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Generation of an isogenic set of HD model hESC lines

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ABSTRACT

Huntington's disease (HD) is a fatal neurodegenerative disease caused by expansion of CAG repeats in the Huntingtin gene (HTT). Neither its pathogenic mechanisms nor the normal functions of HTT are well understood. To model HD in humans, we engineered a genetic allelic series of isogenic human embryonic stem cell (hESC) lines with graded increases in CAG repeat length. Neural differentiation of these lines unveiled a novel developmental HD phenotype: the appearance of giant multinucleated telencephalic neurons at an abundance directly proportional to CAG repeat length, generated by a chromosomal instability and failed cytokinesis over multiple rounds of DNA replication. We conclude that disrupted neurogenesis during development is an important, unrecognized aspect of HD pathogenesis. To address the function of normal HTT protein we generated HTT+/- and HTT-/- lines. Surprisingly, the same phenotype emerged in HTT-/- but not HTT+/- lines. We conclude that HD is a developmental disorder characterized by chromosomal instability that impairs neurogenesis, and that HD represents a genetic dominant-negative loss of function, contrary to the prevalent gain-of-toxic-function hypothesis. The consequences of developmental alterations should be considered as a new target for HD therapies.

ATTACHMENTS

dfqwbiqa7.pdf

DOI

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KEYWORDS

Huntington's disease, CRISPR, Human embryonic stem cells, Disease modeling, Chromosomal instability, Neurogenesis, DNA damage and repair

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MATERIALS TEXT

RUES2 hESC line (NIH hESC-09-0013)

pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A) **addgene Catalog #42335**

N2B27 serum-free medium:

A	B
ROCK inhibitor	20 µM
SB-431542	10 µM
LDN-193189	0.2 µM
FGF8	5 ng/ml

Wash buffer (PBS+0.1% Triton X-100):

A	B
Glycine	100 mM
Sodium azide	0.1%
Normal donkey serum	10%

Primary antibodies:

Purified anti-Pax-6 Antibody **BioLegend Catalog #PRB-278P**

Purified Mouse anti-Human Pax-6 **BD Biosciences Catalog #561462**

Human/Mouse/Rat SOX1 Antibody **R&D Systems Catalog #AF3369**

Anti-Nestin Human Antibody **Emd Millipore Catalog #ABD69**

Anti-FOXG1 antibody (ab18259) **Abcam Catalog #ab18259**

Recombinant Anti-Pericentrin antibody [mAbcam 28144] - Centrosome Marker (ab28144) **Abcam Catalog #ab28144**

Anti-Ctip2 antibody [25B6] (ab18465) **Abcam Catalog #ab18465**

Anti-MAP2 antibody (ab5392) **Abcam Catalog #ab5392**

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Anti-Aurora B antibody (ab2254) **Abcam Catalog #ab2254**

Huntingtin (D7F7) XP® Rabbit mAb #5656 **Cell Signaling Technology Catalog #5656S**

Purified anti-human CD325 (N-Cadherin) Antibody **BioLegend Catalog #350802**

Monoclonal Anti-Tubulin Acetylated antibody produced in mouse **Sigma Aldrich Catalog #T-6793**

Human/Mouse/Rat SOX2 Antibody **R&D Systems Catalog #AF2018**

Antibodies:

A	B	C	D
Name	**Supplier**	**Catalog number**	**Dilution Factor**
PAX6	Covance	PRB-278	200
PAX6	BD Biosciences	561462	200
SOX1	R&D	AF3369	1000
NES	EMD Millipore	ABD69	500
FOXG1	Abcam	ab18259	200
HTT (D7F7)	Cell Signaling	5656S	100
N-cadherin	BioLegend	350802	100
Acetylated tubulin	Sigma-Aldrich	T-6793	200
Pericentrin	Abcam	ab28144	200
SOX2	R&D	AF2018	1000
CTIP2	Abcam	ab18465	200
MAP2	Abcam	ab5392	5000
Aurora b kinase	Abcam	ab2254	100
Alpha-tubulin	Abcam	ab89984	500
CREST	Antibodies Incorporated	15-235-0001	100

Generation of an isogenic set of HD model hESC lines

1 In order to establish an optimal platform for understanding the effects of HD mutations, use CRISPR/Cas9 technology to generate a set of isogenic hESC lines that would differ only in the HTT locus, bearing different lengths of the CAG tract.

