Exome sequencing of individuals with Huntington’s disease implicates FAN1 nuclease activity in slowing CAG expansion and disease onset

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The age at onset of motor symptoms in Huntington’s disease (HD) is driven by HTT CAG repeat length but modified by other genes. This study, in which we used exome sequencing of 683 patients with HD with extremes of onset or phenotype relative to CAG length to identify rare variants associated with clinical effect. We discovered damaging coding variants in candidate modifier genes identified in previous genome-wide association studies associated with altered HD onset or severity. Variants in FAN1 clustered in its DNA-binding and nuclease domains and were associated predominantly with earlier-onset HD. Nuclease activities of purified variants in vitro correlated with residual age at motor onset of HD. Mutating endogenous FAN1 to a nuclease-inactive form in an induced pluripotent stem cell model of HD led to rates of CAG expansion similar to those observed with complete FAN1 knockout. Together, these data implicate FAN1 nuclease activity in slowing somatic repeat expansion and hence onset of HD.

HD is an autosomal dominant neurodegenerative disorder affecting approximately one in 8,000 individuals. Neuronal loss in the brain leads to a progressive movement disorder alongside functionally debilitating neuropsychiatric and cognitive decline. There is no disease-modifying treatment, and premature death typically occurs 10–30 years after symptom onset.

HD is caused by an expanded CAG repeat tract of at least 36 trinucleotides in the HTT gene. Longer inherited CAG repeat tracts are associated with earlier onset of motor symptoms but account for only ~50% of the observed variation. Approximately 40% of the residual age at motor onset is heritable, powering genome-wide association studies (GWASs) that have identified modifiers of disease course. The most recent Genetic Modifiers of HD (GeM-HD) GWAS identified 21 independent signals at 14 genomic loci after accounting for only ~50% of the observed variation. GWASs can identify common genetic variation associated with a disease or trait. However, understanding pathogenic mechanisms through common variants is difficult: over 90% of GWAS signals are in linkage disequilibrium and LIG1, all of which function in DNA repair. In addition, the pure CAG length of the pathogenic HTT repeat, rather than the length of encoded polyglutamine, is most strongly associated with motor onset. A leading hypothesis is that somatic (non-germline) expansion of the pathogenic CAG repeat in susceptible brain neurons drives symptom onset. In support, the largest somatic expansions are observed in the striatal neurons that degenerate earliest in HD in both human brain and mouse HD models, and the size of expansions in postmortem human HD cortex correlates with age at motor onset. Furthermore, in mouse HD models, knockout of Msh3, Mlh1 or Mlh3 ablates somatic expansions of the CAG repeat, whereas knockout of Fan1 increases expansions, in agreement with directions of effect predicted by human genetics.
non-coding and mostly index gene expression changes\textsuperscript{21}. The combined effect of all GWAS single-nucleotide polymorphisms (SNPs) usually accounts for a maximum of two-thirds of trait heritability, with genome-wide-significant SNPs contributing a small percentage\textsuperscript{22}. Some of the missing heritability is due to rare, damaging, non-synonymous coding variants of larger effect size that are not well captured by GWAS. Identifying such variants has given direct insight into molecular pathogenesis in diseases such as schizophrenia\textsuperscript{23}, Alzheimer’s disease\textsuperscript{24} and amyotrophic lateral sclerosis\textsuperscript{25}. In this study, we sequenced the exomes of 683 patients with HD with extremes of phenotype relative to CAG repeat length and identified rare coding variant modifiers of disease.

**Results**

An HD study population with extremes of clinical phenotype. To maximize power to detect rare modifier variants in our cohort, we included individuals of European origin with extreme HD phenotypes from two independent longitudinal studies. First, we stratified 6,086 participants from the retrospective REGISTRY-HD study by residual age at motor onset, the difference between actual age at motor onset and that predicted by pure CAG length alone\textsuperscript{26}, and selected \textasciitilde4\% at each extreme for investigation (Fig. 1a; 250 early onset, 250 late onset; ‘REGISTRY-HD group’). Second, we selected participants from the prospective PREDICT-HD study\textsuperscript{27} for investigation based on extreme cognitive or motor phenotypes, extreme predicted early or late onset or both (Fig. 1b–d; \textasciitilde11\% at each extreme; total \(n = 238/1,069\); ‘PREDICT-HD group’). CAG lengths used in initial selections came from standard polymerase chain reaction (PCR) fragment length assays that assumed a canonical CAG repeat sequence.

**HTT repeat sequences are associated with altered HD onset.** The canonical polyglutamine-encoding CAG repeat in HTT exon 1 is followed by a glutamine-encoding CAACAG and then a polyproline-encoding CCG/CCA/CCT repeat (Fig. 2, allele groups a and b). This region of HTT 3’ of the CAG tract is highly polymorphic\textsuperscript{8,12,13}. To investigate associations of this polymorphic sequence with motor onset, we sequenced the HTT repeat locus in the REGISTRY-HD group using ultra-high-depth MiSeq sequencing. Samples from 419 individuals passed quality control and were associated with motor onsets at least 5 years earlier or later than expected after correcting for accurate CAG lengths from MiSeq\textsuperscript{12}.

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**Fig. 1 | Selection of HD study population with extremes of onset or phenotype.**

\(a\), REGISTRY-HD group. Age at motor onset against inherited pure CAG length for 6,086 patients with HD with 40–55 CAGs in the REGISTRY-HD study, using repeat lengths previously determined by PCR fragment length analysis. Individuals with very early (orange, \(n = 250\)) or very late (green, \(n = 250\)) motor onset given their inherited CAG length were selected for analysis.

\(b\), c, PREDICT-HD group, extremes of phenotype. Individuals with more severe (red dots) or less severe (blue dots) clinical phenotypes in the PREDICT-HD cohort were selected for analysis. Residuals from LOESS were used to identify individuals using TMS (\(n = 117\)) (\(b\)) or SDMT (\(n = 85\)) (\(c\)) and are plotted against CAP score to visualize age and CAG effects. Higher CAP scores represent greater disease burden.

\(d\), PREDICT-HD group, extremely early or late onset (predicted). A time-to-onset model was used to stratify the PREDICT-HD population and select a further cohort of predicted extreme early (red dots) or late (blue dots) onset individuals (\(n = 119\) selected).
We identified 16 independent HTT repeat structures downstream of the pure CAG repeat, eight of which occurred exclusively on pathogenic, expanded HTT alleles (Fig. 2b). A canonical CAACAG followed the pure CAG tract in 94% of all alleles (Fig. 2, allele groups a and b). The proportion of non-canonical glutamine-encoding repeats was enriched in alleles containing an expanded CAG repeat (41/419 (9.8%)) relative to those with an unexpanded repeat (9/419 (2.1%); \( \chi^2 = 21.8, P = 3.1 \times 10^{-4} \), Fig. 2, allele groups c–h). Within the expanded alleles, the distributions of individual non-canonical sequences were highly skewed. Alleles lacking CAACAG were observed only in 21 individuals with early HD onset relative to their CAG tract lengths (Fig. 2, allele groups c and d; 21/213 early (9.9%) and 0/206 late, \( \chi^2 = 19.4, P = 1.0 \times 10^{-5} \); mean earlier onset = 10.2 years). In contrast, alleles containing an extra CAACAG, CAA or CAC were observed only in 20 individuals with late HD onset relative to their CAG tract lengths (Fig. 2, allele groups e–h; 0/213 early and 20/206 late (9.7%), \( \chi^2 = 19.6, P = 9.0 \times 10^{-5} \); mean later onset = 10.4 years).

GeM-HD GWAS reported SNPs tagging two non-canonical HTT repeat sequences: rs764154313 associated with (CAG)\(_6\)(CCG)\(_{12}\)(CCT)\(_7\) (Fig. 2b, group c, top) and rs183415333 associated with (CAG)\(_6\)(CAACAG)CCGCCCA(CCG)\(_7\)(CCT)\(_2\) (Fig. 2b, group e)\(^3\). Comparing GWAS SNP data to our HTT sequencing results in those individuals with both measures (\( n = 398 \)), rs764154313 tagged six of the nine (CAG)\(_6\)(CCG)\(_{12}\)(CCT)\(_7\) alleles but no other allele lacking CAACAG. rs183415333 tagged (CAG)\(_6\)(CAACAG)CCGCCCA(CCG)\(_7\)(CCT)\(_2\) alleles in 15/16 individuals carrying this sequence in an expanded allele. Overall, SNP data identified 28.6% (6/21) of alleles lacking CAACAG and 75.0% (15/20) of expanded alleles with an extra CAACAG, CAA or CAC, suggesting that non-canonical repeat alleles in HD are significantly underestimated by GWAS.

