreno,[1] Christine Sato,[1] Paul M. McKeever,[1,5] Raphael Schneider,[1,5] Julia Keith,[3] Nicolae Petrescu,[3,6] Paul Fraser,[1] Maria Carmela Tartaglia,[1,6] Matthew C. Baker,[2] Neill R. Graff-Radford,[7] Kevin B. Boylan,[7] Dennis W. Dickson,[2] Ian R. Mackenzie,[8] Rosa Rademakers,[2] Janice Robertson,[1,5] Lorne Zinman,[3,6,*] and Ekaterina Rogaeva[1,6,*]

An expanded G_4C_2 repeat in *C9orf72* represents the most common known genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). However, the lower limit for pathological expansions is unknown (the suggested cutoff is 30 repeats). It has been proposed that the expansion might have occurred only once in human history and subsequently spread throughout the population. However, our present findings support a hypothesis of multiple origins for the expansion. We report a British-Canadian family in whom a ~70-repeat allele from the father (unaffected by ALS or FTLD at age 89 years) expanded during parent-offspring transmission and started the first generation affected by ALS (four children carry an ~1,750-repeat allele). Epigenetic and RNA-expression analyses further discriminated the offspring's large expansions (which were methylated and associated with reduced *C9orf72* expression) from the ~70-repeat allele (which was unmethylated and associated with upregulation of *C9orf72*). Moreover, RNA foci were only detected in fibroblasts from offspring with large expansions, but not in the father, who has the ~70-repeat allele. All family members with expansions were found to have an ancient known risk haplotype, although it was inherited on a unique 5-Mb genetic backbone. We conclude that small expansions (e.g., 70 repeats) might be considered "pre-mutations" to reflect their propensity to expand in the next generation. Follow-up studies might help explain the high frequency of ALS- or FTLD-affected individuals with an expansion but without a familial history (e.g., 21% among Finnish ALS subjects).

It is now recognized that amyotrophic lateral sclerosis (ALS [MIM: 612069]) and frontotemporal lobar degeneration (FTLD [MIM: 600274]) have significant clinico-pathological overlap. ALS symptoms are caused by the degeneration of motor neurons in the cerebral cortex, brainstem, and spinal cord and lead to paralysis and death, most commonly as a result of respiratory failure;[1] FTLD symptoms are caused by the degeneration of the frontal and temporal lobes of the cerebral cortex and lead to a behavioral and/or language disorder.[2] Several overlapping genes have been associated with ALS and FTLD, providing strong evidence of common pathological mechanisms.[3] An expanded G_4C_2 repeat in the non-coding region of *C9orf72* (MIM: 614260) represents the most common known genetic cause of both ALS and FTLD in populations of European ancestry.[4,5] It has been detected in up to 29% of FTLD and 50% of ALS subjects, as well as 88% of subjects with both FTLD and ALS symptoms, including familial and simplex individuals.[4,6,7]

The size of expansions is very variable, and whether these alleles have the same pathological significance is unknown. Even the lower limit for pathological expansions is not yet established, and the majority of studies use the originally suggested cutoff of 30 repeats.[4,5] Because most of *C9orf72* genotyping is done by repeat-primed PCR (rp-PCR), the exact size of the expansion cannot be determined beyond a certain length (i.e., when it is larger than 50–70 repeats). When measured by Southern blot, the estimated size of large expansions can vary from several hundred to thousands of repeats;[8–10] however, some individuals show relatively small expansions (30–150 repeats).[6,10,11] Whether these small expansions have pathogenic significance needs to be studied carefully. For example, we reported a Parkinson-disease-affected individual who carries 39 repeats that do not segregate with disease.[6]

The suggested *C9orf72*-related disease mechanisms are complex. One possibility is loss of function through haploinsufficiency, supported by the fact that individuals with expansions show ~50% fewer *C9orf72* transcripts,[4,5] possibly as a result of epigenetic alterations. Indeed, we previously demonstrated that DNA hypermethylation of a CpG island adjacent to the 5′ end of the G_4C_2 repeat is expansion specific.[12,13] Moreover, a higher degree of methylation was associated with familial ALS and shorter disease duration.[12] Given that hypermethylation was only

[1]Tanz Centre for Research in Neurodegenerative Diseases, University of Toronto, 60 Leonard Street, Toronto, ON M5T 2S8, Canada; [2]Department of Neuroscience, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, USA; [3]Sunnybrook Health Sciences Centre, 2075 Bayview Avenue, Toronto, ON M4N 3M5, Canada; [4]Department of Speech Language Pathology, University of Toronto, 500 University Avenue, Toronto, ON M5G 1V7, Canada; [5]Department of Laboratory Medicine and Pathobiology, University of Toronto, 27 King's College Circle, Toronto, ON M5S 1A1, Canada; [6]Division of Neurology, Department of Medicine, University of Toronto, 1 King's College Circle, Toronto, ON M5S 1A8, Canada; [7]Department of Neurology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, USA; [8]Department of Pathology and Laboratory Medicine, University of British Columbia, 2329 West Mall, Vancouver, BC V6T 1Z4, Canada
[9]These authors contributed equally to this work
*Correspondence: lorne.zinman@sunnybrook.ca (L.Z.), ekaterina.rogaeva@utoronto.ca (E.R.)
http://dx.doi.org/10.1016/j.ajhg.2015.04.016.

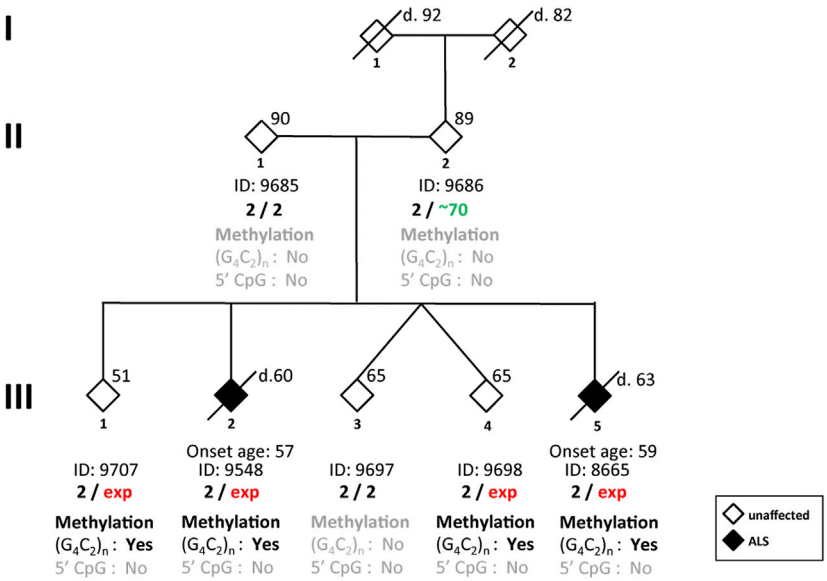


Figure 1. Pedigree of Family PED25
Individual ID and *C9orf72* genotype are shown beneath the corresponding diamond. Arabic numbers indicate the repeat number, and "exp" represents the expansion allele. The age at time of examination is shown in the upper right corner. The age of death is indicated by a prefix of "d." The age of onset is indicated for individuals with disease above the ID number. For protecting confidentiality, the gender of family members is masked. The results of methylation analyses for the 5′ CpG island and for $(G_4C_2)_n$ methylation are shown beneath the *C9orf72* genotype. "Yes" represents the presence of methylation, and "no" represents the absence of methylation.

detected in a portion of individuals with expansions (36% in ALS), we recently tested whether the G_4C_2 expansion itself could be the main methylation site. Using a qualitative assay, we demonstrated that the G_4C_2 expansion is generally methylated in 100% of unrelated individuals with alleles of >90 repeats, whereas the small or intermediate alleles (2–90 repeats) are completely unmethylated.[14]

The search for the origin of the *C9orf72* repeat expansion identified an ~200-kb risk haplotype (known as the "Finnish" risk haplotype) in all individuals with expansions.[15–19] It led to the common founder hypothesis proposing that the expansion occurred as a single event and subsequently spread throughout the population.[18,20] In contrast, a multiple-origins hypothesis suggests that the risk haplotype formed a permissive allele associated with repeat instability and predisposed the G_4C_2 repeat to expand to a pathogenic allele on multiple occasions in human history.[20,21] However, only a limited number of *C9orf72* studies have used Southern blotting—in which a wild-type allele (2–30 repeats) gives a single band and large expansions appear as a smear—to size the expansion. Somatic instability can lead to very different repeat sizes within and among tissues of the same individual.[9] This was recently confirmed in a report of an ALS subject with ~3,000 repeats in CNS tissues but only ~90 repeats in blood.[21] Of note, this individual had the same risk haplotype as other subjects with *C9orf72* repeat expansions, supporting the permissive nature of an ~90-repeat allele to expand.

Observations of somatic instability of the G_4C_2 repeat are in favor of multiple origins of expansions, but direct evidence of this hypothesis could come from the investigation of unaffected parents of subjects with *C9orf72* repeat expansions and affected by ALS or FTLD. In the current genetic and epigenetic study, we present a unique family in whom the repeat stretches from small to large expansions when passed to the offspring (the first generation affected by ALS).

