Intronic ATTTC repeat expansions in \textit{STARD7} in familial adult myoclonic epilepsy linked to chromosome 2

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Familial Adult Myoclonic Epilepsy (FAME) is characterised by cortical myoclonic tremor usually from the second decade of life and overt myoclonic or generalised tonic-clonic seizures. Four independent loci have been implicated in FAME on chromosomes (chr) 2, 3, 5 and 8. Using whole genome sequencing and repeat primed PCR, we provide evidence that chr2-linked FAME (FAME2) is caused by an expansion of an ATTTC pentamer within the first intron of \textit{STARD7}. The ATTTC expansions segregate in 158/158 individuals typically affected by FAME from 22 pedigrees including 16 previously reported families recruited worldwide. RNA sequencing from patient derived fibroblasts shows no accumulation of the AUUUU or AUUUC repeat sequences and \textit{STARD7} gene expression is not affected. These data, in combination with other genes bearing similar mutations that have been implicated in FAME, suggest ATTTC expansions may cause this disorder, irrespective of the genomic locus involved.

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FAME (also referred to as Familial Cortical Myoclonic Tremor and Epilepsy or Benign Adult onset Familial Myoclonic Epilepsy [OMIM phenotypic series: PS601068]) is characterised by cortical myoclonic tremor and overt myoclonic and later generalised tonic-clonic seizures (GTCS).\(^1\) Onset of symptoms occurs in the second to third decade with variable expressivity within and between families; anticipation has been noted in some families.\(^1\) The frequency of GTCS varies from 15 to 100% in some families.\(^1\) We previously showed using whole-exome sequencing to identify ATTTT and ATTTC repeat expansions in the FAME2 linkage interval. We screen for an intronic ATTTC expansion in the first intron of STARD7 by repeat-primed PCR and show it segregates with FAME in 158 affected individuals from 22 families. We use long-read sequencing to suggest the ATTTT and ATTTC expansions may be somatically unstable. We analyse clinical data and show evidence of anticipation over multiple generations of a large FAME family. Finally, we demonstrate that the presence of the ATTTC repeat has no effect on protein or mRNA expression levels of STARD7 in available patient cell lines. These data suggest the repeat sequence alone is pathogenic, independent of an effect on the coding sequence of the encompassing gene.

### Results

**Discovery of a repeat expansion in STARD7.** We analysed Illumina HiSeq X-10 whole-genome sequencing data initially from two individuals from a large Australian–New Zealand FAME family, one from an Italian family and three from a French–Spanish family (Table 1 and Supplementary Table 1; Families 1, 3 and 19, respectively)\(^1,2\) with two repeat expansion detection methods, ExpansionHunter and exSTRa\(^14,15\), to look for similar combined ATTTT and ATTTC repeat expansions on both the forward and reverse chromosome strands within the FAME2 interval. This revealed an expansion of an ATTTT repeat and insertion of an ATTTC repeat in the context of the reverse strand of chr2 within the first intron of STARD7 (STAR-related lipid transfer domain-containing 7) in all FAME samples tested (Fig. 1a, Supplementary Fig. 1). The endogenous ATTTT repeat in intron 1 of STARD7 was also found to be variable in length in the normal population but not expanded to the same extent as repeats found in individuals with FAME. The ATTTC repeat was not present in any whole-genome sequencing data from 69 control samples.