Much of the apparent effect of non-canonical glutamine-encoding HTT repeat sequences on HD onset has been considered spurious, attributable to inaccurate residual age-at-onset calculations based on assumed canonical allele sequences\(^4\). In our cohort, we found...
that polyglutamine length accounted for 31.0% \((P=7.2 \times 10^{-4})\) of the variance in motor onset, rising to 37.2% \((P=3.1 \times 10^{-8})\) when regressing on pure CAG length (Table 1; \(n=558, 463\) REGISTRY-HD and 95 PREDICT-HD). However, non-canonical HTT repeat sequences in expanded alleles remained significantly associated with age at onset even after accounting for pure CAG tract lengths. Expanded alleles lacking the CAACAG sequence were associated with ~16% decrease in age at motor onset, increasing \(R^2\) by 1.0% \((P=4.0 \times 10^{-3})\). In contrast, expanded alleles with an extra CAACAG, CAA or CAC were associated with ~26% increase in age at motor onset, increasing \(R^2\) by 1.4% \((P=2.0 \times 10^{-4})\). Together, these effects increased \(R^2\) by 2.1% (Table 1). The effects of non-canonical repeat sequences on phenotype were also observed in a larger, dichotomous sample (logistic regression with Firth's correction: \(n=637\); 421 REGISTRY-HD and 216 PREDICT-HD; cohort details below). Lack of the CAACAG sequence was significantly associated with early/more severe phenotype \((P=1.2 \times 10^{-7})\). Additional CAACAG, CAA or CAC were significantly associated with late/less severe phenotype \((P=1.5 \times 10^{-4})\). Therefore, changes in the canonical polyglutamine-encoding HTT sequences explain a small, but significant, proportion of the variance in HD onset not explained by CAG repeat length.

**FANI coding variation is associated with altered onset of HD.** Using data from GeM-HD GWAS, we calculated that all SNPs accounted for 25.3% \((\pm 4.5\%)\) of the residual age at motor onset in HD compared to a reported heritability of 40%. To investigate whether rare protein coding variants not captured by GWAS could account for some of this missing heritability, we used exome sequencing and called sequence variants \((n=683\) after quality control, 465 REGISTRY-HD and 218 PREDICT-HD; Supplementary Figs. 1 and 2). Two groups of exomes were used for downstream association analyses, adjusted for accurate CAG repeat lengths from sequencing (Extended Data Fig. 1a). A dichotomous group \((n=637)\) was divided into early onset/more severe phenotype \((n=315)\) and late onset/less severe phenotype \((n=322)\), whereas a continuous phenotype group included only those with a calculable age at motor onset residual \((n=558)\). We assessed the association between rare non-synonymous coding variants (minor allele frequency \((\text{MAF})<1\%)\) and clinical phenotype in the 13 candidate modifier genes (other than HTT) identified in GeM-HD GWAS using linear regression in the dichotomous group and linear regression in the continuous group. Independent analyses were performed with three groups of variants: all non-synonymous variants; non-synonymous, damaging variants (combined annotation-dependent depletion (CADD) score \(\geq 20\), indicating in top 1% of predicted damaging variants in the genome; NSD20 group); and loss-of-function variants such as nonsense, frame-shift and splice donor/acceptor variants. Covariates corrected for population stratification, sequencing depth, baseline variant rate and study group (REGISTRY-HD or PREDICT-HD). The presence or absence of non-canonical glutamine-encoding HTT repeat sequences (Fig. 2 and Supplementary Table 1) was an additional covariate in logistic analyses. FANI showed a significant signal in the dichotomous non-synonymous analysis after multiple testing correction \((P=2.3 \times 10^{-4})\) and approached significance for NSD20 variants in both analysis groups (Table 2). The associations with non-synonymous and the NSD20 variants in the dichotomous group remained nominally significant \((P=3.8 \times 10^{-4}\) and \(P=4.4 \times 10^{-3}\), respectively) even after the removal of the R377W and R507H variants previously identified in GeM-HD GWAS (Supplementary Table 3a). Furthermore, the burden of rare, damaging variants was not significantly associated with any of the lead FANI variants from GeM-HD GWAS after removal of R377W and R507H (Supplementary Table 3b). Taken together, these results indicate that rare, damaging variation in FANI influences HD onset independently of modifiers previously identified by GWAS.

Five other candidate modifier genes had nominally significant associations in at least one analysis: PMS1, MSH3, TCERG1, RRM2B and LIG1 (Table 2) and were the most significant at their respective genomic loci when assessing all genes (Supplementary Table 4). There were marked skews in the distribution of predicted damaging coding variants in FANI, PMS1 and MSH3; those in FANI were often associated with early/more severe phenotype, whereas those in PMS1 and MSH3 were often associated with late/less severe phenotype (Supplementary Table 5). Rare, damaging coding variants in PMS1 occurred in 14 individuals with late/less severe phenotype, including one loss-of-function frameshift variant, but in just two individuals with early/more severe disease (Supplementary Table 6). Although the distribution of predicted deleterious MSH3 variants was less skewed (eight in the early/more severe group and 14 in the late/less severe group; Supplementary Table 5), loss-of-function variants were found exclusively in seven individuals with late/less severe phenotype. These MSH3 variants were all extremely rare and included four splice acceptor variants and three truncations.

**Exome-wide association analysis highlights FANI.** We explored whether an exome-wide analysis of all genes with sufficient rare, damaging variation (NSD20) would highlight new HD modifiers. Analysis of the dichotomous and continuous phenotype groups did not show any significant exome-wide associations (Supplementary Table 7). FANI was the only gene in the top ten of both dichotomous and continuous analyses \((P<1.0 \times 10^{-8}\) in each case). A candidate pathway analysis, using significant pathways \((q<1.0 \times 10^{-3})\) from GeM-HD GWAS, identified one significant pathway \((q=0.0042578)\); phosphoric ester hydrolyase activity, containing FANI; \(P=9.7 \times 10^{-3}\) and several nominally significant pathways, suggesting that damaging, rare variation may be important in pathways previously associated with common variation (Supplementary Table 8). For example, GO:0006281 (DNA repair) is significantly enriched for damaging.

### Table 1 | The polyglutamine-encoding HTT repeat sequence is significantly associated with age at HD onset after accounting for pure CAG length

| Model | Covariate | \(B\) | \(P\) | \(R^2\) |
|-------|-----------|------|------|------|
| Ln(AMO) - PolyQ length | PolyQ | -0.083 | 7.15 \(E-47\) | 0.310 |
| Ln(AMO) - PolyCAG length | CAG | -0.092 | 3.09 \(E-58\) | 0.372 |
| Ln(AMO) - PolyCAG length + \(I_0\) | CAG | -0.091 | 1.90 \(E-57\) | 0.382 |
| Ln(AMO) - PolyCAG length + \(I_2\) | CAG | -0.092 | 2.67 \(E-59\) | 0.386 |
| Ln(AMO) - PolyCAG length + \(I_0 + I_2\) | CAG | -0.091 | 1.68 \(E-58\) | 0.393 |
| Ln(AMO) - PolyCAG length + \(I_0 + I_2\) | \(I_0\) | -0.168 | 5.93 \(E-03\) | |
| Ln(AMO) - PolyCAG length + \(I_0 + I_2\) | \(I_2\) | 0.225 | 2.97 \(E-04\) | |

Linear regression of the natural logarithm of age at motor onset (AMO) on polyglutamine (PolyQ) or pure CAG (PolyCAG) length with or without covariates to represent non-canonical allele sequences. Covariate \(I_0\) indicates the presence (1) or absence (0) of an expanded HTT allele lacking a CAACAG after the pure CAG tract (Fig. 2, allele groups c and d). Covariate \(I_2\) indicates the presence (1) or absence (0) of an expanded HTT allele with an additional, non-canonical CAACAG or extra CAA or CAC trinucleotides (Fig. 2, allele groups e–h). All samples passing quality control, including ultra-high–depth MiSeq sequencing or capillary electrophoresis (GeneScan) data and allele sequences confidently called from exome sequencing data were included (total \(n=558\); REGISTRY-HD \(n=463\) and PREDICT-HD \(n=95\) where motor onset has occurred). Significant \(P\) values after multiple testing correction for five models \((P<0.01)\) are in bold.
Table 2 | Candidate gene analysis shows that rare coding variation in FAN1 is associated with modified HD phenotype

| Chr | Gene | NS (n = 637) | NSD20 (n = 558) | LoF |
|-----|------|-------------|-----------------|-----|
| 2A  | PMS1 | 2.72E-02    | 2.65E-03        | 3.13E-01 |
| 3A  | MLH1 | 1.72E-01    | 5.58E-02        | NA  |
| 5A  | DHFR | 1.05E-01    | 9.83E-02        | NA  |
| 5A  | MSH3 | 5.96E-02    | 2.78E-01        | 9.51E-03 |
| 5B  | TERC1 | 1.25E-02    | 4.54E-01        | NA  |
| 7A  | PM52 | 1.00E+00    | 9.00E-01        | 5.55E-01 |
| 8A  | RRM28 | 1.69E-01    | NA              | NA  |
| 8A  | UBR5 | 7.47E-01    | 7.23E-01        | NA  |
| 11A | CCDC82 | 5.27E-01   | NA              | NA  |
| 11B | SYT9 | 1.05E-01    | 1.13E-01        | NA  |
| 15A | FAN1 | 2.32E-04    | 6.61E-04        | 2.17E-01 |
| 16A | GSG1 | 9.05E-01    | 9.13E-01        | NA  |
| 19A | LIGI | 9.47E-02    | 7.07E-02        | 5.09E-01 |

SKAT-O of rare coding variants (MAF < 1%) and HD phenotypes for 13 candidate modifier genes from GeM-HD GWAS. Gene-wide variant numbers were regressed on either dichotomous early/more severe or late/less severe phenotypes (n = 637; logistic regression) or a continuous phenotype of residual age at motor onset (n = 558; linear regression). Phenotypes were corrected for common variant association in GeM-HD GWAS. Three different variant groups were tested: all non-synonymous (NS), non-synonymous and predicted damaging to protein function (NSD20), and non-synonymous and loss of function (LoF). Chromosomal loci from GeM-HD GWAS are indicated. Significant associations are in bold (P < 6.4 × 10⁻⁵; Bonferroni correction for 13 genes and six tests); nominally significant associations are in italics (P < 0.05). See also Supplementary Table 4. NA, not applicable due to insufficient variation in the study population.