In accordance with the ethical review board, informed consent was obtained from all study participants. PED25 is a Canadian family of British origin (Figure 2). The parents, 9685 (II-1) and 9686 (II-2), had five children, two of whom, 9548 (III-2) and 8665 (III-5), were diagnosed with ALS at Sunnybrook Health Sciences Centre according to the revised El Escorial Criteria.[1] Both parents (90 and 89 years old) are alive and, according to neurological assessments, do not show symptoms of ALS or FTLD. The detailed clinical findings can be found in the Supplemental Data ("Clinical Descriptions").

Samples of blood DNA were collected from seven family members in two generations (Figure 1). The *C9orf72* G_4C_2 repeat was genotyped by a two-step strategy as described previously[6] with minor modifications. In the first step, undiluted PCR products were resolved on a 3100 DNA analyzer (amplicon-length analysis). In the second step, rp-PCR was used for determining the presence of large expansions.[4,5]

The rp-PCR revealed repeat expansions in five family members, including the unaffected 89-year-old father (9686 [II-2]) and his four children, two of whom are affected by ALS (Figures 1 and 2). Notably, the amplicon-length analysis revealed that the father has a small expansion of ~70 repeats, whereas his children have larger expansions beyond a detectable range (Figure 2). Issues of paternity were excluded by genotyping for eight randomly selected autosomal short tandem repeat (STR) markers (D2S2166, D5S406, D7S493, D10S1651, D11S908, D15S978, D18S452, and D21S266; data not shown). Sanger sequencing of other ALS- and FTLD-associated genes (*SOD1*, *FUS*, and *GRN*) in the ALS-affected proband (9548 [III-2]) was performed as reported previously[22] and did not reveal additional pathogenic mutations. So far, no clinical sign of ALS or dementia was found for two of four children with a large expansion: 9698 (III-4; age 65 years) and 9707 (III-1; age 51 years). This is most likely

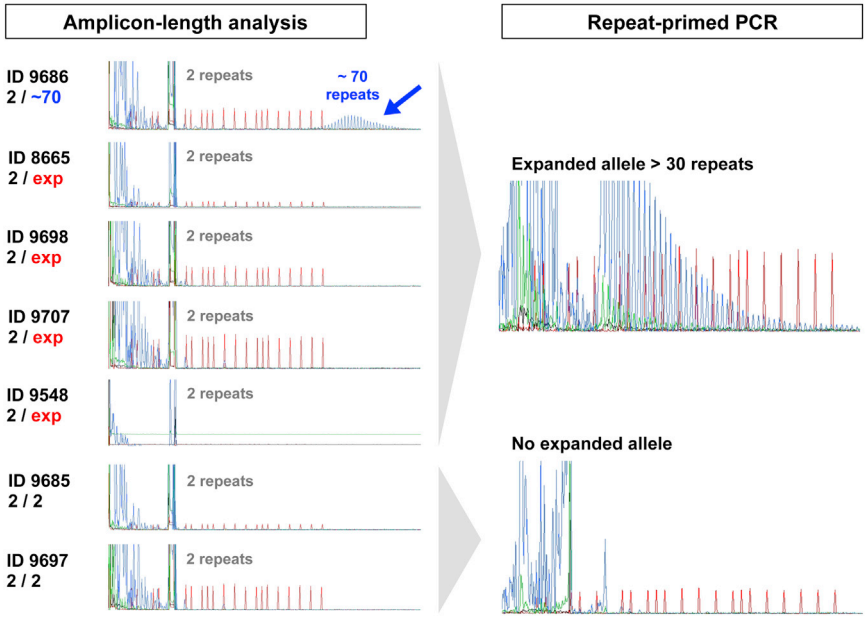


Figure 2. Results of *C9orf72* Genotyping
PCR products of amplicon-length analysis and rp-PCR were separated on an ABI3100 DNA Analyzer and visualized by GeneScan software. Although five family members were identified to have expansions in the rp-PCR, only individual 9686 had a small expansion of ~70 repeats.

due to the high clinical heterogeneity, including a variable age of onset, in *C9orf72*-related diseases. For instance, we previously reported a pair of monozygotic twins who both have large expansions and have been discordant for ALS for at least 6 years.[23] However, the penetrance of the pathological expansion in *C9orf72* reaches ~100% by age 80 years.[15,24]

To estimate the size of the G_4C_2 repeat, we conducted Southern blotting as previously described.[9] In brief, a total of 7–10 μg of DNA was digested with XbaI (Promega) and electrophoresed on 0.8% agarose gel. Subsequently, DNA was transferred to a positively charged nylon membrane (Roche), cross-linked, and then hybridized with a digoxigenin (DIG)-labeled probe. Expansions were visualized with anti-DIG antibody (Roche) and CDP-star substrate (Roche) on X-ray film. DNA sufficient for Southern blotting of six PED25 family members (not 8665) was obtained. For one deceased family member (9548), DNA was also obtained from multiple autopsy tissues (cerebellar vermis, orbitofrontal cortex, primary motor cortex, superior temporal gyrus, hippocampus, and medulla dissected from the fresh right hemisphere, as well as the spinal cord, heart, kidney, liver, and skin). In addition, Southern blotting was done for fibroblasts derived from skin biopsy of three family members with large expansions (9698, 9707, and 9548). The fibroblasts were generated from 3-mm dermal explants and cultured in 6-well plates pre-coated with 0.1% gelatin. Cells were maintained in DMEM with high glucose (11995, Invitrogen), supplemented with 20% (v/v) fetal bovine serum (GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO). Cells were then maintained at 37°C and 5% CO_2 in a humidified incubator.

Southern blot analysis of blood DNA confirmed the substantial difference in expansion size between the two generations. The father showed two distinct bands corresponding to a wild-type allele (two repeats) and a small expansion (~70 repeats). In contrast, four of his children demonstrated a large smear similar in length, and the most abundant expansion sizes were ~650 and ~1,750 repeats (Figure 3). Large expansions were also revealed in all investigated autopsy tissues of ALS-affected individual 9548, in whom repeat numbers over 3,500 were detected in CNS tissues; in contrast, the cerebellum showed smaller expansions (~1,750 repeats; Figure 4), a general phenomenon observed in subjects with *C9orf72* repeat expansions.[9] Fibroblasts showed a relatively small expansion as a well-defined band (~750 repeats; Figure 4). In the liver, we observed an expansion band corresponding to ~3,000 repeats; in other non-CNS tissues, we detected a higher level of somatic instability (not shown) comparable to that seen in blood (Figure 4).

To study the epigenetic profile of *C9orf72* in family PED25, we used an aliquot from the same DNA preparation as for Southern blot. In addition to generating fibroblasts from 9698, 9707, and 9548, we generated fibroblasts for two more family members (9697 and 9686) and two unrelated healthy control individuals.

The methylation level of the CpG island 5′ to the G_4C_2 repeat was estimated via direct bisulfite sequencing as described previously.[12] In brief, each DNA sample was sequenced after bisulfite conversion, after which all unmethylated C nucleotides were converted to T nucleotides, and methylated C remain unchanged. A total of 26 CpG sites were studied at the CpG island 5′ to the G_4C_2 repeat and were classified as either unmethylated (T peak) or methylated (T-C double peaks). None of the family members demonstrated increased DNA methylation at the CpG island 5′ to the repeat in blood (Figure 1).

Methylation of the G_4C_2 repeat itself was studied with our recently reported qualitative $(G_4C_2)_n$-methylation assay.[14] In brief, each sample was amplified by rp-PCR with primers specific to methylated or unmethylated DNA after bisulfite conversion. The primers were labeled by FAM for amplification of methylated DNA (blue channel) and HEX for amplification of unmethylated DNA (green channel). Data were visualized by Genotyper Software (version 3.6, Applied Biosystems). If the G_4C_2 repeat was methylated in all DNA copies, only the blue channel was expected to have products, whereas if the G_4C_2 repeat was unmethylated in all DNA copies, only the green

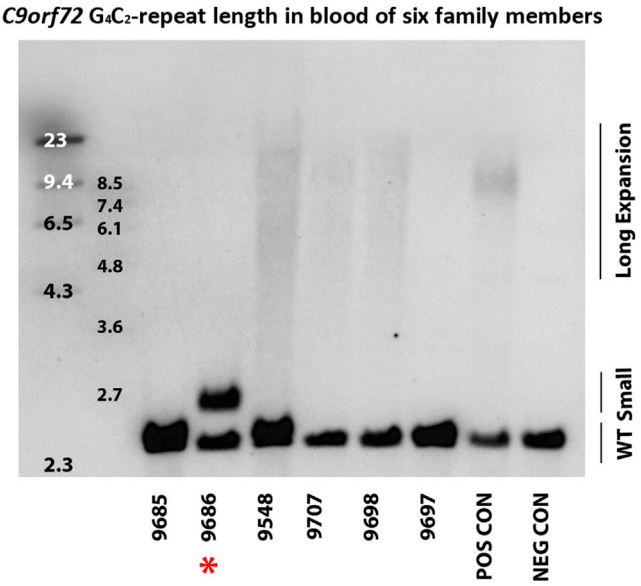


Figure 3. Southern Blot Results from Members of Family PED25
Southern blot analysis using blood DNA confirmed the presence of a small expansion in 9686 (highlighted by the red star), the presence of large expansions in 9698, 9707, and 9548, and the absence of an expansion in 9685 and 9697. The molecular-weight marker is shown on the left side of the Southern blot. "POS CON" and "NEG CON" refer to positive and negative control individuals, respectively.