### Table 1 Clinical summaries of 22 investigated FAME families

| Family | Nationality | Total affected | Mean onset [range] | Myoclonus/CT | TCS | Focal Sz | References |
|--------|-------------|----------------|--------------------|--------------|-----|---------|------------|
| 1      | Australian/New Zealand of European ancestry | 55 | 18.6 y [4-59.60 y] | 55/55 (100%) | 8/55 (15%) | 2/55 (4%) | 2 |
| 2      | Italian     | 2 | 15-25 y | 2/2 (100%) | 2/2 (100%) | 0/2 (0%) | 12 |
| 3      | Italian     | 4 | 2-18y | 4/4 (100%) | 4/4 (100%) | 2/4 (50%) | 14 |
| 4      | Italian     | 11 | 22.3 y [12-49.50 y] | 11/11 (100%) | 11/11 (100%) | 3/11 (27%) | 4,17 |
| 5      | Italian     | 25 | 26.6 y [5-39.40 y] | 25/25 (100%) | 10/25 (40%) | 0/25 (0%) | 38 |
| 6      | Italian     | 12 (3 studied) | 12 y [8-17.18 y] | 11/12 (91.6%) | 6/12 (50%) | 1/12 (8.3%) | 9,39 |
| 7      | Italian     | 4 | 22.75 y [10-35.36 y] | 4/4 (100%) | 3/4 (75%) | 0/4 (0%) | 40 |
| 8      | Italian     | 10 (6 studied) | 18.5 y [17-19.20 y] | 6/6 (100%) | 4/6 (66.6%) | 0/6 (0%) | 41 |
| 9      | Italian     | 13 (11 studied) | 17 y [12-21.22 y] | 11/11 (100%) | 9/11 (81.1%) | 0/11 (0%) | 41 |
| 10     | Italian     | 16 (14 studied) | 15.8 y [13-19.20 y] | 14/14 (100%) | 10/14 (71%) | 0/14 (0%) | 41 |
| 11     | Italian     | 10 (5 studied) | 15.5 y [13-17.18 y] | 5/5 (100%) | 4/5 (80%) | 0/5 (0%) | 42 |
| 12     | Italian     | 21 (17 studied) | 39.2 y [24-55.56 y] | 17/17 (100%) | 13/17 (76.4%) | 0/17 (0%) | 43,44 |
| 13     | Italian     | 3 | 17.7 y [12-22.23 y] | 3/3 (100%) | 1/3 (33%) | 0/3 (0%) | 4,17 |
| 14     | Italian     | 3 | 16.3 y [15-18 y] | 3/3 (100%) | 3/3 (100%) | 0/3 (0%) | 44,45 |
| 15     | Italian     | 4 | 30.3 y [18-48.49 y] | 4/4 (100%) | 3/4 (75%) | 2/4 (50%) | 46,57 |
| 16     | Iraqi of Sephardic Jewish ancestry | 15 (10 studied) | 21 y [12-31.32 y] | 10/10 (100%) | 4/10 (40%) | 2/10 (20%) | 47 |
| 17     | Israeli of Sephardic Jewish ancestry | 2 | 21 y [21 y] | 2/2 (100%) | 2/2 (100%) | 0/2 (0%) | 48 |
| 18     | South African of European ancestry | 24 (15 studied) | 15.8 y [11-19.20 y] | 15/15 (100%) | 7/15 (47%) | 1/15 (7%) | 49 |
| 19     | French/ Spanish | 13 | 41 y [30-59.60 y] | 13/13 (100%) | 8/13 (62%) | 0/13 (0%) | 50,51 |
| 20     | French      | 7 (2 studied) | 20 y (n = 1) Childhood | 2/2 (100%) | 1/2 (50%) | 0/2 (0%) | 52 |
| 21     | Syrian      | 1 | 20 y (n = 1) | 1/1 (100%) | 1/1 (100%) | 0/1 (0%) | 53,54 |
| 22     | Italian     | 11 (10 studied) | 25.1 y [14-39.40 y] | 9/10 (90%) \(a\) | 4/10 (40%) | 1/10 (10%) | 55 |

CT: cortical tremor, Focal Sz: focal seizures, TCS: tonic-clonic seizures, y: years, n: number of individuals

\(a\) One family member last evaluated at 9 years of age.
samples (Supplementary Fig. 1), nor is it reported in the Simple Repeats track in the UCSC genome browser (build hg38). Segregation of STARD7 ATTTC expansions by repeat-primed PCR. We developed a repeat-primed PCR (RP-PCR) assay to rapidly identify the expansion in 137/137 affected individuals from 16 independently reported FAME2 families worldwide (Fig. 1c, d, Table 1, Supplementary Table 1, Supplementary Data 1, Supplementary Fig. 2; Families 1, 3–6, 8–10, 12–16, 19, 20 and 22). Of the 24 individuals tested in these families that did not have a FAME diagnosis, two were positive for the ATTTC expansion; both were from younger generations and likely pre-symptomatic. We tested an additional 72 individuals (52 unrelated and 20 cases from six families with multiple affected individuals) with clinical similarity to FAME. We tested an additional 72 individuals (52 unrelated and 20 cases from six families with multiple affected individuals) with clinical similarity to FAME. Of these, 20/20 familial and 1/52 singleton cases were positive for an ATTTC repeat expansion in familial and 1/52 singleton cases were positive for an ATTTC repeat expansion. Of these, 20/20 familial and 1/52 singleton cases were positive for an ATTTC repeat expansion, two were positive for the ATTTC repeat insertion (bottom panel). Full screening results are provided in Supplementary Data 1.