FAF1 variants that modify HD cluster in functional domains. FAN1 is the most significant gene in both common variant modifier GWAS and our candidate exome analysis. In our cohort, we identified 65 rare non-synonymous FAN1 variants across 62 individuals, including 28 different variants (Fig. 3a) and six previously unreported mutations (K168N, P366R, D498N, D702E, L7131 and R969L). Focusing on variants predicted to be most damaging to protein function (CADD score ≥ 20 or loss of function), we found that 6.8% of our cohort (43/637) were heterozygous for at least one such variant in FAN1 (Fig. 3a,b). Two individuals carried two such variants, although these could not be phased. One individual carried a loss-of-function frameshift variant, ST186SX (15:31197095;G:A). Of those carrying damaging FAN1 variants, significantly more had an early/more severe phenotype than a late/less severe phenotype (odds ratio = 3.43, 95% confidence interval, 1.66–7.09, P = 8.9 × 10⁻⁴; Fig. 3c). Those variants associated mostly with early/more severe HD clustered in the DNA-binding and nuclease domains of FAN1, whereas a small cluster of variants associated with late/less severe HD mapped to the protein–protein interaction domain (Fig. 3b,d). The R377W and R507H variants detected in GeM-HD GWAS, and also found here, map on the DNA-binding domain of FAN1 and are found mostly in individuals with early/more severe disease. Of the seven individuals with late/less severe HD but carrying FAN1 R377W or R507H, one had an extra CAACAG in the expanded HTT repeat tract. Five were also germlinetype in GeM-HD GWAS: one was homozygous and two were heterozygous for the common I219V variant in MLH1 (rs1799977) associated with later-onset HD; one had high predicted FAN1 expression and one had low predicted MSH3 expression, consistent with later-onset HD. Finally, five extremely rare (MAF < 1.0 × 10⁻⁴) damaging variants in the C-terminal nuclease domain were exclusively associated with early/more severe disease. Overall, these data implicate FAN1 DNA-binding and nuclease activities in delaying HD onset.

FAF1 variant nuclease activity correlates with HD onset. The nuclease activity of FAN1 is required for its functions in DNA repair but had no significant effect on CAG repeat expansion in a U2OS cell model system. We first assessed whether four lymphoblastoid cell lines derived from individuals with HD heterozygous for the R507H FAN1 variant were as efficient in ICL repair as four age-matched and CAG-length-matched controls with wild-type FAN1 alleles. Each R507H line was more sensitive to mitomycin C than its matched counterpart (Supplementary Fig. 3a), and, overall, the mean IC₅₀ for the R507H lines was significantly lower (Fig. 4a; P = 1.3 × 10⁻³), implying that R507H is deleterious to FAN1 function, as previously suggested. Next, we selected six predicted deleterious FAN1 variants identified by exome sequencing for in vitro biochemical analysis of nuclease activity. These variants (R377W, R507H, D702E, K794R, R982C and C1004G), as well as wild-type FAN1 and a known nuclease-inactive variant (D981A R982A), were expressed and partially purified from *Escherichia coli* as NusA-His-tagged full-length proteins (Supplementary Fig. 3b,c). The flap endonuclease activities of wild-type and variant FAN1 proteins were assayed on canonical FAN1 substrates with short 5' flaps. Wild-type FAN1 converted 66% of substrate to product in a 10-minute reaction (Fig. 4b, lane 2). The nuclease-inactive double mutant lacked all nuclease activity, as expected (Fig. 4b, lane 7). The predicted damaging FAN1 variants all had reduced nuclease activity compared to wild-type (Fig. 4c), but variants associated mostly with early/more severe phenotype (R377W, R507H, R982C and C1004G) had much reduced activity compared to the two variants found in individuals with late/less severe phenotype (D702E and K794R). There was a significant correlation between mean residual age at onset for individuals harboring each variant and nuclease activity of that variant (P = 2.7 × 10⁻²; Fig. 4d), suggesting that FAN1 nuclease activity mediates its modifier role in HD. Individuals carrying a FAN1 variant with low nuclease activity (<50% of wild-type) are more likely to have early-onset HD relative to that predicted by CAG length alone (Fig. 4e). The low FAN1 nuclease activity in the two individuals with 38 and 39 CAGs, respectively, might be contributing to the full penetrance of these HTT alleles.
FAN1 requires nuclease activity to slow CAG repeat expansion.

Only one HD-induced pluripotent stem cell (iPSC) line, reprogrammed using lentivirus from a patient with 109 CAGs, has previously demonstrated inherent and reproducible HTT CAG expansion in culture32. To identify isogenic lines for assaying repeat expansion, we first confirmed CAG repeat expansion in three further independent iPSC lines derived from the same patient (but reprogrammed using non-integrating vectors): CS09iHD109-n1, CS09iHD109-n4 and CS09iHD109-n5 (ref. 33). CAG repeat lengths ranged from 92 to 122, indicative of a mosaic population and CAG repeat instability, and expanded over time in culture (Supplementary Fig. 4). To establish isogenic iPSC models of HTT CAG repeat expansion, the parent CS09iHD109-n1 and CS09iHD109-n5 (Q109) lines were used for CRISPR genome editing. We investigated the effect of FAN1 by measuring CAG expansion rates in multiple independent isogenic Q109 sub-clones with and without FAN1 knockout (Fig. 5a).
and Extended Data Fig. 2a–c). The HTT CAG repeat expanded significantly faster in Q109-n1 iPSCs lacking FAN1; each modal CAG unit increase occurred in 8.9 days in FAN1−/− cells compared to 33.1 days in FAN1+/+ cells (P = 1.5 × 10⁻⁸; Fig. 5b,c). Expansion in differentiated neurons (Supplementary Fig. 4d) showed a similar effect, although expansion rates were slower (Fig. 5d). Each CAG unit increase occurred in 20.6 days in FAN1−/− neurons compared to 73.0 days in FAN1+/+ cells (P = 2.7 × 10⁻⁷). Despite the slower rate of CAG expansion seen in neurons compared to iPSCs, the ratio of expansion rates FAN1+/−:FAN1−/− was remarkably similar at 3.7× in iPSCs and 3.5× in neurons.

To investigate the importance of FAN1 nuclease activity in slowing HTT CAG repeat expansion, we used CRISPR to introduce a D960A FAN1 point mutation into Q109-n5 iPSCs (Extended Data Fig. 2e–g). D960 coordinates an essential divalent cation, usually Mg²⁺, in the nuclease active site of FAN1. Mutation to D960A is well established to abolish nuclease activity but retain wild-type-DNA-binding capacity, allowing the role of FAN1 nuclease activity to be specifically assayed. Somatic instability of the HTT CAG tract was then assessed in FAN1+/+, FAN1+/−D960A, FAN1+/−D960A and FAN1−/− Q109-n5 iPSCs (Fig. 5e) and showed a nominally significant difference between genotypes (P = 4.5 × 10⁻²). Cells carrying homozygous or heterozygous FAN1D960A alleles had significantly greater rates of change in modal CAG length compared to wild-type cells (P = 2.3 × 10⁻²), but there was no detectable difference among Q109 FAN1+/−D960A, FAN1+/−D960A and FAN1−/− cells (P = 2.3 × 10⁻²). Adding one CAG took 23.9 days in FAN1+/+ cells but 19.3 days in FAN1−/−D960A cells and 15.6 and 16.3 days in FAN1−/−D960A/D960A.
and FAN1+/− cells, respectively. These data suggest an additive effect of FAN1 nuclease activity on expansion.

We also found a significant difference between genotypes in rates of expansion in a single clone of each FAN1D960A genotype in neural precursor cells (NPCs) grown for 42 days (Fig. 5f). A significant effect of genotype on rate of change in modal CAG was observed (P=6.0×10−3). Adding one CAG took 19.1 days in FAN1+/+ cells, 14.8 days in FAN1+/D960A cells and 10.8 days in FAN1D960A/D960A cells. The rate of CAG expansion in FAN1+/+ cells was significantly lower than that in FAN1D960A/D960A cells (P=6.0×10−3), whereas the rate of CAG expansion in FAN1+/− cells trended toward being lower than that in FAN1+/D960A cells (P=6.6×10−2). Fitting an additive model of D960A to CAG expansion rates increases significance over the general model (P=3.0×10−2), suggesting that there is a dose-dependent effect of the D960A mutation on CAG expansion rate, consistent with the effect seen in iPSCs.