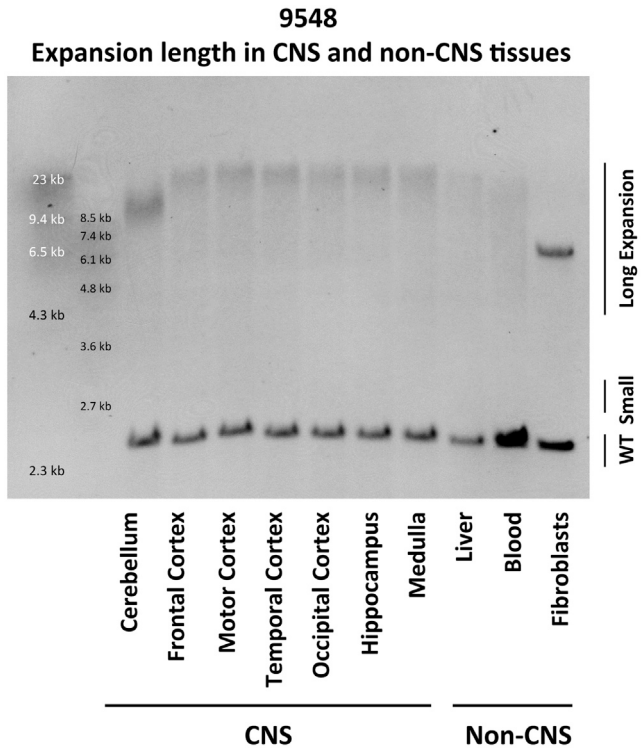


Figure 4. Southern Blot Results from Autopsy Tissues of Individual 9548
Southern blot analysis using genomic DNA extracted from autopsy tissues confirmed the presence of large expansions in CNS and non-CNS tissues from individual 9548.

channel was expected to have products. If the G_4C_2 repeat was methylated in some DNA copies, both channels would have products. We used a yes/no test to evaluate the methylation of ~30 repeats at the 5′ end of the expansion (this test was sensitive enough to detect methylation in a mixture of DNA standards containing only 5% highly methylated DNA).[14]

The $(G_4C_2)_n$-methylation assay demonstrated that all four children with large expansions had CpG methylation of the G_4C_2 repeat itself (Figures 1 and 5). The autopsy tissues of 9548 were also shown to have methylation of the expanded G_4C_2 repeat (Figure S1). In contrast, $(G_4C_2)_n$ methylation was not detected in the blood DNA of the two family members without an expansion (9685 and 9697) or of the father (9686), who has the ~70-repeat allele (Figure 5). Fibroblasts generated from the skin of the father did not show $(G_4C_2)_n$ methylation either (Figure S2). Surprisingly, fibroblasts from all three family members with large expansions (9698, 9707, and 9548) also did not have $(G_4C_2)_n$ methylation (Figure S2). Importantly, for the deceased individual (9548), we obtained enough skin DNA, which clearly demonstrated $(G_4C_2)_n$ methylation (Figure S1B). Our results suggest that *C9orf72*-related phenotypes in fibroblast-based functional studies should be interpreted with caution because the epigenetic profile has been reset in such cell lines.

For assessing *C9orf72* expression, total RNA was extracted from blood with the RNeasy Mini Kit (QIAGEN) and reverse transcribed to cDNA with oligo dT primers and the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies). RNA integrity was checked on an Agilent 2100 Bioanalyzer, and only samples with an RNA integrity number > 7 were used. Real-time PCR was performed on an ABI Prism 7500 System (Life Technologies). We used inventoried TaqMan expression assays for *C9orf72* (Hs00376619_m1) and five housekeeping genes including *HPRT1* (MIM: 308000; Hs99999909_m1), *UBC* (MIM: 191340; Hs00824723_m1), *B2M* (MIM: 109700; Hs99999907_m1), *GAPDH* (MIM: 138400; Hs00266705_g1) and *TBP* (MIM: 600075; Hs00427620_m1) (Applied Biosystems). Samples were run in triplicate. All tested housekeeping genes were evaluated for expression stability via the geNorm algorithm incorporated in the software qbase$^+$ (Biogazelle),[25] which determined *HPRT1*, *UBC*, and *B2M* to be the suitable reference genes for the blood samples and *GAPDH* and *UBC* to be suitable reference genes for the fibroblasts samples. Relative quantification was calculated with the ddCt method after normalization to the corresponding reference genes.

The inventoried TaqMan gene-expression assay detected all three known *C9orf72* transcripts in blood from five family members, four of whom (in addition to two normal control individuals) also had fibroblasts available to be tested (Figure 6). Expression of *C9orf72* was correlated with the status of $(G_4C_2)_n$ methylation. Blood RNA from family members with methylated large G_4C_2-repeat expansions (n = 3) showed less gene

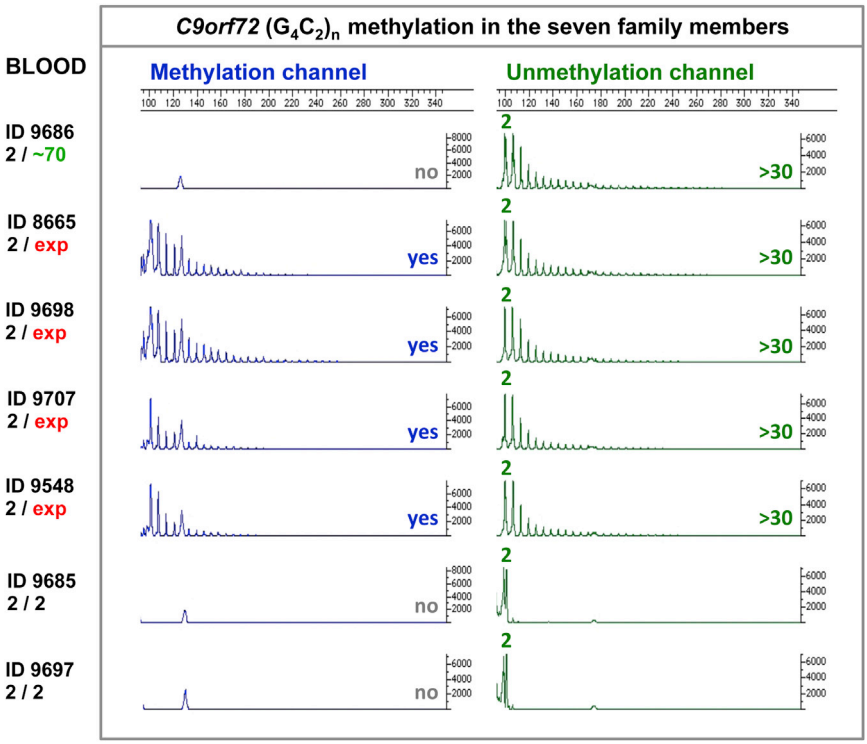


Figure 5. Chromatograms of the $(G_4C_2)_n$-Methylation Assay in Blood Samples from Family PED25

Methylated and unmethylated G_4C_2-repeat alleles were detected by a methylation channel and a non-methylation channel, respectively. Detectable repeat size was marked along with the corresponding peak.

expression than did blood RNA from family members with normal alleles (n = 2). In contrast, fibroblast DNA from the same three individuals with large expansions was not methylated at the G_4C_2 repeat and expressed *C9orf72* at a level similar to that in fibroblast DNA from individuals with normal alleles (Figure 6). Surprisingly, *C9orf72* mRNA levels were higher in the blood and fibroblasts of the father (who has the unmethylated 70-repeat allele) than in those of the control individuals (Figure 6). A follow-up test confirmed that his *C9orf72* expression in blood cells was higher than that in three additional unrelated normal control individuals (9446, 9593, and 9600; Figure S4).

To assess RNA foci in individuals with wild-type alleles and G_4C_2 expansions, we carried out RNA fluorescence in situ hybridization on fibroblasts by using a locked nucleic acid probe recognizing the sense strand of the repeat expansion in *C9orf72* according to previously published protocols[26,27] (Figure S5). Fibroblasts from an unrelated healthy control individual were examined alongside those of the PED25 family members, including 9697 (with normal alleles), 9686 (with the 70-repeat allele), and 9698, 9707, and 9548 (with large expansions). RNA foci were detected in the fibroblasts from all three individuals with large expansions: 9698 (26.0% of cells, n = 3 experiments), 9707 (20.4% of cells, n = 3 experiments), and 9548 (27.5% of cells, n = 3 experiments) (Figure S5). These values are comparable to those in a published report, which found that 15%–45% of fibroblasts from subjects with expansions were positive for sense-strand RNA foci.[26] In contrast, no or negligible levels of RNA-foci-positive cells were detected in fibroblasts from 9686, who has the 70-repeat allele (0.006% of cells, n = 2 experiments); similar results were observed for the control individual (0% of cells, n = 3 experiments) and 9697 (0% of cells, n = 3 experiments) (Figure S5). These results further support that the 70-repeat allele is not likely to be pathogenic.