**Fig. 1** Identification of an expanded pentameric ATTTC repeat causing FAME2. a Estimated sizes of the AAATG repeats in two affected individuals from Family 1 (red, orange), one from Family 3 (brown) and three affected individuals from Family 19 (blue, green, purple), compared to 69 individuals without FAME using TruSeq Nano (grey) or KAPA Hyper (tan) library preparation. Left panel shows empirical cumulative distribution functions from exSTRa panel and right panel shows the estimated repeat size by Expansion Hunter (the sum of both alleles suggests repeat sizes of 0.75–2.3 kb). Data underlying this part of the figure are available in Source Data. b WGS data from two individuals in Family 1 and one from Family 3 show reads suggesting expansion of AAAAT and insertion of AAATG repeats in the chr2 linkage interval. c Upper section shows the location of the repeat in the context of chr2. The approximate location of the FAME2 minimal linkage interval is shown above the ideogram with two blue arrow heads. The STARD7 gene is on the reverse chromosome strand and the endogenous AAAAT repeat is found in the first intron of the gene. Schema in the lower section shows the primers used in the RP-PCR to detect the ATTTC “3’ assay” and ATTTC “5’ assay” expanded repeats, respectively. d Example results of the RP-PCR 5’ assay obtained in an individual negative for the ATTTC insert (top panel) and in an individual affected by FAME, positive for the ATTTC repeat insertion (bottom panel). e Summary of 184 individuals from 22 families tested with the RP-PCR assay. Individuals under category (+) tested positive for the ATTTC repeat and individuals under category (−) tested negative for the repeat.

Long-read sequencing reveals the repeat structure. The total numbers of repeats could not be determined by the RP-PCR assay, therefore we investigated some of these with long-read sequencing (Fig. 2). In one individual from the Australian-New Zealand family (Family 1: IV-98) a single molecule real-time (SMRT) read and a single Oxford Nanopore read were found that spanned the repeat. The SMRT read generated to 99% base accuracy by circular consensus calling was comprised of four subreads and contained 274 AAATG and 387 AAAAT repeats, without interruption from other sequences. The Oxford Nanopore read contained 345 AAATG and 390 AAAAT repeats with some interruptions, suggesting somatic variation of repeat sizes.
clinical anticipation in FAME111, we searched for evidence of dynamic mutations of ATTTC repeats, and the demonstration of the discovery that FAME2 and FAME1 are caused by similar Evidence of anticipation in a large FAME2 family

This included two families with the previously identified ADRA2B; c.675_686delTGGTGGGGCTTTinsGTTTGGCAG; p. H225_L229delinsQ225_F_G_R228 variant strongly suggesting that allele is not causative (Table 1; Family 4 & 15)17.

Evidence of anticipation in a large FAME2 family. In view of the discovery that FAME2 and FAME1 are caused by similar dynamic mutations of ATTTC repeats, and the demonstration of clinical anticipation in FAME111, we searched for evidence of anticipation in our pedigrees. We examined the median onset age of any relevant symptom, where available, for each generation in the Australian/New Zealand family (Family 1). We found evidence of anticipation; generation III had a median onset of 30 years (range 14–60 y, n = 6), in generation IV median onset was 17 years (8–50 y, n = 30) and the median onset in generation V was 12 years (4–19 y, n = 16). The remaining families were either too small or onset data were unavailable for anticipation to be robustly assessed.