**Discussion**

Inherited pathogenic HTT CAG repeat length is a stronger determinant of age at onset of HD than polyglutamine length. Here we have additionally shown that non-canonical glutamine-encoding HTT repeat sequences are significantly associated with HD onset, and that FAN1+/− neurons demonstrate significantly faster rates of HTT CAG repeat expansion than FAN1+/+ neurons (P=1.1×10−2). FAN1+/+ HD-iPSCs show dose-dependent increase in HTT CAG repeat expansion over time in culture (n=3 clones per genotype, each cultured in triplicate wells). Genome editing performed in Q109-n5 iPSCs. D960A mutations enhance HTT CAG repeat expansions in a dose-dependent manner in NPCs derived from FAN1+/+, FAN1+/+D960A and FAN1D960A/D960A iPSCs (n=1 clone per genotype, cultured in triplicate wells). Values are expressed as mean ± s.e.m.
even after correcting for accurate CAG repeat lengths (Table 1). Expanded repeat tracts lacking the canonical 3’ CAACAG were exclusively associated with earlier-onset disease; tracts with extra CAACAG, CAA or CAC were exclusively associated with later-onset disease. GeM-HD GWAS suggested that much of this association is spurious, attributable to mis-sizing of CAG tracts when a canonical repeat sequence is assumed8. The difference in results probably arises from both our extreme phenotype study design, enriching for rare, non-canonical sequences of large effect size, and the underestimation of non-canonical HTT repeat sequences by the SNP genotyping used in GWAS. Non-canonical repeats likely mediate their effects through altered somatic expansion of the genomic pathogenic CAG repeat, with tracts lacking CAACAG expanding more easily and those with extra CAACAG, CAA or CAC expanding less easily. However, alternative mechanisms, including effects on splicing or translation of RNA, and the influence of flanking repeats, are possible.

DNA repair genes that modify HD onset are also likely to function through modulation of somatic expansion of the HTT CAG repeat in the brain36, although an effect on global genomic stability cannot be discounted. Assessing candidate modifier genes from GeM-HD GWAS through exome sequencing, we identified significant numbers of rare, predicted deleterious coding variants in FAN1 and nominally significant numbers in PMS1, MSH3, TCERG1, RRM2B and LIG1 (Table 2). Directions of effect were consistent with GeM-HD GWAS, further implicating these genes as the disease modifiers at their respective loci. Although variant effect sizes in the wider HD population cannot be determined from our extreme phenotype cohort, they are likely to be large. For example, GeM-HD GWAS showed that each FAN1R507H or FAN1R377W allele is associated with 5.2 years or 3.8 years earlier onset of HD, respectively8.

The coding variants we identify are heterozygous in affected individuals, suggesting that either wild-type protein levels are rate limiting for somatic expansion and the variants are hypomorphic or that the variants have dominant-negative or gain-of-function effects. Transcriptome-wide association studies in HD have implicated expression levels of FAN1 and MSH3 in modification of motor

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**Fig. 6 | Model of how FAN1 nuclease activity might prevent repeat expansions.** The fully base-paired (CAG)n(CTG)n tract is in dynamic equilibrium with a four-way junction that includes loop-outs of (CAG)n and (CTG)n on their respective strands. Under normal cellular conditions, most repeats are in their native double-stranded conformation. However, when a longer repeat tract (>35 CAG) is present, it can adopt a more stable four-way structure that can be further bound and stabilized by MSH2/MSH3 (MutSβ) (1). This four-way junction can be cleaved on both strands in either of two orientations (A or B) by MutL complexes (2). The resulting DNA products have long overhangs, either 3’ (A) or 5’ (B), and they can either anneal fully to re-form the starting genomic DNA with no change in repeat tract length (top) or they can slip before partial reannealing (3). Slipped products can have 5’ or 3’ flaps, and these are a substrate for FAN1 nuclease cleavage (bold arrows) and subsequent ligation, yielding repeat contractions (4a). Alternatively, the slipped products can have gaps, and these are substrates for gap-filling DNA polymerases, with subsequent ligation yielding repeat expansions (4b).
onset and progression, respectively, probably through effects on somatic expansion. Notably, the seven individuals carrying a heterozygous loss-of-function variant in MSH3 here all had a late/less severe phenotype. These variants might limit or disrupt the formation of the MSH2/MSH3 (MutSβ) heterodimers that are required to bind and stabilize slipped DNA structures formed by trinucleotide repeats before repeat expansion. Rare variants in PMS1 were also mostly found in patients with late/less severe phenotype (Supplementary Table 5), implicating PMS1 in repeat expansion, as previously suggested in a murine cell model of Fragile X disorders. PMS1 forms a heterodimer with MLH1 (MutLβ), but, unlike PMS2 and MLH3, the other two binding partners of MLH1, PMS1 lacks a known catalytic activity and does not participate directly in mismatch repair. It remains unclear how PMS1 might facilitate repeat expansions.

Previous genetic studies have strongly implicated FAN1 as the HD modifier gene at the chromosome 15 locus, with at least four independent GWAS signals, including the variants encoding R377W and R507H. Our exome-wide association study highlighted FAN1 as the only gene with \( P < 1.0 \times 10^{-4} \) in both dichotomous and continuous analyses (Supplementary Table 7), showing that its association is robust to which HD phenotypes are used. Removing the variants encoding R377W and R507H reduced, but did not ablate, the significance of these associations. Furthermore, the remaining burden of rare, damaging variation in FAN1 in our cohort was not associated with known GWAS signals (Supplementary Table 3b), providing more evidence that rare, damaging variation in FAN1 modifies HD onset in addition to genetic effects captured by GWAS. The rare, predicted damaging variant burden in FAN1 was three-fold higher than expected from population frequencies (genoMA\(^{-2}\)), further suggestive of a modifier effect. Notably, although FAN1[R377W] and FAN1[R507H] are mostly found in those with early/more severe phenotype, they are also seen in seven individuals with late/less severe phenotype (Fig. 3), suggesting that the effects of these variants are themselves modifiable. Indeed, six of these individuals carried an additional modifier associated with later HD onset.

FAN1 has cellular functions in ICL repair, replication fork restart and maintenance of genomic stability, each of which requires its structure-specific nuclease activity. The clustering of rare modifier variants in FAN1 domains provides new insight into how FAN1 might modify HD onset. One cluster between residues 658–794 of FAN1, or its variants, leads to binding, sequestration and effective inactivation of expansion-promoting proteins, such as PMS1, MLH1 and MLH3 (refs. \(^{39,40}\)). Conversely, when FAN1 is expressed from its endogenous promoter, it could be rate limiting for repeat stability and require its nuclease activity to prevent expansions.

Recent evidence highlighting how FAN1–MLH1 interaction might promote accurate repair of DNA loop-outs and slow repeat expansion fits such a model.

The substrate preference of FAN1 nuclease is short DNA flaps. We propose that FAN1 mediates its anti-expansion effects through cleavage of such flaps in expansion intermediates. Overall, this would shift the equilibrium between repeat expansion and contraction at an expanded CAG repeat toward the latter, helping to maintain or reduce repeat lengths. This model predicts that factors favoring repeat expansions, such as MSH3, MLH1, and an expansion-prone \( HTT \) CAG repeat, would be epistatic to FAN1 in determining repeat stability, consistent with our non-canonical repeat observations (Fig. 2) and recent data from HD mouse models. FAN1 nuclease likely has a similar anti-expansion, disease-modifying function in other repeat expansion disorders. For example, \( FAN1 \) variants are associated with altered age at onset of CAG expansion-related spinocerebellar ataxias, and Fan1 inhibits somatic expansion in mouse models of Fragile X disorders.

In conclusion, the genetic architecture of the onset modifier trait in HD is similar to that of other oligo-genic or poly-genic diseases or traits, consisting of both common variants of small effect and rarer variants of larger effect. Our study population was solely of European origin. Rare variant studies in other populations could prove valuable and reveal new genetic modifier signals, as demonstrated for common variants in a Venezuelan HD cohort. Our finding that single, heterozygous coding variants in modifier genes can be associated with clinically relevant changes in HD onset or severity suggests that drugs targeting individual modifiers, or their regulators, could be effective. Such therapeutics are already in development.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at [https://doi.org/10.1038/s41593-022-01033-5](https://doi.org/10.1038/s41593-022-01033-5).

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Methods

Clinical sample selection and CAG length determination. Human subjects were selected from two HD patient cohorts of European origin. The first cohort was from the European REGISTRY-HD study58, which primarily enrolled individuals with HD and clinical onset. In total, 6,086 individuals had both a known inherited pure CAG length (40–55 CAGs) and an age at onset of motor symptoms. Of these, 3,046 had CAG lengths determined in line with REGISTRY protocols (https://www.enroll-hd.org/enrollhd_documents/2016-10-R1/REGISTRY-protocol-3.0.pdf), and 3,040 had CAG lengths determined by local laboratories. CAG lengths on which samples were selected were determined by a standard PCR fragment length assay and assumed a canonical glutamine-encoding repeat sequence in HTT. The residual age at motor onset was calculated for each participant by subtracting the expected age at onset given pure CAG length from the observed age at motor onset43,44. Expected age at onset was estimated using pure CAG length based on the Langbehn equation that was derived from the HD cohort39. For 38,39, 40 CAGs, we extrapolated in 59 years, respectively, extrapolating from the Langbehn model. Observed age at motor onset was determined as follows: when onset was classified as motor, oculomotor or mixed, the rating clinician’s best estimate for motor onset was used (exrater). For non-motor onsets, or where the clinician’s onset estimate was missing, the motor component of the clinical characteristics questionnaire was used instead (ccmtrage). The REGISTRY-HD population was stratified by residual age at onset and 250 (4–4%) at each extreme of the distribution selected for analysis (exact samples depending on DNA availability), along with seven technical exome-sequencing controls of different CAG repeat lengths and residuals of less than 1 year. After targeted HTT CAG repeat sequencing using MiSeq (Fig. 2), we determined accurate age at motor onset for the REGISTRY-HD population using sequenced pure CAG lengths. These corrected residuals were used in all subsequent analyses.