To examine whether PED25 members share a haplotype, we studied six STR markers (D9S171, D9S1679, D9S259, D9S2154, D9S161, and D9S319) spanning a 5-Mb region flanking *C9orf72*. STR markers were amplified by PCR with one FAM-labeled primer, diluted, and analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems). Alleles were scored with GeneMapper Software version 5 (Applied Biosystems) and normalized to CEPH standard genotypes. STR genotypes were obtained for six members of family PED25 and two expansion-affected individuals from the published family VSM-20.[4] In addition, we genotyped three unrelated persons with small expansions in *C9orf72* and 11 subjects with repeats in the long wild-type range (21–30 repeats). The details of all STR assays are available upon request. TaqMan SNP-genotyping assays were performed for rs4879515 (C_31009935_20) and rs3849942 (C_27515934_20; Thermo Fisher Scientific) on a 7900HT Fast Real-Time PCR System; genotype calls were made with SDS 2.4 software (Applied Biosystems).

Haplotype analysis showed that the father (9686, who has the small expansion) and his children with large expansions (9548, 9707, and 9698), but not family members without expansions (9685 and 9697), share a 5-Mb haplotype flanking *C9orf72* (Table 1). Interestingly, STR alleles in PED25 family members with expansions were different from those observed in family VSM-20[4] (Table 1) or in any of the investigated subjects with small expansions or with repeats in the long wild-type range (Table S1 and Figure S3). Assessment of SNPs mapping within 100 kb of the expansion and tagging the reported "Finnish" *C9orf72* risk haplotype[4] revealed that the father and his children with large expansions do share a much smaller haplotype with family VSM-20. We also detected the same risk haplotype in subjects with small expansions and subjects with repeats in the long wild-type range, including control individuals (Table S1).

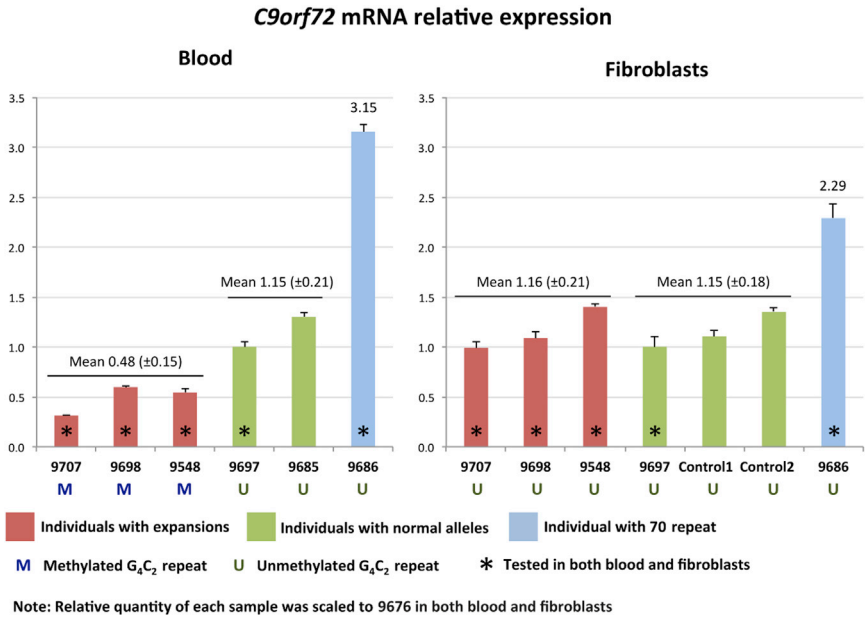


Figure 6. Relative Quantification of *C9orf72* mRNA in Blood and Fibroblast Samples from Family PED25

Both blood and fibroblasts of individuals with expansions (9548, 9698, and 9707) were tested. We also tested blood of individuals with normal alleles (9685 and 9697), as well as fibroblasts of 9697 and two normal control individuals. The relative quantity of each sample was scaled to that of individual 9676. Error bars represent the SE of the triplicate reactions.

In summary, we report a British-Canadian family in whom a paternal ~70-repeat allele in *C9orf72* expanded considerably in the next generation and passed large G_4C_2 expansions (~1,750 repeats) on to four children. Notably, it is possible that an individual can carry a small expansion in blood but a large expansion in the CNS.[9] Because the father is alive, we can only study DNA from his blood and fibroblasts; however, it is unlikely that a large expansion would occur in his CNS tissues, given that he remains unaffected by ALS or FTLD even at 89 years of age and that penetrance of pathological G_4C_2 expansions was found to be almost 100% by 80 years of age.[15,24] Thus, the possibility that he will develop ALS or FTLD in the coming years is low. In contrast, two of his children with large expansions developed ALS in their 50s. Importantly, one of them (the deceased individual) showed large expansions in all studied CNS and non-CNS tissues. Our observation that ALS segregates with large but not small expansions demands a better-defined cutoff for pathogenic repeat number. Small expansions could be considered premutations because of their potential instability, which could lead to larger expansions in the next generation.

Although we do not have access to a sperm sample from the elderly father, the expansion event most likely happened in his germ cells rather than occurring four times separately in each child and reaching a similar size (Figure 3). Notably, many diseases caused by repeat expansions are predominantly inherited through paternal transmissions, most likely as a result of germ-cell-specific mutations.[28] Our study is in favor of the multiple-origins hypothesis of *C9orf72* expansions.[21] Indeed, it does not seem plausible that the expansion occurred just once in human history, given that such events are still ongoing (i.e., in family PED25).

It was shown that wild-type *C9orf72* alleles (<20 repeats) are stable between generations.[6] In contrast, intermediate

Table 1. STR Markers and SNPs for Members of Family PED25

		Family PED25															
		9685		9686		9548		9707		9698		9697		VSM-20 (a)		VSM-20 (b)	
Marker	Mb	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2
D9S171	24.53	167	185	167	167	167	185	167	167	167	185	167	185	185	183	185	185
D9S1679	24.78	135	135	131	137	131	135	131	135	131	135	137	135	135	135	135	131
D9S259	26.02	288	292	288	280	288	292	288	288	288	292	280	292	288	280	288	288
D9S2154	26.17	138	150	142	148	142	150	142	138	142	150	146	150	146	150	146	134
rs4879515*	27.48	C	C	T	C	T	C	T	C	T	C	C	C	T	T	T	C
rs3849942*	27.54	G	G	A	G	A	G	A	G	A	G	G	G	A	A	A	G
*C9orf72**	27.57	2	2	EXP	2	EXP	2	EXP	2	EXP	2	2	2	EXP	2	EXP	5
D9S161	27.63	119	127	119	127	119	119	119	127	119	119	127	119	117	117	117	129
D9S319	29.56	159	159	163	171	163	159	163	159	163	159	171	159	167	167	167	163

Segregation of the repeat expansion in *C9orf72* and genetic markers flanking the area of the repeat expansion was observed in family PED25. The "*C9orf72*" row displays the number of repeats on allele 1 (a1) and allele 2 (a2). Repeat expansions are indicated with "EXP." Asterisks denote the shared *C9orf72* risk haplotype.

alleles (20–30 repeats) and small expansions (30–150 repeats) were reported to be susceptible to unfaithful inheritance[8,10] or somatic instability.[9,29,30] This might suggest a mechanism for the enlargement of repeat number, implying that the critical repeat length makes it difficult for the DNA machinery to maintain the precise number of repeats. Another mechanism could involve the *C9orf72* risk haplotype as a prerequisite for the repeat expansion to occur. Both mechanisms support the multiple-origins hypothesis of *C9orf72* expansions. In family PED25, we observed an ancient *C9orf72* risk haplotype inherited on a unique genetic backbone. The father and offspring with expansions each carry a distinct 5-Mb haplotype that is not observed in family VSM-20 or other investigated subjects, but a smaller, ~100-kb region that is part of a known risk haplotype was present in family PED25 and in VSM-20 family members with large expansions or alleles with 21–30 repeats. Notably, SNPs tagging the risk haplotype are common in white individuals; for instance, rs3849942, a surrogate marker for the repeat expansion, has a minor allele frequency of 23% in control individuals.[4] Sequencing of the entire risk haplotype in subjects with *C9orf72* repeat expansions and normal control individuals with the minor rs3849942 allele might reveal the variant(s) that predispose individuals to G_4C_2-repeat instability.