STARD7 transcript and protein abundance are not altered. Reverse transcriptase, quantitative PCR using primer pairs spanning the repeat containing intron between exons one and two and a second pair spanning between exons three and four showed no significant differences in STARD7 transcript expression in patient-derived fibroblast cell lines (Fig. 3a). Protein abundance was also unaltered, confirmed by western blotting using an antibody to STARD7 protein that was previously validated using STARD7-knockout cell lines (Fig. 3b)18. RNA-Seq data from six patient-derived fibroblasts (four from Family 1 and two from Family 5) showed there was no significant difference in gene expression of STARD7 between affected and unaffected individuals along the entire length of the gene (Supplementary Fig. 4; p = 0.838; False Discovery Rate = 1). Reads containing ATTTC repeats were not present in the RNA-Seq data despite robust expression of...
DAB1 phatidylcholine24. Previously, increased levels of choline have functions including intra-mitochondrial lipid transfer (transfer) domain-containing family of lipid transfer proteins with absence of epilepsy in individuals with SCA37. FAME thus far are more broadly expressed throughout the brain https://www.gtexportal.org23 shows that known genes implicated with pathogenic ATTTC dynamic mutation21. In zebra fish, there is suggested to be RNA toxicity21. The mechanism of disease pathogenesis has been hypothesised that the thymidine to cytosine transition occurs after expansion of the endogenous ATTTC repeat to ~200 copies followed by further expansion of the mutant ATTTC sequence22. The ATTTT/ATTTC strand of the repeat is aligned with the direction of gene expression in all genes reported thus far, regardless of their chromosomal orientation. The mechanism of disease pathogenesis has been suggested to be RNA toxicity21. In zebrafish embryos, direct injection of RNA containing 58 copies of the AUUUC repeat was lethal or caused developmental defects in 81%, while the effect of injecting RNA containing 139 AUUUU repeats was not significantly different from controls21. Accumulation of AUUUC repeat containing RNA was observed in the brain of some individuals with FAME1, but we did not have access to similar biopsy tissue from FAME2, these findings may have important therapeutic implications, including RNA-targeting treatments, such as antisense oligonucleotides or RNA-targeting Cas9 (RCas9)26.

**Discussion**

The pathogenic ATTTC insertion and expansion was always accompanied by the endogenous ATTTC pentanucleotide repeat in all cases of FAME2 that we describe here, replicating the findings in the cases of FAME with expansions in SAMD12, TNRC6A, RAPGEF210,11,19 and the report of a similar expansion in MARCH6 causing chr5-linked FAME20. The same observation also holds for spinocerebellar ataxia 37 (SCA37, OMIM: 615945), which is caused by the same repeat expansion in the first intron of DAB221. For SCA37, it has been hypothesised that the thymidine to cytosine transition occurs after expansion of the endogenous ATTTC repeat to ~200 copies followed by further expansion of the mutant ATTTC sequence22. The ATTTT/ATTTC strand of the repeat is aligned with the direction of gene expression in all genes reported thus far, regardless of their chromosomal orientation. The mechanism of disease pathogenesis has been suggested to be RNA toxicity21. Zebrafish embryos, direct injection of RNA containing 58 copies of the AUUUC repeat was lethal or caused developmental defects in 81%, while the effect of injecting RNA containing 139 AUUUU repeats was not significantly different from controls21. Accumulation of AUUUC repeat containing RNA was observed in the brain of some individuals with FAME1, but we did not have access to similar biopsy tissue from FAME221. While we found no significant change in expression of STARD7 in patient-derived cell lines, it is possible that expression of this gene is regulated differently in the non-proliferating cells of the brain. Profiling expression of all known genes implicated with pathogenic ATTTC dynamic mutations using gene expression data from the GTEx portal https://www.gtexportal.org25 shows that DAB1 has high expression specifically in cerebellum while the five genes implicated in FAME thus far are more broadly expressed throughout the brain (Fig. 4). This difference in expression may partly explain the absence of epilepsy in individuals with SCA37.

**STAR**

STAR is a member of the START (STAR-related lipid transfer) domain-containing family of lipid transfer proteins with functions including intra-mitochondrial lipid transfer of phosphatidylcholine24. Previously, increased levels of choline have been detected by proton magnetic resonance spectroscopy (1H-MRS) in the cerebellum of 11 individuals from three Italian families all shown here to have the ATTTC dynamic mutation25 (Table 1). This observation may be peculiar to FAME2 families since the SAMD12, RAPGEF2, TNRC6A and MARCH6 genes do not have overlapping molecular functions.