The second cohort (n = 238) was from the global (mainly US) PREDICT-HD study (total n = 1,069) that enrolled healthy-at-risk HTT mutation carriers and prospectively followed them to clinical onset over 10 years27. Phenotypic extremes (consistently in the top or bottom 20% were selected as ‘consistent phenotypic extremes’ for the exome analysis58. For each visit, individuals were ranked by the residuals, and those who were extreme for non-canonical alleles, as unexpanded CAG lengths were short (<1 year). After targeted HTT CAG repeat sequencing using MiSeq (Fig. 2), we determined accurate age at motor onset for all PREDICT-HD samples, reads were extracted from whole-exome data using SAMTools59 using region 4:3073000–308000. Reads were mapped to the human genome (hg19; UCSC Genome Browser, UCSC Genome Browser (http://genome.ucsc.edu). Pooled libraries were normalized to 2 nM, and flow cell cluster amplification and sequencing were performed according to the manufacturer’s protocols using the HiSeq 2500. Each run was a 75-bp paired end with a dual eight-base index barcode read. Data were analyzed using the Broad Picard Pipeline, which includes de-multiplexing and data aggregation.

For the PREDICT-HD cohort, an in-solution DNA probe based hybrid selection method was used to generate Illumina exome sequencing libraries from blood DNA60,61. The exome library specifically targets approximately 37.7 Mb of mainly exonic territory made up of all targets from Agilent SureSelect V2 kit (Illumina). Whole-exome sequencing. A formal power calculation for exome sequencing of 80 samples taken from the extremes of the REGISTRY-HD population, based on residual age at onset, gave 88% power to detect individual variants of similar effect size to those reported in GeM-HD GWAS at a significance level of 5%. Ultimately, we sequenced as much larger sample as was feasible. For the REGISTRY-HD cohort (n = 307), sequencing was performed at Cardiff University. Patient DNA derived from low-passage lymphoblastoid cells was obtained from the European HD Network (EHDN, projects 0791 and 0803; DNA prepared by BioKeip). Whole-exome libraries were generated using TrueSeq rapid exome library kits (Illumina). Libraries were pooled in equimolar amounts in groups of 96 and run over eight lanes on a HiSeq 4000 patterned flow cell. Clustering used Illumina ExAmp reagents from a HiSeq 3000/4000 PE cluster kit (Illumina) on a cBot system. Sequencing used a 2 × 75bp end run with a HiSeq 3000/4000 SBS kit for 150 cycles (Illumina). Note that the exome sequencing data include significant coverage of promoter and untranslated regions due to the use of the TrueSeq rapid exome library kit (Illumina).

For Sanger sequencing, all FAN1 variants identified by exome sequencing were confirmed by Sanger sequencing in the REGISTRY-HD sample. Amplifications for Sanger sequencing were performed using MyAq (BioLine) using ~20–60 ng of template DNA, and reactions were cleaned up with QIAquick PCR purification columns (Qiagen). Then, 1.5 µl of the relevant sequencing primer (25 µM) was added to each purified sample (Supplementary Table 9), and sequencing was performed using the Eurofins Genomics LIGHTRUN service.

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FAN1 structural modeling. FAN1 structure annotation (Fig. S4) was performed using I-TASSER in complex with DNA62. This structure (Protein Data Bank ID: 4R81) contained residues 370–1,017. Two small loop sections (510–518 and 799–810) were missing and added to the model using the homology model building tools within the Molecular Operating Environment.

Lymphoblastoid cell line culture and mitomycin C survival assay. Lymphoblastoid cell lines from individuals with HD were obtained from EHDN (projects 0791 and 0803). Lines were cultured in RPMI-1640 GlutaMAX (Thermo Fisher Scientific) supplemented with 15% FBS and 1% penicillin-streptomycin and passaged three times per week. For the mitomycin C survival assays, cell lines (viability > 80%) were seeded in triplicate at 20,000 cells per well in 12-well plates, treated once with mitomycin C (0–150 nM, SelleckChem) and cultured for a further 7 days before viable cell counts were determined by trypan blue staining (Countess II, Thermo Fisher Scientific). Each experiment was independently repeated three times. Differences in means was tested using Student’s t-test (two-tailed). Data distribution was assumed to be normal, but this was not formally tested.
Protein purification and nuclease assays. Full-length Nus-His-FAN1 proteins were expressed in E. coli and partially purified in one step using cobalt agarose®. Further purification of FAN1 proteins was attempted, but active protein yields were low. iPSCs were cultured on Geltrex-coated plates (37 °C, 1 hour) (Life Technologies) in Essential 8 Flex medium (Life Technologies) under standard culturing conditions (37 °C, 5% CO2). iPSCs were passaged into replicate plates using Gentle Cell Dissociation Reagent (STEMCELL Technologies). For screening, DNA was extracted using QuickExtract (Cambio) (room temperature, 10 minutes; 65 °C, 6 minutes; 95 °C, 2 minutes) and PCR amplified using two primer pairs amplifying exom 2 of FAN1; FAN-KO, 5′-CCTGTTTGTATTTGCTGACAGA-3′ and 5′-CCATTCCAGACGAGGCTG-3′ and FAN1-T7, 5′-TCAGATGTTGCTTCTTCCCT-3′ and 5′-GATGCCTACCCGTTCAGCA-3′. Amplicons were visualized on a 1.5% agarose gel on the Gel Doc XR system (Bio-Rad). Sanger sequencing was used to confirm successful editing.

FAN1 D960A variant. D960A was introduced by using cellular homology-directed repair (HDR). A single gRNA sequence (5′-GGGGGCTTCCTCGTTTCTC-3′) and a 122-bp repair template containing the desired edit (5′-AGGaGGCCTCCCCGcGCTGTG-3′) was used for HDR. The HDR template contained two silent mutations (5′-AGGaGGCCTCCCCGcGCTGTG-3′) which was in the PAM sequence to prevent further excision, and introduced restriction sites allowing efficient screening. Nucleofection was carried out as above. The DNA sample was extracted using QuickExtract and PCR amplified using two primer pairs: Faul-D960A, 5′-TCACGGAGGAAAGTGCTCAAC-3′ and 5′-GCCAAACGACCTCAGAAAGAT-3′ and Stul-D960A, 5′-TCACGGAGGAAAGTGCTCAAC-3′ and 5′-GCAACTCCGACGCGCGTGCG-3′. Cloning was performed by EcoR1 and BamHI restriction digestion, and transformed with Faul or Stul in CutSmart Buffer (NEB). Amplicons were separated on a 1.5% agarose gel and visualized on the Gel Doc XR system (Bio-Rad). Sanger sequencing was used to confirm successful editing.

PCR-based fragment size analysis of HTT exon 1 repeat loci in Q109 iPSCs. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen), and 200 ng (50 ng/µl) was used for genotyping. Samples were genotyped on the Infinium PsychArray-24 Kit (Ilumina) or the Infinium Global Screening Array-24 (Illumina) and scanned using the iScan System (Ilumina). Data were exported from Genome Studio and analyzed using PennCNV®. Sample-level quality control was applied based on the standard deviation of log R ratio set at 0.3, minimum SNP number of 10 and minimum region size of 100,000 bp.

HD iPSC model. The human iPSC lines CS09HD109-n1, CS09HD109-n4 and CS09HD109-n5 (herein referred to as Q109-n1, Q109-n4 and Q109-n5) are independent lines generated previously from a human HD fibroblast line, ND39258 (RRID:CVCL_ZC78), with an expanded HTT allele initially containing 109 repeats. iPSCs were passaged every 3–4 days using ReLeSR (STEMCELL Technologies) and seeded at a split ratio of 1:12. Full media changes were performed every 1–2 days. For differentiations, iPSC colonies were dissociated into a single-cell suspension using Accutase (Life Technologies), seeded into 12-well plates coated with Growth Factor Reduced Matrigel (0.5 ng ml−1 of BSA and 0.2 mM ascorbic acid). After 14 days in SJB medium, cells received half changes were performed daily up until day 16. Day 16 NPCs were passaged into replicate wells of a 96-well plate, which, after 7 days, were passaged into replicate plates using Gentle Cell Dissociation Reagent (STEMCELL Technologies). For screening, DNA was extracted using QuickExtract and PCR amplified using two primer pairs amplifying exom 2 of FAN1; FAN-KO, 5′-CCTGTTTGTATTTGCTGACAGA-3′ and 5′-CCATTCCAGACGAGGCTG-3′ and FAN1-T7, 5′-TCAGATGTTGCTTCTTCCCT-3′ and 5′-GATGCCTACCCGTTCAGCA-3′. Amplicons were visualized on a 1.5% agarose gel on the Gel Doc XR system (Bio-Rad). Sanger sequencing was used to confirm successful editing.

Cell immunocytochemistry. iPSCs and neurons were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Cells were permeabilized in 0.1% Triton-X in PBS for 20 minutes at room temperature and blocked in 3% BSA with 0.1% goat serum for 1 hour. Primary antibodies used were OCT4 (Abcam, ab19857, 1:100), β-III-tubulin (UpState, 05-661, mouse monoclonal, 1:10,000). Donkey anti-mouse and donkey anti-rabbit Alexa Fluor 568 and Alexa Fluor 488 (Invitrogn, A11001, 1:50) and Alexa Fluor goat anti-rabbit antibody 568 (Invitrogn, A11011, 1:800) were used as secondary antibodies.