Results of the epigenetic, expression, and RNA-foci analyses further discriminate large expansions from the 70-repeat allele, arguing against its pathogenic nature. In contrast to the large expansions in the offspring, the 70-repeat allele is not methylated (Figure 5). Intriguingly, detecting methylation in CNS tissues required more than twice the amount of input DNA than in non-CNS tissues, which might indicate a lower level of methylation in the CNS. Therefore, it is tempting to speculate that methylation of the repeat expansion is a protective mechanism to minimize the production of toxic RNA and dipeptides, which lead to collateral damage (haploinsufficiency). Indeed, we demonstrated that the presence of $(G_4C_2)_n$ methylation is linked to decreased expression of *C9orf72*. In blood, methylation of large G_4C_2 expansions was associated with reduced *C9orf72* mRNA levels, whereas in fibroblasts derived from the same subjects, $(G_4C_2)_n$ methylation was not detected, and *C9orf72* was expressed at normal levels (Figure 6). Remarkably, *C9orf72* mRNA levels were increased in both blood and fibroblasts from the PED25 father (who has the unmethylated 70-repeat allele), reminiscent of what was seen for *FMR1* (MIM: 309550), a gene in which mutations cause fragile X mental retardation syndrome (MIM: 300624) as a result of a CGG-repeat expansion. Methylation of a fully expanded pathogenic *FMR1* allele (>200 CGG repeats) led to transcriptional silencing of *FMR1*,[31] whereas the level of *FMR1* mRNA was elevated by 2- to 5-fold in unaffected individuals with pre-mutations (55–200 CGG repeats).[32] For *C9orf72*, the 70-repeat allele might also be considered a "pre-mutation" to reflect its propensity for substantial expansion in the next generation.

In conclusion, our data suggest that the repeat expansion in *C9orf72* might have occurred on multiple occasions in human history and encourage the investigation of families similar to PED25. Specifically, it would be important to investigate the unaffected parents of individuals with disease to establish a better cutoff for pathological repeat number, which is critical in the clinical utility of genetic screening, as well as for functional studies of *C9orf72*. Such investigations might help explain the high frequency of simplex cases of *C9orf72* repeat expansions, each of which could be the first generation of a familial form of ALS and/or FTLD. Indeed, the incidence of *C9orf72* repeat expansions in subjects without a family history of ALS or FTLD is unusually high for a monogenic disorder (e.g., 21% among Finnish individuals with ALS).[5]

Supplemental Data

Supplemental Data include clinical descriptions, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2015.04.016.

Acknowledgments

This work was supported by the W. Garfield Weston Foundation (E.R., Z.X., J.R., J.K., and L.Z.), the James Hunter Family ALS Initiative (L.Z. and J.R.), an ALS Canada Bernice Ramsey Discovery Grant (Y.Y. and L.Z.), and NIH grant R01 NS080882. J.R. is a Tier 2 Canada Research Chair in ALS; P.M.M. holds an Alzheimer Society of Canada Doctoral Award; P.M. holds an American ALS Association Milton Safenowitz Postdoctoral Fellowship; J.R.M. is a James Hunter Senior Research Fellow; and M.v.B. is supported by the Milton Safenowitz Post-Doctoral Fellowship for ALS Research from the ALS Association.

Received: March 12, 2015
Accepted: April 22, 2015
Published: May 21, 2015

References

1. Brooks, B.R., Miller, R.G., Swash, M., and Munsat, T.L.; World Federation of Neurology Research Group on Motor Neuron Diseases (2000). El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. Amyotroph. Lateral Scler. Other Motor Neuron Disord. *1*, 293–299.
2. Neary, D., Snowden, J.S., Gustafson, L., Passant, U., Stuss, D., Black, S., Freedman, M., Kertesz, A., Robert, P.H., Albert, M., et al. (1998). Frontotemporal lobar degeneration: a consensus on clinical diagnostic criteria. Neurology *51*, 1546–1554.
3. Hardy, J., and Rogaeva, E. (2014). Motor neuron disease and frontotemporal dementia: sometimes related, sometimes not. Exp. Neurol. *262* (Pt B), 75–83.
4. DeJesus-Hernandez, M., Mackenzie, I.R., Boeve, B.F., Boxer, A.L., Baker, M., Rutherford, N.J., Nicholson, A.M., Finch, N.A., Flynn, H., Adamson, J., et al. (2011). Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron *72*, 245–256.

5. Renton, A.E., Majounie, E., Waite, A., Simón-Sánchez, J., Rollinson, S., Gibbs, J.R., Schymick, J.C., Laaksovirta, H., van Swieten, J.C., Myllykangas, L., et al.; ITALSGEN Consortium (2011). A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron *72*, 257–268.
6. Xi, Z., Zinman, L., Grinberg, Y., Moreno, D., Sato, C., Bilbao, J.M., Ghani, M., Hernández, I., Ruiz, A., Boada, M., et al. (2012). Investigation of c9orf72 in 4 neurodegenerative disorders. Arch. Neurol. *69*, 1583–1590.
7. Cruts, M., Gijselinck, I., Van Langenhove, T., van der Zee, J., and Van Broeckhoven, C. (2013). Current insights into the C9orf72 repeat expansion diseases of the FTLD/ALS spectrum. Trends Neurosci. *36*, 450–459.
8. Beck, J., Poulter, M., Hensman, D., Rohrer, J.D., Mahoney, C.J., Adamson, G., Campbell, T., Uphill, J., Borg, A., Fratta, P., et al. (2013). Large C9orf72 hexanucleotide repeat expansions are seen in multiple neurodegenerative syndromes and are more frequent than expected in the UK population. Am. J. Hum. Genet. *92*, 345–353.
9. van Blitterswijk, M., DeJesus-Hernandez, M., Niemantsverdriet, E., Murray, M.E., Heckman, M.G., Diehl, N.N., Brown, P.H., Baker, M.C., Finch, N.A., Bauer, P.O., et al. (2013). Association between repeat sizes and clinical and pathological characteristics in carriers of C9ORF72 repeat expansions (Xpansize-72): a cross-sectional cohort study. Lancet Neurol. *12*, 978–988.
10. Dols-Icardo, O., García-Redondo, A., Rojas-García, R., Sánchez-Valle, R., Noguera, A., Gómez-Tortosa, E., Pastor, P., Hernández, I., Esteban-Pérez, J., Suárez-Calvet, M., et al. (2014). Characterization of the repeat expansion size in C9orf72 in amyotrophic lateral sclerosis and frontotemporal dementia. Hum. Mol. Genet. *23*, 749–754.
11. Dobson-Stone, C., Hallupp, M., Loy, C.T., Thompson, E.M., Haan, E., Sue, C.M., Panegyres, P.K., Razquin, C., Seijo-Martínez, M., Rene, R., et al. (2013). C9ORF72 repeat expansion in Australian and Spanish frontotemporal dementia patients. PLoS ONE *8*, e56899.
12. Xi, Z., Zinman, L., Moreno, D., Schymick, J., Liang, Y., Sato, C., Zheng, Y., Ghani, M., Dib, S., Keith, J., et al. (2013). Hypermethylation of the CpG island near the G4C2 repeat in ALS with a C9orf72 expansion. Am. J. Hum. Genet. *92*, 981–989.
13. Xi, Z., Rainero, I., Rubino, E., Pinessi, L., Bruni, A.C., Maletta, R.G., Nacmias, B., Sorbi, S., Galimberti, D., Surace, E.I., et al. (2014). Hypermethylation of the CpG-island near the C9orf72 G_4C_2-repeat expansion in FTLD patients. Hum. Mol. Genet. *23*, 5630–5637.
14. Xi, Z., Zhang, M., Bruni, A.C., Maletta, R.G., Colao, R., Fratta, P., Polke, J.M., Sweeney, M.G., Mudanohwo, E., Nacmias, B., et al. (2015). The C9orf72 repeat expansion itself is methylated in ALS and FTLD patients. Acta Neuropathol. *129*, 715–727.
15. Majounie, E., Renton, A.E., Mok, K., Dopper, E.G., Waite, A., Rollinson, S., Chiò, A., Restagno, G., Nicolaou, N., Simon-Sanchez, J., et al.; Chromosome 9-ALS/FTD Consortium; French research network on FTLD/FTLD/ALS; ITALSGEN Consortium (2012). Frequency of the C9orf72 hexanucleotide repeat expansion in patients with amyotrophic lateral sclerosis and frontotemporal dementia: a cross-sectional study. Lancet Neurol. *11*, 323–330.
16. Ishiura, H., Takahashi, Y., Mitsui, J., Yoshida, S., Kihira, T., Kokubo, Y., Kuzuhara, S., Ranum, L.P., Tamaoki, T., Ichikawa, Y., et al. (2012). C9ORF72 repeat expansion in amyotrophic lateral sclerosis in the Kii peninsula of Japan. Arch. Neurol. *69*, 1154–1158.
17. Ratti, A., Corrado, L., Castellotti, B., Del Bo, R., Fogh, I., Cereda, C., Tiloca, C., D'Ascenzo, C., Bagarotti, A., Pensato, V., et al.; SLAGEN Consortium (2012). C9ORF72 repeat expansion in a large Italian ALS cohort: evidence of a founder effect. Neurobiol. Aging *33*, e7–e14.
18. Smith, B.N., Newhouse, S., Shatunov, A., Vance, C., Topp, S., Johnson, L., Miller, J., Lee, Y., Troakes, C., Scott, K.M., et al. (2013). The C9ORF72 expansion mutation is a common cause of ALS+/-FTD in Europe and has a single founder. Eur. J. Hum. Genet. *21*, 102–108.
19. van der Zee, J., Gijselinck, I., Dillen, L., Van Langenhove, T., Theuns, J., Engelborghs, S., Philtjens, S., Vandenbulcke, M., Sleegers, K., Sieben, A., et al.; European Early-Onset Dementia Consortium (2013). A pan-European study of the C9orf72 repeat associated with FTLD: geographic prevalence, genomic instability, and intermediate repeats. Hum. Mutat. *34*, 363–373.
20. Pliner, H.A., Mann, D.M., and Traynor, B.J. (2014). Searching for Grendel: origin and global spread of the C9ORF72 repeat expansion. Acta Neuropathol. *127*, 391–396.
21. Fratta, P., Polke, J.M., Newcombe, J., Mizielinska, S., Lashley, T., Poulter, M., Beck, J., Preza, E., Devoy, A., Sidle, K., et al. (2015). Screening a UK amyotrophic lateral sclerosis cohort provides evidence of multiple origins of the C9orf72 expansion. Neurobiol. Aging *36*, e1–e7.
22. Rubino, E., Rainero, I., Chiò, A., Rogaeva, E., Galimberti, D., Fenoglio, P., Grinberg, Y., Isaia, G., Calvo, A., Gentile, S., et al.; TODEM Study Group (2012). SQSTM1 mutations in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Neurology *79*, 1556–1562.
23. Xi, Z., Yunusova, Y., van Blitterswijk, M., Dib, S., Ghani, M., Moreno, D., Sato, C., Liang, Y., Singleton, A., Robertson, J., et al. (2014). Identical twins with the C9orf72 repeat expansion are discordant for ALS. Neurology *83*, 1476–1478.
24. Williams, K.L., Fifita, J.A., Vucic, S., Durnall, J.C., Kiernan, M.C., Blair, I.P., and Nicholson, G.A. (2013). Pathophysiological insights into ALS with C9ORF72 expansions. J. Neurol. Neurosurg. Psychiatry *84*, 931–935.
25. Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. *8*, R19.
26. Lagier-Tourenne, C., Baughn, M., Rigo, F., Sun, S., Liu, P., Li, H.R., Jiang, J., Watt, A.T., Chun, S., Katz, M., et al. (2013). Targeted degradation of sense and antisense C9orf72 RNA foci as therapy for ALS and frontotemporal degeneration. Proc. Natl. Acad. Sci. USA *110*, E4530–E4539.
27. Mizielinska, S., Grönke, S., Niccoli, T., Ridler, C.E., Clayton, E.L., Devoy, A., Moens, T., Norona, F.E., Woollacott, I.O., Pietrzyk, J., et al. (2014). C9orf72 repeat expansions cause neurodegeneration in Drosophila through arginine-rich proteins. Science *345*, 1192–1194.
28. Pearson, C.E. (2003). Slipping while sleeping? Trinucleotide repeat expansions in germ cells. Trends Mol. Med. *9*, 490–495.
29. Waite, A.J., Bäumer, D., East, S., Neal, J., Morris, H.R., Ansorge, O., and Blake, D.J. (2014). Reduced C9orf72 protein levels in frontal cortex of amyotrophic lateral sclerosis and frontotemporal degeneration brain with the C9ORF72