In conclusion, we have identified the molecular basis of FAME2 is an inserted expanded ATTTC repeat in the first intron of the STARD7 gene, in 22 pedigrees with 266 affected individuals. The insertion segregates with disease status in 100% of individuals tested from families with linkage or suggestive linkage to chromosome 2 providing substantial genetic evidence that this mutation is causal in this syndrome. The FAME2 locus is one of the few that has been linked to familial cases of FAME2. This is consistent with the observations from lymphoblastoid cell lines (LCLs) derived from individuals with FAME1, where no reads with repeats were found11.

**Methods**

**Ethics.** This study was approved by the Human Research Ethics Committees of the University of Melbourne and the University of Adelaide. Written, informed consent was obtained from all participants in the study.

**Whole-genome sequencing.** Adelaide: Human genomic DNA extracting from peripheral blood lymphocytes was prepared from two individuals in Family 1 (IV-3 and V-118) for sequencing using the TrueSeq Nano DNA Library Preparation Kit (Illumina). Mapping of 150 bp, paired-end sequence reads to the UCSC hg19 build of the genome and calling of single nucleotide variants from whole-genome sequencing (WGS) data generated using an Illumina HiSeqX10 platform (Kinghorn Centre for Clinical Genomics, Sydney, Australia), was performed as previously described with the minor modification of using the Genome Analysis Toolkit (GATK) version 3.8 software27,28. Filtering of both coding and non-coding variants within the chr2 linkage interval shared between both individuals under a dominant model and absent from the gnomAD variant database29 at a frequency >0.001 was performed using the bcftools isec command from htslib v1.9. Single nucleotide variants and indels were annotated with ANNOVAR30. Reads
containing the expanded repeat were visualised using the Integrative Genomics Viewer (IGV) v2.4.5 with soft-clipped reads unmapped.

Rome: WGS library was prepared from the genomic DNA of the individual (PM195; Family 4) by using TruSeq DNA PCR-Free KIT (Illumina, San Diego, CA, USA) and sequenced 150 bp paired-end reads on an Illumina HiSeq producing 470,174,247 fragments, corresponding to about 39X coverage after mapping and removal of duplicated reads. Reads were quality filtered and aligned to the reference human genome sequence (GRCh38/hg38) with BWA-MEM v.0.7.15.32. Resulting BAM files underwent local realignment around insertion-deletion sites, duplicate marking and recalibration steps with GATK v3.8.28. Variant calling was performed with HaplotypeCaller v3.8 with standard parameters, and output VCF files were recalibrated with VariantRecalibrator from GATK v3.8. Genomic variant annotation was carried out with VarSeq v1.4.7 (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com) and only variants with a minimum read depth of 5X were included in the downstream analysis. Thereafter, only variants in the pericentromeric region of interest of chr2 (chr2: 91,800,000–106,700,000) were considered.

Prioritisation of variants of potential interest was carried out through three distinct analyses. For the first analysis, all variants reported to be pathogenic or potentially pathogenic in the clinical databases of ClinVar, HGMD Professional v2017.2 and/or Centogene CentroMD v4.1 were retained. For the second analysis, we focused on variants in exonic regions without a reported clinical annotation. We excluded variants with a population frequency above 1% in the databases of 1000 Genomes Project, National Heart, Lung and Blood Institute (NHLBI, https://www.nhlbi.nih.gov/ Exome Sequencing Project (ESP, http://evs.gs.washington.edu/), ExAC (Exome Aggregation Consortium, http://exac.broadinstitute.org/) and gnomAD (The Genome Aggregation Database, https://gnomad.broadinstitute.org/), along with variants recorded in the Personal Genomics internal database. We retained all the non-synonymous variants predicted to alter the protein structure or function by at least three of the following in silico prediction tools: Mutation Taster, SIFT, Polyphen-2, Mutation Assessor and FATHMM. For the third analysis, we prioritised the variants outside exonic regions by considering rare variants (frequency below 1% in frequency population databases, including the Personal Genomics internal database) and with a predicted significant effect on the protein structure or function by at least three of the in silico prediction tools. Variants were then prioritised by considering their presence in regulatory regions as reported in the ENCODE database (https://www.encodeproject.org/). The manual inspection of the BAM files, by using Integrative Genomics Viewer (IGV), allowed us to evaluate the coverage of the variants and the quality of the aligned reads.