Immunoblotting. Cells were washed once in DPBS and lysed with RIPA buffer (Sigma-Aldrich) containing Complete, EDTA-free Protease Inhibitor Cocktail Tablets (Merck). Protein samples were denatured at 70 °C for 10 minutes in 4× NuPAGE LDS Sample Buffer (Life Technologies). Next, 40 µg of cell protein extract per sample was separated on NuPAGE 4–12% Bis-Tris gradient gel (Life Technologies) according to PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific) using MOPS SDS NuPAGE Running Buffer (Invitrogn) for 200 V for 50 minutes. Where purified proteins were run, 30 ng of protein per lane was loaded. Gels were then transferred to methanol-activated Immobilon-P PVDF membranes (Sigma-Aldrich) using NuPAGE Transfer Buffer (Invitrogn) at 120 V for 45 minutes. The membrane was blocked in 5% milk in PBS-T and incubated overnight at 4 °C with anti-FAN1 (CHD1, sheep polyclonal, 1:1,000) and anti-β-tubulin (UpState, 05-661, mouse monoclonal, 1:10,000). Donkey anti-mouse IgG Alexa Fluor 680 (Invitrogn, A27828, 1:10,000) and IRDye 800CW donkey anti-goat IgG secondary antibody (LI-COR Biosciences, 926-32214, 1:15,000) were used as secondary antibodies. Immunoblots were visualized with the Odyssey CLx Imaging System using β-tubulin as a loading control.

Reagents. The sequences of all oligonucleotides used in this project are provided in Supplementary Table 9. The names and catalog numbers (where appropriate) of all reagents used in this project are provided in Supplementary Table 10.

Quantification and statistical analyses. Exome sequencing: alignment and variant calling. We used a standard Genome Analysis Toolkit (GATK, version 3) best practices workflow for the alignment and variant calling of both sets of exomes® [1]. Read alignment and indel calling was performed using Picard (https://github.com/broadinstitute/picard). Alignment used BWA-MEM® to the hg19/GRCh37 genome assembly. Local insertion/deletion realignment used GATK, and
duplicate reads were marked and reads aggregated across lanes with Picard. Base quality scores were recalibrated using GATK's base quality score recalibration (BQSR). Germline SNPs were called with GATK's haplotype caller. Variant quality score recalibration (VSQR) was performed on both SNPs and insertions/deletions using GATK's recommended parameters for exome sequencing.

Exome sequencing: quality control and annotation. Whole-exome data were subject to a multi-step quality control pipeline (Extended Data Fig. 1a). Picard's Collective HydroMet was used to assess target exome coverage. Samples with conflicting duplicates were marked and reads aggregated across lanes with Picard. Base coverage was retained. Sex imputation used Peddy77; samples with conflicting imputed and recorded sex were excluded. One individual with originally unknown sex was kept. Ancestry was estimated using Peddy by principal component analysis (PCA) against genomes from the 1000 Genomes Project phase 3 (refs. 7, 26). Samples were excluded if they were either (1) predicted to have non-European ancestries by Peddy or (2) outside the primary cluster of European samples in Supplementary Fig. 1. We focused on cohorts of European origin because HD is more common in these populations, and most of the large longitudinal studies, such as REGISTRY-HD and PREDICT-HD, have individuals of non-European ancestries to power rare variant association analyses. First-degree relatives were identified using Hall's genetic relatedness matrix function, with a cutoff of 0.125. For each pair of related individuals, the individual with the most extreme uncorrected residual age at motor onset was retained.

Exomes underwent a multi-step annotation pipeline (Extended Data Fig. 1b). Exomes were annotated from ADNPS version 2.1.4.0 (refs. 78, 79), dNSpS version 2.1.1 (refs. 80, 8), and Variant Effect Predictor (VEP) version 95 (ref. 3). Homozygotes were defined as having ≥90% reads of either the reference or the alternative allele, whereas heterozygotes were defined as having between 25% and 75% of the reference or alternative allele. Loss-of-function calls were defined as 'HIGH'-impact calls by VEP and non-synonymous, damaging calls were defined as 'either 'HIGH' or 'MODERATE' calls. Non-synonymous, damaging calls included loss-of-function calls or non-synonymous calls with ≥20 CADD score. Hall was used for PCA. Baseline variant rates were determined for each exome as the total number of variant classes at various MAFs.

In total, 683 exomes passed quality control. After annotation, we identified 311,960 high-confidence (≥75% call rate and ≥98.50 VQSRS) non-reference variants (Supplementary Fig. 2), with a mean of 35,808 variants per individual. There were 150,139 different non-synonymous variants (moderate- and high-impact variants) in our cohort with a mean of 12,700 such variants per individual, similar to those reported in the ExAC study76. Where there were duplicate samples, the exome with the highest quality score recalibration (VQSR) was performed on both SNPs and insertion/deletion variants using GATK’s recommended parameters for exome sequencing.

Exome sequencing: association analyses of rare variation. Coding variants that modify phenotype are likely to be deleterious to protein function. Therefore, our primary association analyses used variants meeting either of the following two criteria: (1) loss-of-function variants (frameshifts, start/stop lost, premature stop codons and splice donor/acceptor variants) or (2) non-synonymous variants with a CADD-PHIRED score ≥20 (that is, the 1% predicted most damaging in the human genome)74. Additionally, variants were required to have MAF <1%, as defined by the European cohort of gnomAD (version 2.1.1)25. To be included, variants required a call rate ≥75% and VSQR ≥98.50. Secondary analyses were performed on loss-of-function and all non-synonymous variants, separately.

Given that codon variants of interest are individually rare, we collapsed qualifying coding variants on genes in the exome and tested each gene with at least ten variants (n = 3,912 genes (dichotomous group); n = 3,198 genes (continuous group)) for association with residual HD onset using the Optimal Sequence Kernel Association Test (SKAT-Ω)87. SKAT-Ω combines elements of both a burden test and a Sequence Kernel Association Test (SKAT) and, therefore, does not assume that all variants in a gene behave the same direction or effect. The dichotomous group (n = 637) was analyzed using logistic regression, with late/less severe phenotype coded as 0 and early/more severe phenotype coded as 1. The continuous group (n = 558) was analyzed using linear regression. We included as covariates population principal components 1–5 (to correct for population stratification), the baseline variant rate (number of variants per variant class examined), mean sample depth and study group (REGISTRY-HD or PREDICT-HD). Additionally, the presence or absence of non-canonical HTT repeats in the expanded allele was a covariate in logistic analyses. Baseline variant rate was calculated for each individual and represented the total number of variants observed in the exome that passed quality control at the particular MAF/damaging filter being used. Multiple testing correction was performed using Bonferroni correction for the number of genes tested in each analysis. A burden test was additionally run using the same cutoffs and covariates as SKAT-O on the logistic patient group (n = 637) using a Wald logistic burden regression test, implemented in Hail.

Note that SKAT-O analysis without correcting for non-canonical HTT repeat sequences and accurate CAG repeat lengths from sequencing found one exome-wide significant signal in NOP14 on chromosome 4, 130 kbp upstream of HTT (P = 8.3 × 10−14, continuous analysis). We found that NOP14 R697C (c.42943419G>A) is in strong linkage disequilibrium with the pathogenic CAG6 (CAACAG), HTT allele (Fig. 2, allele group e, R² = 0.902), explaining the association of NOP14 variation with HD phenotype. Correction for HTT repeat sequence and accurate CAG length ablated this signal.

We also tested whether the association of rare variation with onset observed in FAN1 was independent of the previous GWAS results in two ways (Supplementary Table 3). First, we ran the SKAT-O analysis on the logistic patient group (n = 637) after removing the R377W and R307H variants that were reported as being associated with GWAS. Second, we tested whether the association of rare, damaging variation on each of the four lead variants identified by GWAS by logistic regression. This analysis was performed in the 441 individuals with both exome sequencing and GWAS data. The burden of rare, damaging variation was defined both including and excluding R377W and R307H.

Statistical modeling and analysis of iPSC data. Data collection and analysis were not performed blinded to the conditions of the experiments. No data points were excluded from analyses. The primary outcome measure was change in modal CAG from its initial value. Secondary analyses looked at changes in expansion and instability index. All outcome measures are zero when time is zero, requiring regression models without intercepts to be fitted. These are detailed below.

D960-D42 iPSC data: data consisted of three wild-type, three FAN1+/−D960A heterozygous, three FAN1−/−D960A clonal lines and two FAN1−/− knockout lines. Each clonal line was cultured in triplicate wells that remained independent from one another for the duration of the experiment and were repeatedly measured at different time points. Observations are, therefore, correlated if they are taken from the same line and/or well, and it is important that statistical analyses take these correlations into account. This was done by performing mixed effects linear regression using the lmer() function in R, fitting random effects for the variation in rate of change of outcome between lines and wells. The models fitted were:

\[
m_0 \sim lmer(change \sim 0 + time + (0+time/line) + (0+time/well))
\]

\[
m_1 \sim lmer(change \sim 0 + time + timegeno + (0+time/line) + (0+time/well))
\]

The significance of different genotypes on rate of change of outcome is calculated by anova(m1,m0).