hexanucleotide repeat expansion. Neurobiol. Aging *35*, 1779.e5–1779.e13.

30. Buchman, V.L., Cooper-Knock, J., Connor-Robson, N., Higginbottom, A., Kirby, J., Razinskaya, O.D., Ninkina, N., and Shaw, P.J. (2013). Simultaneous and independent detection of C9ORF72 alleles with low and high number of GGGGCC repeats using an optimised protocol of Southern blot hybridisation. Mol. Neurodegener. *8*, 12.
31. Pieretti, M., Zhang, F.P., Fu, Y.H., Warren, S.T., Oostra, B.A., Caskey, C.T., and Nelson, D.L. (1991). Absence of expression of the FMR-1 gene in fragile X syndrome. Cell *66*, 817–822.
32. Tassone, F., Hagerman, R.J., Taylor, A.K., Gane, L.W., Godfrey, T.E., and Hagerman, P.J. (2000). Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. Am. J. Hum. Genet. *66*, 6–15.

The American Journal of Human Genetics

Supplemental Data

Jump from Pre-mutation to Pathologic Expansion in *C9orf72*

Zhengrui Xi, Marka van Blitterswijk, Ming Zhang, Philip McGoldrick, Jesse R. McLean, Yana Yunusova, Erin Knock, Danielle Moreno, Christine Sato, Paul M. McKeever, Raphael Schneider, Julia Keith, Nicolae Petrescu, Paul Fraser, Maria Carmela Tartaglia, Matthew C. Baker, Neill R. Graff-Radford, Kevin B. Boylan, Dennis W. Dickson, Ian R. Mackenzie, Rosa Rademakers, Janice Robertson, Lorne Zinman, and Ekaterina Rogaeva

Clinical Descriptions

None of the seven family members of PED25 have history of physical trauma, illegal drug abuse or pesticide exposure.

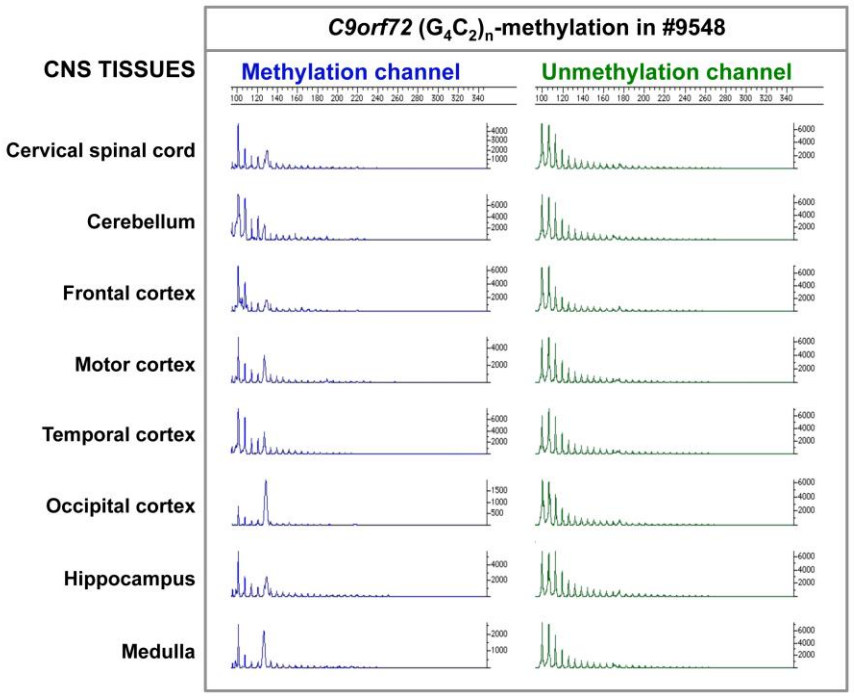
Both parents of the proband are alive without symptoms of ALS or FTLD, based on neurological assessments. The 90-year-old mother (#9685) has recent onset of mild memory loss. The 89-year-old father (#9686) has a medical history of chronic obstructive pulmonary disease and heart disease. He was an officer in the British and Canadian navy, "always mentally sharp" as reported by his children. He is still independent for all activities of daily living. His performance fell within the normal range in the Social Norm Questionnaire with a score of 16/22, but he noted some difficulty with short-term memory over the last year (at age 89), which was reflected in a MoCA score of 23/30, losing 2 points for mistakes with the clock drawing test and 5 points for delayed recall. Although the MoCA score of #9686 is within the range of mild cognitive impairment (18-26), it is still above the average (21) of the general elderly population of 70-80 years old [1]. His mild cognitive impairment might indicate early disease symptoms; however the MoCA score is still above the average for his age-group. Of note, 65% of Canadians older than 85 have cognitive impairment [2].

The proband (#9548) developed symptoms of bulbar onset at age 57 without any evidence of cognitive deficit on a Montreal Cognitive Assessment (MoCA) [3] (29/30). This individual had a college education and worked as an emergency unit clerk. The medical history included hypothyroidism, chronic obstructive pulmonary disease, cholecystectomy, incontinence, hysterectomy and a hernia. Individual #9548 smoked a pack of cigarettes per day for 37 years starting at age 14 and drank a glass of red wine daily. At age 59 individual #9548 passed away and autopsy results confirmed the diagnosis of ALS with pathological signs of early stage FTLD.

One of the four proband's siblings (#8665) also developed ALS at age 59 and passed away at age 62 (autopsy was not performed). Individual #8665 had lumbar onset ALS with primary symptoms in the upper limbs (bulbar symptoms developed at the end stage of the disease). At the time of diagnosis individual #8665 was completing an MBA degree, and MoCA score was within normal range (27/30), with one point missed for attention, orientation and delayed recall, respectively. The medical history was significant for bipolar affective disorder treated by Lithium. Individual #8665 had a history of heavy smoking (up to two packs daily) and drinking (two or more drinks daily).