The identification of putative genomic expansions, structural variants or copy number variations was carried out by using Lumpy v0.2.13.35 and Manta v1.2.24 software. The ExpansionHunter tool v2.5.3.14 was adopted to estimate the size of potential repetitions of short unit sequences.

Long-read sequencing. DNA was extracted for all long-read sequencing protocols using the QIAasympomy system from skin fibroblasts (passage 6) cultured in Dulbecco’s modified Eagle’s Medium (DMEM; Life Technologies) with 10% fetal calf serum. Pacific Biosciences (PacBio) single molecule real-time (SMRT) sequencing data were obtained in two batches: In the first batch, two Australian FAME2 carriers (Family 1: IV-44 and IV-98) were sequenced with two flow cells per sample. Resulting bam files were converted to fastq using the SMRT Link software v5.1.0 bam2fastq program. Resulting fastq files were either mapped directly to the human genome hg38 build using NGM-LR 28 with structural variants called by Sniffles 35 or used as input for de novo assembly with Canu v1.7. In the second batch, a single sample (Family 1: IV-98) was sequenced. DNA fragment sizes were determined with the Fermo Pulse capillary electrophoresis system (Agilent Technologies, Santa Clara, CA). DNA fragments of size greater than 6 kb were selected with BluePippin (Sage Science, Beverly, MA) pulsed field gel electrophoresis system. Sequencing was carried out for 20 h per SMRT cell on the Sequel system with Binding Kit 3.0 (PacBio, 101–500–400) and Sequencing Kit 3.0 (PacBio 101–427–800). Circular consensus calling was performed using CCS 3.2.1 software. Reads were mapped to the GRCh38 build of the human genome using pbmm2 with “-c 0 -L 0.01” for CCS reads and “-c 0 -L 0.1” for subreads.

Oxford nanopore data were obtained for DNA samples extracted from fibroblasts from two individuals from Family 1, as described above, and two from Family 5 (II-37 and IV-29 Fig. S2e). For each of the four participant samples, 3 μg of DNA was prepared for Oxford Nanopore 1D genomic sequencing by ligation using the SQK-LSK108 kit and was run on a FLO-MIN106 flow cell for 48 h. Basecalling was performed on MinKNOW 18.01.06 with MinKNOW Core 1.11.5 and Albacore v2.1. Data were either mapped with NGM-LR or assembled with Canu v1.7 as described below, using suggested settings for nanopore sequencing reads.

De novo whole-genome assembly of one individual with input of both PacBio and nanopore sequencing from one individual from Family 1 was carried out using the Canu v1.7 assembler with default starting parameters for a genome size of 3.6 Gbp. Recalibrated reads from Canu were mapped to the hg38 build of the human genome using NGM-LR as described above.

Repeat expansion analysis. WGS was performed for two affected individuals from Family 1 on the Illumina HiSeq X10 platform, one individual from Family 3 as described above, and three affected individuals from Family 19 on the Illumina HiSeq platform. A cohort of 69 individuals without FAME were used for comparison, with 150 bp paired-end sequencing performed on the Illumina HiSeq X platform (Kinghorn Centre for Clinical Genomics, Sydney, Australia). Library preparation for 53 of the samples used the Illumina TruSeq Nano DNA HT Library Preparation Kit; the other 16 samples used KAPA Hyper Prep Kit PCR-free library preparation.