Gene expression was initially scaled as a four-level factor: 1 = WT/WT, 2 = R960A/WT, 3 = R960A/R960A and 4 = −/−, giving a 3-df test. Post hoc analyses on the pattern of genotype differences were performed by fitting models with restrictions on the genotype effects and comparing (via ANOVA) to the general 3-df model. Estimates of expansion rates for each genotype were produced by the R command m2 (ref. 83), and six lines of N1−/−FAN1 knockout lines and two FAN1−/− knockout lines were included with the m2().regression models that were used for the D960A-D42 iPSC experiment. FAN1 knockout (neuronal data): Data consisted of seven wild-type (FAN1+/+) lines and six FAN1 knockout (FAN1−/−) lines in this experiment. Three of the FAN1−/− lines and two of the FAN1−/− lines were cultured in triplicate wells, the remainder in single wells. Wells remained independent from one another for the duration of the experiment and were repeatedly measured at different time points. Effects of genotype on the rate of change of the different phenotypes were tested using the same regression models that were used for the D960A-D42 iPSC experiment. FAN1 knockout (neuronal data): Data consisted of five lines of N1−/−FAN1−/− and four lines of N1−/−FAN1−/−. A separate, independent well was taken from each line at each time point. So, observations are correlated only through shared line, not shared well.

Effect of genotype (+/− versus −/−) on outcome was tested by fitting the following two zero intercept mixed effect linear models:

\[
m_0 \sim lmer(change \sim 0 + time + (0+time/line))
\]

\[
m_1 \sim lmer(change \sim 0 + time + time + (0+time/line))
\]

Again, the effect of genotype on the rate of change of the measures is tested by anova(m0,m1).
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Author contributions

T.H.M. and L.J. conceived the overall study. M.E.M., J.D.L., E.H.A., J.F.G., N.D.A., L.J. and T.H.M. conceived the exome sequencing. B.M., J.D., C.S.B., S.P., U.C., G.E., J.S., L.-N.S., G.M., M.C., A.M., G.M., J.M.C., E.P.H., J.F.G., M.E.M., V.C.N., P.H. and T.H.M. performed data analysis. D.L., B.L., G.B.L., A.E.R., J.S.P. and REGISTRY and T.H.M. performed data analysis. D.L., B.L., G.B.L., A.E.R., J.S.P. and REGISTRY and T.H.M. wrote the original manuscript, with all authors reviewing and editing subsequent drafts.

Competing interests

V.C.W. is a scientific advisory board member of Triplet Therapeutics, a company developing new therapeutic approaches to address triplet repeat disorders such as Huntington's disease and myotonic dystrophy. V.C.W.'s financial interests in Triplet Therapeutics were reviewed and are managed by Massachusetts General Hospital and Mass General Brigham in accordance with their conflict of interest policies. V.C.W. is also a scientific advisory board member of LoQus23 Therapeutics and has provided paid consulting services to Alnylam. J.-M.L. is on the scientific advisory board of GenEdit. J.D.L. is a paid advisory board member for E. Hoffmann-La Roche and uniQure biopharma and is a paid consultant for Vaccinex, Wave Life Sciences, Genentech, Triplet Therapeutics and PTC Therapeutics. E.H.A. serves on a Data Safety Committee for fragment analysis.

Reporting Summary

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

Data availability

Phenotypic data, variant call files and MiSeq data are available from the European Genome-phenome Archive (EGA). BAM files of exome sequencing data are available from EGA (REGISTRY-HD) or dbGap (PREDICT-HD) https://www.ncbi.nlm.nih.gov/gap/, accession number phs000371.v2.p1). Access to the EGA datasets will be provided on reasonable request through a data access committee coordinated by the corresponding authors. Biological materials derived in this work (edited iPSC lines and FAN1 expression plasmids) are available upon reasonable request from the corresponding authors. Source data are provided with this paper.

Code availability

Whole-exome sequences were aligned and variants were called using a GATK-based pipeline (https://gatk.broadinstitute.org/hc/en-us), and variant annotation and quality control was performed with a hybrid pipeline of the Manta package (https://github.com/Leeshawn/SKAT/), and MiSeq data used ScaleHD (https://scalehd.readthedocs.io/en/latest/). Finally, analyses of GeneScan data used AutogeneFy (https://github.com/BrandufMul/AutoGenescan).

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Monitoring Board for Roche. G.B.L. has provided consulting services, advisory board functions, clinical trial services and/or lectures for Allergan, Alnylam, Amarin, AOP Orphan Pharmaceuticals, Bayer Pharma, the CHDI Foundation, GlaxoSmithKline, F. Hoffmann-La Roche, Ipsen, Isis Pharma, Lundbeck, Neurosearch, Medesis, Medivation, Medtronic, NeuraMetrix, Novartis, Pfizer, Prana Biotechnology, Sangamo/Shire, Siena Biotech, Temmler Pharma and Teva Pharmaceuticals. G.B.L. has also received research grant support from the CHDI Foundation, the Bundesministerium für Bildung und Forschung, the Deutsche Forschungsgemeinschaft and the European Commission (EU-FP7, JPND). His study site in Ulm has received compensation in the context of the observational Enroll-HD Study from Teva, Isis, F. Hoffmann-La Roche and the Gossweiler Foundation. He receives royalties from Oxford University Press and is employed by the State of Baden-Württemberg at the University of Ulm. A.E.R. is chair of the European Huntington’s Disease Network executive committee and is the global PI for Triplet Therapeutics. J.S.P. has provided consulting services, advisory board functions and clinical trial services for Acadia, F. Hoffman-La Roche, Wave Life Sciences and the CHDI Foundation. J.F.G. is a scientific advisory board member and has a financial interest in Triplet Therapeutics. His National Institutes of Health-funded project is using genetic and genomic approaches to uncover other genes that significantly influence when diagnosable symptoms emerge and how rapidly they worsen in Huntington’s disease. The company is developing new therapeutic approaches to address triplet repeat disorders, such as Huntington’s disease, myotonic dystrophy and spinocerebellar ataxias. His interests were reviewed and are managed by Massachusetts General Hospital and Mass General Brigham in accordance with their conflict of interest policies. J.F.G. has also been a consultant for Wave Life Sciences. Within the last 5 years, D.G.M. has been a scientific consultant and/or received honoraria/stock options/research contracts from AMO Pharma, Charles River Laboratories, LoQus23, Small Molecule RNA, Triplet Therapeutics and Vertex Pharmaceuticals. L.J. is a member of the scientific advisory boards of LoQus23 Therapeutics and Triplet Therapeutics. T.H.M. is an associate member of the scientific advisory board of LoQus23 Therapeutics. B.M., J.D., C.S.B., S.P., U.C., G.E., J.S., S.I., L.E., L.-N.S., E.R., G.M., M.C., A.M., M.J.C., E.P.H., D.L., M.E.M., N.M.W., N.D.A. and P.H. have nothing to disclose.

Additional information
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Extended Data Fig. 1 | Quality control and annotation pipelines for HD exome sequencing data. a, Quality control pipeline showing where and why sequencing samples were removed from the dataset. From an initial 785 sequenced exomes, including some samples re-sequenced due to initial low quality, 683 passed all quality control steps (465 from REGISTRY-HD, 218 from PREDICT-HD). Subgroups of this population were used in downstream analyses: a continuous group (N = 558) containing all individuals with a known age at motor onset and a dichotomous group (N = 637) containing all individuals with an extreme phenotype, either early or late actual or predicted onset of symptoms, or more or less severe motor or cognitive symptom scores. See also Fig. 1 and Supplementary Fig. 1. b, Annotation pipeline indicating the pathway, databases (gnomAD & dbSNFP) and tools used to annotate individual variants across exomes. Key: VEP, variant effect predictor tool. See also Supplementary Fig. 2.
Extended Data Fig. 2 | See next page for caption.
**Extended Data Fig. 2 | FAN1 knockout and D960A editing using CRISPR-Cas9.**

a, Schematic depicting CRISPR-Cas9 targeting of exon 2 of FAN1 in Q109-n1 and Q109-n5 using two guide RNAs (gRNAs) to induce a 94 bp deletion leading to a premature stop codon and FAN1 knockout. The primer pair used for PCR screening of exon 2 after CRISPR is also shown. 
b, Diagnostic PCR screen using primers FAN1-KO-F and FAN1-KO-R showing representative banding patterns for iPSC lines with the three possible FAN1 genotypes after CRISPR: FAN1/+ (230/230 bp; wild-type), FAN1+/− (230/135 bp) and FAN1−/− (135/135 bp). 
c, Sanger sequencing of PCR products demonstrates the targeted 94 bp deletion in exon 2 of FAN1. 
d, Undifferentiated iPSCs stained for the pluripotency marker OCT4. iPSC-derived neurons stained positive for the neuronal marker MAP2 (red) and CTIP2 (green). All nuclei are counterstained with DAPI (blue). 
e, Schematic depicting CRISPR-Cas9 targeting of exon 13 of FAN1 in Q109-n5 using a homology directed repair (HDR) template. A single guide RNA sequence (grey) and a 122 bp HDR template containing the desired gene edit coding for an amino acid change (D960A) were utilised to generate FAN1-nuclease dead clones. The HDR template contained two silent mutations (lowercase) to prevent Cas9 re-cutting of the edited region and to introduce a SfiI restriction site for diagnostic screening. 
f, Restriction digest with SfiI confirms Q109-n5 FAN1+/+, Q109-n5 FAN1+D960A and Q109-n5 FAN1D960A/D960A genotypes. SfiI cleaves the 442 bp PCR product into 124 and 318 bp products only in the presence of the silent 2868 G > A mutation. The parental Q109-n5 line is also shown as a negative control (right). 
g, Sanger sequencing of PCR products confirms successful introduction of D960A variant. 
h, Virtual karyotyping of iPSC lines using copy number variant (CNV) analysis at the time of repeat expansion experiments. CNV analysis reveals small deletions at 2q22.1 and 14q24.3 and duplications at 12q14.2 in all samples. Duplication of chromosome 1 shown in all but one sample.
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Software and code