Three other siblings (#9697, #9698 and #9707) remain asymptomatic at their current age (65, 65, and 51 years, respectively). They all completed a college education and worked professionally (e.g. in healthcare, human resources, or arts). All of them had smoking habits for 6-7 years (~5 cigarettes per week). They did not report any changes in their cognitive abilities, with the exception of #9698, who complained about memory issues, affecting daily activities. All three asymptomatic siblings underwent cognitive screening using MoCA and ALS Cognitive Behavioural Screen [4], showing performance within normal range.

A.



B.

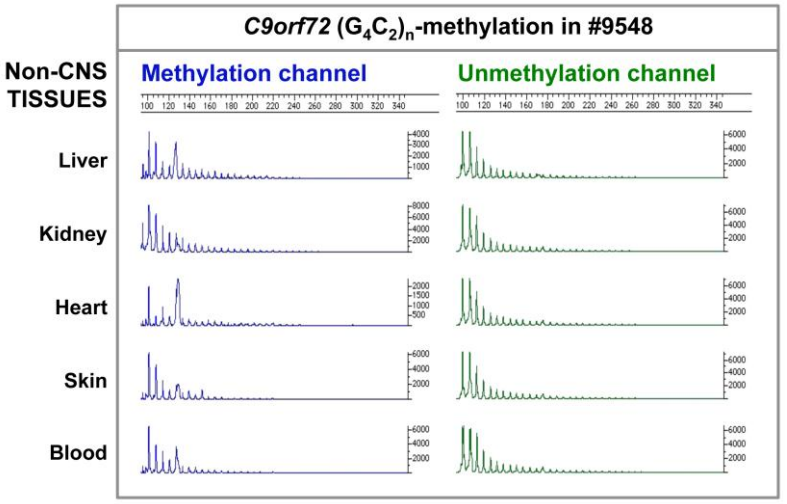


Figure S1. Chromatograms of the $(G_4C_2)_n$-methylation assay for #9548 in multiple autopsy tissues: (A) central nervous system (CNS) tissues (B) non-CNS tissues

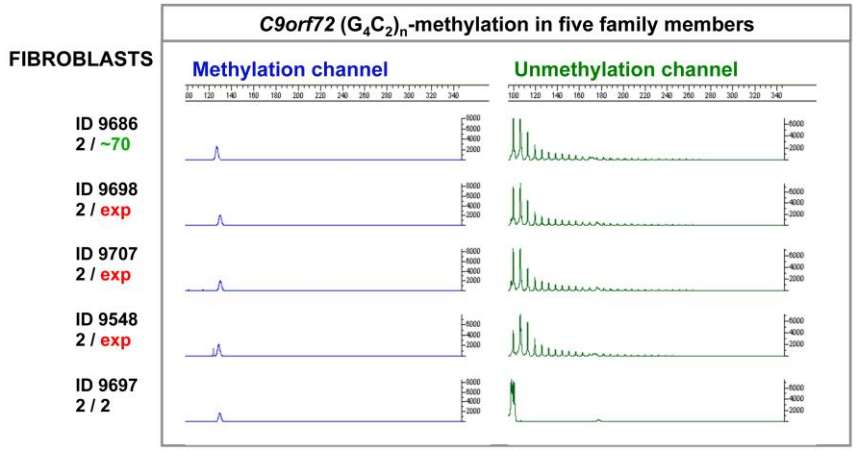


Figure S2. Chromatograms of the $(G_4C_2)_n$-methylation assay for PED25 in fibroblast samples

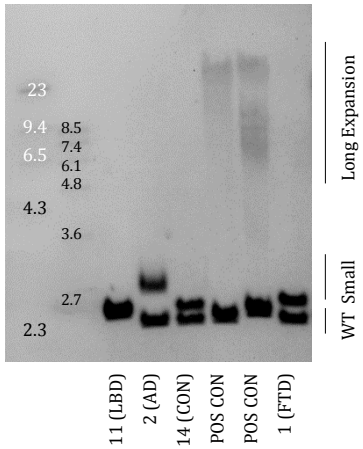


Figure S3. Southern blot results for subjects with small expansions or repeats in the long wild-type range.

Subjects are diagnosed with Lewy Body Dementia (LBD), Alzheimer's disease (AD), frontotemporal dementia (FTD), or they are unaffected (CON). Two positive controls are included with a long expansion on one allele and wild-type alleles of 2 and 11, respectively (POS CON). Case #2 has been described by Wojtas *et al.* [5]. Numbers correspond to Table S1.

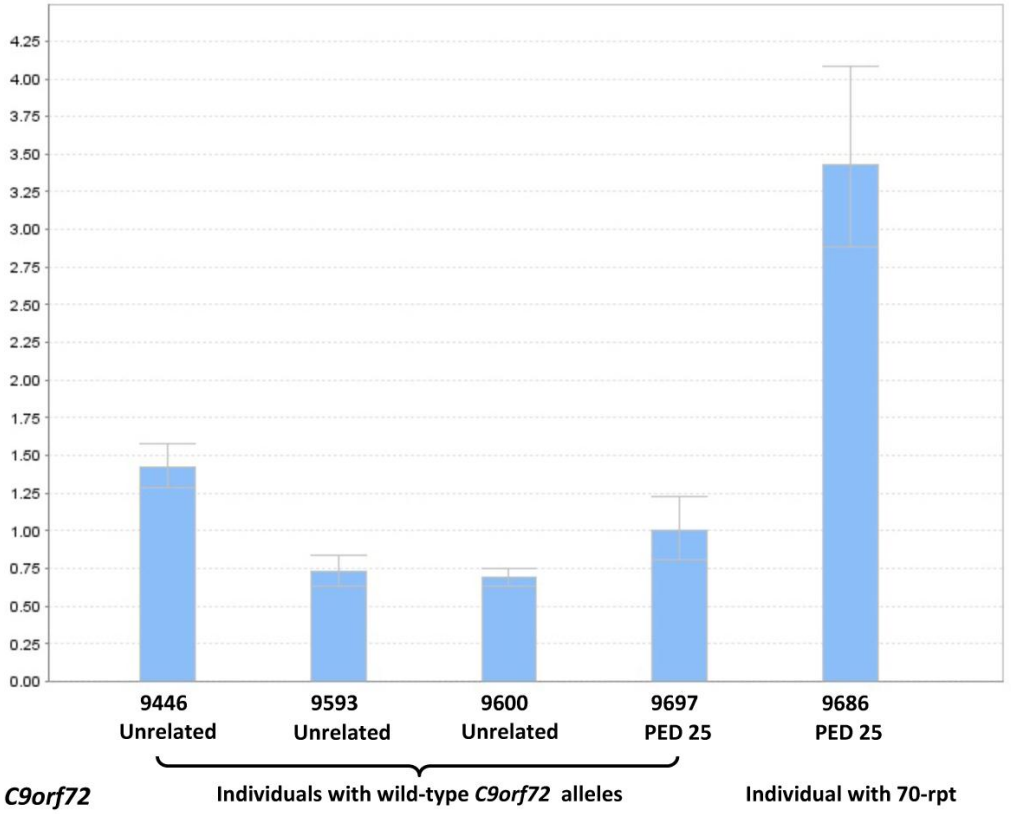


Figure S4. Relative quantification of *C9orf72* mRNA in blood from unrelated normal controls and the individual with the small expansion

The individual with the small expansion #9686 (2/~70) showed increased *C9orf72* gene expression compared to three unrelated normal controls: #9446 (2/22), #9593 (2/8) and #9600 (2/2), as well as PED25 family member #9697 with normal alleles. Bars represent the standard error of the triplicate reactions.

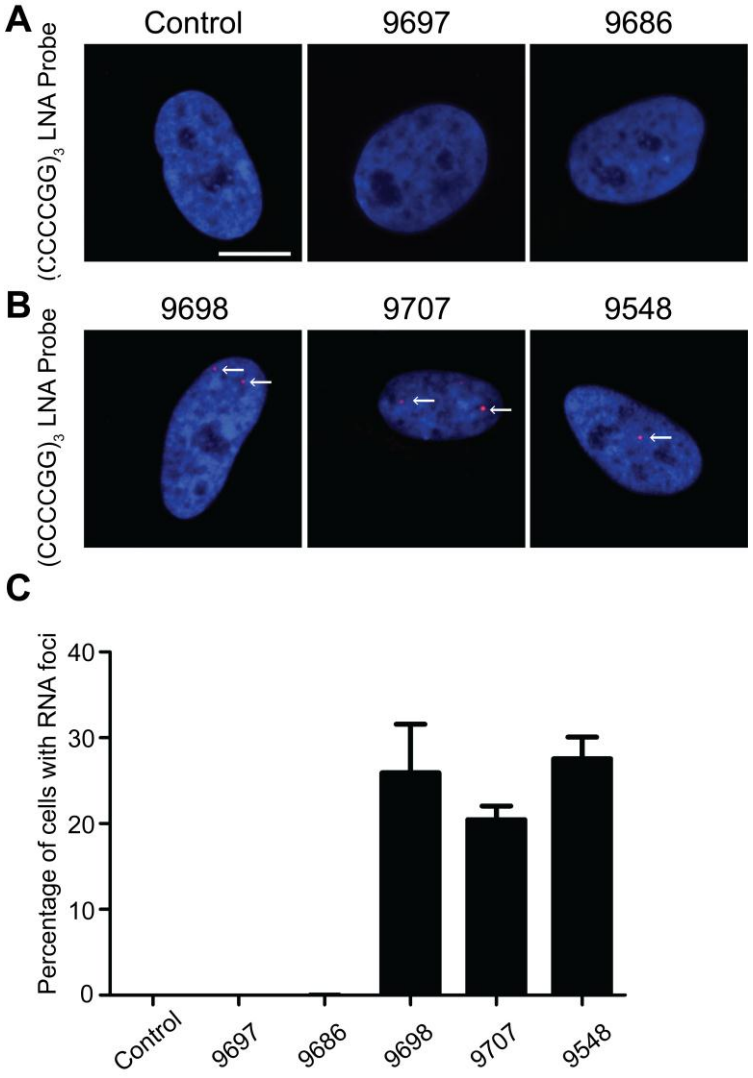


Figure S5. RNA foci were detected in fibroblasts from individuals with large expansions but not from the individual with the 70-repeat allele or unrelated control