Reads were aligned to the hg19 reference genome with BWA-MEM v0.7.17-r118832, then duplicate marking, local realignment and recalibration were performed with GATK v4.0.3.0.34. Repeat expansion analysis targeting two FAME2 loci, the ATTTT repeat and predicted ATTTT insertion in STARD7, was performed using ExpansionHunter v2.5.3.15 and exSTRA v0.88.3 with Bio-STR-exSTRA v1.0.15. Custom files defining the FAME2-AAAAT and FAME2-AAATG repeat loci were created for ExpansionHunter (below) and exSTRA (Supplementary Table 2).
of four Australian/New Zealand FAME, two Italian FAME and four age-matched stranded libraries with 150-bp mean fragment sizes and 50-bp paired-end Genomics Core Facility. The TruSeq v2 kit (Illumina) was used to generate un-preparation and RNA-Seq were performed as a service by the UCLA Neuroscience cDNA standard made from pooled control.

Values of each gene were normalised to relative standard curve method using SYBR green (Bio-Rad; cat# 1725121) with primers at 1 μM and 0.1 mM Na3VO4). Extracts were separated by 4%––94 °C for 10 s, annealing 56 °C for 30 s, elongation 68 °C 3 min) followed by an additional 20 cycles (94 °C for 1 s, annealing 56 °C for 30 s, elongation 68 °C 45 s + 20 s each cycle elongation for each successive cycle). PCR products were sequenced by electrophoresis on 1% agarose gel. DNA was extracted from the agarose gel slice and the number of repeat units was determined by Sanger sequencing (Eurofins Genomics Sequencing Service).

Repeat-primed PCR. Primers for both Adelaide and Rome are shown in Supplementary Table 3. Adelaide Reaction mixes included 100 ng genomic DNA, 0.5 μM FAM-labelled locus specific (RP-PCR-FAME2-P1 or P2) and RP-PCR-P3 primers, and 0.05 μM repeat specific primer (one of RP-PCR-FAME2–4 to 5.48) with Expand Long Template polymerase (Roche, cat# 25524324) or Taq polymerase (Roche, cat# 18697220). The initial RP-PCR step was at 95 °C for 5 min followed by 10 cycles (95 °C for 30 s, 48 °C + 1 °C each cycle for 45 s and 65 °C + 1 °C each cycle for 30 s) (95 °C for 30 s, 58 °C for 1 min and 72 °C for 5 min) and ending with 72 °C for 7 min. Fragment analysis was performed on the RP-PCR products with an ABI3730 DNA analyser.

Rome: The pentanucleotide repeat sequence in STARD7 gene was amplified by ATTTT and ATTTT RP-PCR with the following primers: STARD7R’ 5’ FAM-labelled (locus specific primer), RP-PCR-STARD7-P3 (generic primer) and RP-PCR-STARD7-P4 primers specific for the short pentanucleotide repeat (ATTTT) and for the possible expanded (ATTTC) repeat or possible (ATTTC) repeat interruption. PCR was performed with 100 ng DNA, 1.5 mM MgCl2, 200 μM dNTP, 0.4 μM locus specific primer, 0.4 μM generic primer, 0.2 μM repeat primer, 2.5 U Polymerase Taq in 25 μl volume. The initial PCR step was at 94 °C for 15 min followed by 35 cycles (94 °C for 45 s, 60 °C for 45 s and 72 °C for 2 min) and 72 °C elongation for 30 min. Capillary electrophoresis was performed on ABI310 GEN ANALYZER (Applied Biosystems).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability Source data for Figs. 1a, 3a, 3b, Supplementary Figs. 1a, b and 4b are provided in the Source Data files of this manuscript. RNA-Seq data are available from the NCBI BioProject PRJNA563467. Whole-genome sequencing data are available from the corresponding author on request, subject to human research ethics approval and patient consent.

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(OffTargetRegions) [
  "chr3:151086374151086421",
  "chr4:2171641221716717",
  "chr6:4867170448671855",
  "chr8:119379052119379154",
  "chr9:113463975113464220",
  "RepeatId": "FAME2-AAAAT",
  "RepeatUnit": "AAAAA",
  "TargetRegion": "chr2:9686280596862859"
]

(OffTargetRegions) [
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  "chr4:2171641221716717",
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  "chr8:119379052119379154",
  "chr9:113463975113464220",
  "RepeatId": "FAME2-AAAAT",
  "RepeatUnit": "AAAAA",
  "TargetRegion": "chr2:968622596862826"
]

Supplementary Figure 1 shows the repeat sizes predicted by ExpansionHunter and empirical cumulative distribution function of repeated bases from exSTRA for the two FAME2 loci. Significance testing was performed using the exSTRA tsm test function with 100,000 permutations in case-control mode comparing each affected individual with FAME to the 69 unaffected individuals without FAME. All FAME2 carriers were significant outliers (p < 0.0001 for all individuals) while only four samples were significant outliers (p < 0.05) for the FAME2-AAAAT locus.