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Data analysis

Whole exome sequences were aligned and variants called using a Genome Analysis Toolkit (GATK)-based pipeline (v3.6-0g89b7209; https://gatk.broadinstitute.org/hc/en-us), and variant annotation and quality control used Hail v0.1-5a67787 (https://hail.is/). SKAT-O analyses were performed in R using the SKAT package (v2.0.1; https://github.com/leeshawn/SKAT/), and MiSeq data used ScaleHD (0.322; https://scalehd.readthedocs.io/en/latest/). Analyses of genescan data used Autogenescan (v1.5.1; https://github.com/BranduffMcli/AutoGenescan), which uses the Fragman package (v1.0.9; Giovanny Covarrubias-Pazaran et al. 2016, BMC Genetics 17: 62), and GeneMapper v4.1 (Applied Biosystems). Details and versions of all software packages used are provided in Supplementary Table 10.

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Phenotypic data, variant call files (VCFs) and MiSeq data are available from the European Genome-Phenome Archive through a data access committee (EGA; ega-archive.org, accession number XXX). BAM files of exome sequencing data are available from EGA (REGISTRY-HD) or dbGaP (PREDICT-HD; ncbi.nlm.nih.gov/gap, accession number phs000371.v2.p1). Access to the EGA datasets is contingent on assurances that no attempt will be made to identify individual research...
participants from genetic data. Biological materials derived in this work (edited iPSC lines, FAN1 expression plasmids) are available on request from the corresponding authors.

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Sample size

GeM-HD GWAS identified a number of genome-wide significant genetic modifiers of HD onset (GeM-HD Consortium, 2015). Using the effect size of the lead SNP a formal power calculation showed that exome-sequencing 40 individuals from each extreme of the residual age-at-onset distribution for a HD population of 7000 would have 88% power to detect a modifier variant with an equivalent effect size (5% significance level). The study was extended to include >700 exomes in the end, further increasing power.

Data exclusions

In exome sequencing quality control: exomes >3 standard deviations smaller than the mean of any of the three metrics were excluded for Registry-HD exomes. VerifyBamID76 was used to detect contamination, and samples with a Freemix > 0.075 were excluded, as per the ExAC study. Where there were duplicate samples, the exome with the highest coverage was retained. Sex imputation used Peddy; samples with conflicting imputed and recorded sex were excluded. One individual with originally unknown sex was kept. Ancestry was estimated using Peddy by principal component analysis (PCA) against genomes from the 1000 genomes project phase 379. Samples were excluded if they were either 1) predicted to have non-European ancestries by Peddy or 2) outside the primary cluster of European samples in Supplementary Fig. 1.

Replication

Exome sequencing was performed in two independent clinical samples (Registry-HD and Predict-HD) with consistent results. The two samples were not individually large enough to allow the detection of rare variants in one as a discovery dataset and the other as a replication dataset. Biochemical and cellular assays were repeated at least three times each on different days with fresh reagents, with two or three technical replicates of each individual data point. The results of all experimental replications of biochemical and cellular assays were consistent, and all data are presented in the paper.

Randomization

Randomisation was not relevant to this study. Individuals with HD were assigned to early/more severe and late/less severe phenotype groups on the basis of their clinical phenotypes. In Registry-HD individuals were stratified by residual age at motor onset and those at the extremes of the distribution selected for sequencing. In Predict-HD individuals were stratified by motor or cognitive scores, or predicted time to onset, and extremes selected for sequencing.

Blinding

Researchers were not blinded to clinical phenotype when performing exome/variant association analyses. For nuclease assays, the user was not blinded to FAN1 variant, but they were blinded to the phenotypes associated with patients harbouring that variant. For iPSC assays the user was not blinded to the cell line being used but CAG lengths were called by user-independent software to reduce the risk of peak calling bias.

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Antibodies

Antibodies used:
- Anti-FAN1 (sheep polyclonal, 1:1000, CHDI); Anti-β-Tubulin Antibody, clone AA2 (mouse monoclonal, 1:10000, Upstate 05-661); Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 680 (donkey polyclonal, 1:10000, Invitrogen A32788); IRDye® 800CW Donkey anti-Goat IgG Secondary Antibody (donkey polyclonal, 1:15000, Licor 926-32214); Anti-OCT4 antibody (rabbit polyclonal, 1:100, AbCam ab19857); Anti-MAP2 antibody (rabbit polyclonal, 1:500, AbCam ab32454); Anti-
**Eukaryotic cell lines**

**Policy information about** [cell lines](https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11001); [Goat-anti-Mouse-IgG-H-L-Cross-Adsorbed-Secondary-Antibody](https://www.thermofisher.com/antibody/product/Donkey-anti-Mouse-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A32788); [Alexa Fluor goat anti-rabbit IgG 568](https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11001); [Alexa Fluor goat anti-mouse IgG 488](https://www.thermofisher.com/antibody/product/Goat-anti-Mouse-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A32788). Anti-CTIP2 antibody is validated for use in ICC and cited in over 550 papers (https://www.abcam.com/ctip2-antibody-25b6-ab18465.html); Anti-CTIP2 is validated for use in ICC and cited in over 550 papers (https://www.abcam.com/ctip2-antibody-25b6-ab18465.html); Alexa Fluor goat anti-mouse IgG 488 is cross-adsorbed and validated for use in ICC, being cited over 5000 times (https://www.thermofisher.com/antibody/product/Goat-anti-Mouse-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11001); Alexa Fluor goat anti-rabbit IgG 568 is cross-adsorbed and validated for use in ICC and cited over 1700 times (https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11001).

**Cell line source(s)**

| Human lymphoblastoid cell lines derived directly from HD patient samples were obtained from CHDI (lines generated and banked by BioRep, Italy). Human induced pluripotent stem cell (iPSC) lines, 109 CAGs, as reported in HD iPSC Consortium, 2012, Cell Stem Cell: 11: 264-278 and Mattis et al., 2015, Human Molecular Genetics 24: 3257-3271. Lines available through CHDI. |

**Authentication**

| Lymphoblastoid cell lines derived directly from HD patient samples were received from BioRep, Italy and grown in the lab. HTT CAG repeat lengths were assayed regularly by PCR fragment analysis and by MiSeq and cross-referenced against expected repeat lengths from banked DNA samples from the same individuals. Q109 iPSCs were regularly sized for HTT CAG repeat length, had regular virtual karyotyping by CNV analysis, and were exome sequenced. |

**Mycoplasma contamination**

| Cell lines were regularly checked for mycoplasma contamination and were always negative |

**Commonly misidentified lines**

| No commonly misidentified cell lines were used in this project |

**Human research participants**

**Policy information about** [studies involving human research participants](https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11001). |

**Population characteristics**

| Both patient cohorts (REGISTRY-HD and PREDICT-HD) were studies of Huntington’s disease patients. The main measure used both for analysis and selection of REGISTRY-HD patients was age at motor onset of disease. Using CAG length and the Langbehn model (Langbehn et al, 2004, Clinical Genetics 65:267-277) a residual age at onset was calculated. For PREDICT-HD, those who had phenoconverted to motor onset similarly had a residual age at onset calculated. In addition, predicted ages at onset were calculated as described in the manuscript. Extreme worst or best scorers for single digit modalities test (SDMT) or total motor score (TMS) were also determined as described. The predicted ages at onset and extreme SDMT/TMS scorers were used as binary measures as extreme worst/best measures alongside early/late onset individuals from Registry. Overall, 465 participants from REGISTRY-HD were included (53.4% female, mean age at onset = 48.4 years) and 218 participants from PREDICT-HD (61.9% female, mean age at onset = 47.1 years). See Supplementary Table 1 for details. |

**Recruitment**

| Patients were not recruited directly to this study. The REGISTRY-HD and PREDICT-HD populations were recruited over a number of years and followed longitudinally over time. Participants were research-motivated individuals carrying the HD mutation who provided detailed phenotypic information as well as DNA samples. They gave informed consent at the time of recruitment into the two studies for their data to be analysed by researchers at a later date. This study stratified the two populations as described (Fig. 1) and selected those with extremes of phenotype for exome sequencing. Selections were made on the basis of phenotypic analysis and sample availability and as such there were no inherent selection biases identified. |

**Ethics oversight**

| Ethical approval for REGISTRY-HD was obtained in each participating country. Investigation of deidentified PREDICT-HD subjects was approved by the Institutional Review Board of Partners HealthCare (now Mass General Brigham). Participants from both studies gave written informed consent. All experiments described herein were conducted in accordance with the declaration of Helsinki. Local ethical approval was through Cardiff University School of Medicine SMREC 19/55. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.