RNA FISH using a probe complementary to the *C9orf72*-repeat expansion on fibroblasts from the control (#9697) or the individual with 70-repeat allele (#9686) did not detect RNA foci (**A**; scale bar = 10μm). However, RNA foci were detected in fibroblasts from subjects with large expansions: #9698, #9707 and #9548 (**B**, white arrows). Quantification of the number of RNA foci-positive cells in repeat expansion carriers revealed approximately 20-27% of cells to be positive for RNA foci (**C**). N = 3 experiments per fibroblast line, except 9686 (n = 2). Data are mean ± SEM. RNA FISH was based on previously published protocols [6; 7]. Briefly, fibroblasts plated on 13mm glass coverslips were washed with RNase-free PBS (Ambion) before fixation with 4% paraformaldehyde for 15 minutes at ambient temperature. Cells were permeabilized with PBS containing 0.2% (w/v) Triton X-100 for 10 minutes and washed once with 2 X saline sodium citrate (SSC) (Sigma) buffer. For prehybridization, cells were incubated with 2 X SSC containing 50% (v/v) formamide for 30 minutes at 60°C, followed by incubation with hybridization solution for 30 minutes at 60°C (50% formamide, 2 X SSC, 10% dextran, 0.2% bovine serum albumin, 2mM vanadyl ribonucleoside complex, 1mg/ml tRNA, 1mg/ml single stranded DNA from salmon sperm). A locked nucleic acid (LNA) probe, recognizing the sense strand (CCCCGG3) of the repeat expansion and with a 5'-TYE 563 fluorescent modification (Lagier-Tourenne et al., 2013; Exiqon catalogue number 607323), was denatured for 5 minutes at 85°C before addition to the hybridization solution to create a working concentration of 40nM. Cells were then incubated with probe for 2 hours at 60°C, and protected from light. This

was followed by three 20 minutes washes with 2 X SSC containing 50% formamide (v/v) at 60°C with shaking, and finally three 10 minute washes with 2 X SSC, with shaking. Coverslips were mounted with Prolong gold containing DAPI (Life Technologies). To quantify the number of cells expressing RNA foci, 10 random images were captured at 63x magnification per coverslip using a Leica DMI 6000 microscope with Volocity software (PerkinElmer). The number of RNA foci-positive cells was expressed as a percentage of total cells.

Table S1. Short tandem repeat (STR) markers and single nucleotide polymorphisms (SNPs) for subjects with small expansions or repeats in the long wild-type range

		1 (FTD)		2 (AD)		3 (FTD/MND)	
Marker	**Mb**	a1	a2	a1	a2	a1	a2
D9S171	24.53	175	183	185	167	169	185
D9S1679	24.78	135	131	135	131	131	135
D9S259	26.02	288	292	288	294	288	288
D9S2154	26.17	146	150	150	158	150	150
*rs4879515	27.48	T	C	T	C	T	C
*rs3849942	27.54	A	G	A	G	A	G
**C9orf72*	27.57	EXP	2	EXP	2	EXP	2
D9S161	27.63	129	131	117	129	117	127
D9S319	29.55	171	174	171	171	163	171

		4 (CON)		5 (CON)		6 (MND)		7 (AD)		8 (CON)		9 (CON)		10 (MND)		11 (LBD)		12 (MND)		13 (AD)		14 (CON)	
Marker	**Mb**	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a1	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2
D9S171	24.5	167	175	167	175	175	181	167	183	167	175	169	175	169	179	167	183	183	185	169	175	175	181
D9S1679	24.8	131	135	135	135	135	131	135	137	131	135	131	135	131	135	133	135	131	133	131	137	131	131
D9S259	26.0	288	294	280	280	288	280	280	292	280	292	288	292	280	292	288	292	288	280	292	292	280	288
D9S2154	26.2	158	158	150	154	146	150	154	154	146	150	150	154	150	150	134	150	146	154	154	158	134	150
*rs4879515	27.5	T	C	T	T	T	T	T	C	T	C	T	T	T	C	T	T	T	T	T	T	T	C
*rs3849942	27.5	A	G	A	G	A	G	A	G	A	G	A	A	A	G	A	A	A	A	A	G	A	G
**C9orf72*	27.6	21	2	21	5	21	2	21	2	22	2	23	8	23	2	27	20	27	10	27	5	27	2
D9S161	27.6	119	127	125	127	129	131	117	131	117	129	127	129	119	127	117	127	127	127	127	131	117	117
D9S319	29.5	171	175	167	167	163	167	171	174	167	175	159	167	167	171	167	171	167	171	167	171	171	174

Subjects are diagnosed with frontotemporal dementia (FTD), Alzheimer's disease (AD), FTD with motor neuron disease (FTD/MND), MND, or Lewy Body Dementia (LBD); the remaining subjects are unaffected (CON). The *C9orf72* row displays the number of repeats on allele 1 (a1) and allele 2 (a2). Repeat expansions are indicated with 'EXP'. A Southern blot showing the small repeat expansion for subject #1 and #2 (subject #3 has been published elsewhere [8]); and two representative samples with long wild-type alleles are provided in Figure S3. All samples share a small region that is thought to represent the risk-haplotype (denoted by the asterisks).

Supplemental References

1. Rossetti, H.C., Lacritz, L.H., Cullum, C.M., and Weiner, M.F. (2011). Normative data for the Montreal Cognitive Assessment (MoCA) in a population-based sample. Neurology 77, 1272-1275.
2. Graham, J.E., Rockwood, K., Beattie, B.L., Eastwood, R., Gauthier, S., Tuokko, H., and McDowell, I. (1997). Prevalence and severity of cognitive impairment with and without dementia in an elderly population. Lancet 349, 1793-1796.
3. Nasreddine, Z.S., Phillips, N.A., Bedirian, V., Charbonneau, S., Whitehead, V., Collin, I., Cummings, J.L., and Chertkow, H. (2005). The Montreal Cognitive Assessment, MoCA: a brief screening tool for mild cognitive impairment. J Am Geriatr Soc 53, 695-699.
4. Woolley, S.C., York, M.K., Moore, D.H., Strutt, A.M., Murphy, J., Schulz, P.E., and Katz, J.S. (2010). Detecting frontotemporal dysfunction in ALS: utility of the ALS Cognitive Behavioral Screen (ALS-CBS). Amyotroph Lateral Scler 11, 303-311.
5. Wojtas, A., Heggeli, K.A., Finch, N., Baker, M., Dejesus-Hernandez, M., Younkin, S.G., Dickson, D.W., Graff-Radford, N.R., and Rademakers, R. (2012). C9ORF72 repeat expansions and other FTD gene mutations in a clinical AD patient series from Mayo Clinic. Am J Neurodegener Dis 1, 107-118.
6. Lagier-Tourenne, C., Baughn, M., Rigo, F., Sun, S., Liu, P., Li, H.R., Jiang, J., Watt, A.T., Chun, S., Katz, M., et al. (2013). Targeted degradation of sense and antisense C9orf72 RNA foci as therapy for ALS and frontotemporal degeneration. Proc Natl Acad Sci U S A 110, E4530-4539.
7. Mizielinska, S., Gronke, S., Niccoli, T., Ridler, C.E., Clayton, E.L., Devoy, A., Moens, T., Norona, F.E., Woollacott, I.O., Pietrzyk, J., et al. (2014). C9orf72 repeat expansions cause neurodegeneration in Drosophila through arginine-rich proteins. Science 345, 1192-1194.
8. van Blitterswijk, M., Dejesus-Hernandez, M., Niemantsverdriet, E., Murray, M.E., Heckman, M.G., Diehl, N.N., Brown, P.H., Baker, M.C., Finch, N.A., Bauer, P.O., et al. (2013). Association between repeat sizes and clinical and pathological characteristics in carriers of C9ORF72 repeat expansions (Xpansize-72): a cross-sectional cohort study. Lancet Neurol 12, 978-988.