RNA-Seq. Total RNA was extracted from patient-derived primary skin fibroblasts of four Australian/New Zealand FAME, two Italian FAME and four age-matched controls using QiAGEN RNeasy kits, as per the manufacturer’s protocol. Library preparation and RNA-Seq were performed as a service by the UCLA Neuroscience Genomics Core Facility. The TruSeq v2 kit (Illumina) was used to generate un-stranded libraries with 150-bp mean fragment sizes and 50-bp paired-end sequencing performed using the HiSeq2500 (Illumina). Sequence data were mapped to the GRCh38 build of the human genome using HISAT2 v2.0.6. Read counts were generated with StringTie v1.3.5. Differential expression was determined using the exact test from the DESeq2 package in R v3.6.2. Differentially expressed genes were filtered to false discovery rate (FDR) < 0.05 and log base 2-fold change (LFC) > 1 or <-1.

Quantitative PCR. RNA was extracted from four patient-derived primary skin fibroblast cell lines from Family 1 and four control fibroblast cell lines from adult donors not affected by FAME as described above under RNA-Seq. cDNA were generated from 1 μg of total RNA using the iScript reverse transcription kit (Bio-Rad, Gladesville, NSW, Australia; cat# 1708891), according to the manufacturer’s protocol.

Quantification of differentially expressed transcripts was performed with the relative standard curve method using SYBR green fluorescence intensity for detection. Products were amplified in 1 x iTaq Universal SYBR Green supermix (Bio-Rad; cat# 1725121) with primers at 1 μM final concentration. Each sample and standard was amplified with three technical replicates on an Applied Biosystems StepOnePlus. Expression values were determined relative to a dilution curve of a cDNA standard made from pooled control fibroblast cDNA. Specificity of products was determined by melt curve analysis at the conclusion of each run. Expression values of each gene were normalised to HPRT1 expression values from the same sample.

Western blotting. Fibroblasts were cultured as described in Supplementary methods then lysed with lysis buffer (150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.25% Sodium deoxycholate, 50 mM Tris. Added protease inhibitor, 50 mM Nα, Nβ-L-α, β- E64. Extracts were separated by 4%–12% polyacrylamide gel and transferred to nitrocellulose membrane by electroblotting. STARD7 was detected with rabbit polyclonal anti-human/mouse/ rat STARD7 (Proteintech cat# 15869–1-AP) at 1:500 dilution followed by anti-rabbit IgG conjugated to horse-radish peroxidase (HRP) at 1:12000 (Dako cat# P0448). Enhanced chemiluminescent detection (Bio-Rad cat# 1705061) was visualised with the chemidoc detection system (Bio-Rad). Full blots are available in the Source Data file.

PCR amplification and sequencing of repeats (Rome). Pentanucleotide repeats were analysed in duplicate by long-range PCR with Expand Long Template PCR System (Roche) according to the manufacturer’s recommendation. Some 200 ng genomic DNA were amplified with primers STARD7F and STARD7R (500 nM), dNTP (350 μM) buffer (1×), E64 Buffer (1×, 50 μM) and 0.1 mM Na3VO4). Extracts were separated by 4%–12% polyacrylamide gel and transferred to nitrocellulose membrane by electroblotting. STARD7 was detected with rabbit polyclonal anti-human/mouse/rat STARD7 (Proteintech cat# 15869–1-AP) at 1:500 dilution followed by anti-rabbit IgG conjugated to horse-radish peroxidase (HRP) at 1:12000 (Dako cat# P0448). Enhanced chemiluminescent detection (Bio-Rad cat# 1705061) was visualised with the chemidoc detection system (Bio-Rad). Full blots are available in the Source Data file.
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Competing interests

A.W. and S. Chakraborty are employees and shareholders of Pacific Biosciences. There are no other competing interests to declare.

Additional information

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