2 Use the already established, registered and validated RUES2 hESC line (NIH hESC-09-0013) as the parental line.

Genetic analysis of the HTT gene in RUES2 showed that it contained 20 and 22 CAGs

3 Design the homology donors for the CRISPR/Cas9-mediated homologous recombination to contain ~1 kb homology arms flanking HTT exon 1, a piggyBac transposable element containing a Puro-TK cassette (for both negative and

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positive selections), and various versions of HTT exon 1 containing different lengths of the CAG tract (20, 42, 48, 56, 67, 72 CAGs).

4 To construct the homology donors, first generate a parental plasmid using Gibson assembly, in which we could easily swap the length of the CAG tract.

5

Amplify all fragments needed for the assembly using Q5 high-fidelity polymerase (NEB) or the GC-RICH PCR System (SigmaAldrich) using the primers listed in Table S3.

Table S3. Primers

Fragment	Fw primer sequence	Rv primer sequence	Template
pUC57-Kan bb	CTCCAGCTTTTGTTCCCTTT	CCAATTCGCCCTATAGTGAGTC	pUC57-Kan plasmid
Left homology arm	AAAGGGAACAAAAGCTGGAGgggtcacacttggggtcct	TCTAGGGTTAAaagcagaacctgagcggc	gDNA from RUES2 (WT)
ePB-PUTK	aggttctgcttTTAACCCTAGAAAGATAGTCTGC	GGGCCGCAGGTTAACCCTAGAAAGATAATCATATTG	ePB-CAG-PUTK-pA plasmid
Right homology arm	TTTCTAGGGTTAACCTGCGGCCCAGAGCCC	GACTCACTATAGGGCGAATTGGCCTCCCCATCAGCAACGTGT	gDNA from RUES2 (WT)

This resulting 'base' homology donor plasmid contained a 20 CAG repeat tract, which could easily be swapped using the flanking XmnI and BbsI sites.

6 To create the homology donor plasmids with expanded CAG lengths, amplify PCR the HTT exon 1 region from genomic DNAs obtained from iPSCs or fibroblasts derived from HD patients, and clone them into the 'base' plasmid using XmnI and BbsI.

The primers used for the CAG tract amplification are polyCAG_Fw CCAAGATGGACGGCCGCTC and polyCAG_Rv AGGACAAGGGAAGACCCAAG. The origin of the templates used for each CAG length are summarized in Table S4.

Table S4. Templates used for each CAG length.

polyQ length	Origin of genomic DNA template	Obtained from
44	ND38548 iPSC	Coriell Biorepository
50	GENEA20 hESC	GENEA
58	QS-001 fibroblasts	Tabrizi lab
69	QS-004 fibroblasts	Tabrizi lab
75	QS-004 fibroblasts	Tabrizi lab

7 For the CRISPR/Cas9-mediated targeting, design multiple sgRNAs to recognize sequences near the CAG tract, but gene targeting attempts using a single sgRNA were unsuccessful.

However, an optimized strategy that combined two sgRNAs targeting sequences that flanked the CAG tract proved to be much more efficient in generating the desired homologous recombination: hHTT_sgRNA25 and hHTT_sgRNA14 (Table S5).

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Table S5. sgRNAs

sgRNA	Protospacer + PAM sequence
hHTT_sgRNA25	GGTAAAAGCAGAACCTGAG CGG
hHTT_sgRNA22	CTGCTGCTGGAAGGACTTGA GGG
hHTT_sgRNA14	GCTGCACCGACCGTGAGTTT GGG

8 Clone these two sgRNAs into a Cas9 nickase expression vector (pX335 from the Feng Zhang lab, Addgene plasmid #42335).

9 Nucelofect both CRISPR plasmids, together with the appropriate homology donor into RUES2 cells using a Nucleofector II instrument and Cell Line Nucleofector Kit L (Lonza).

10 

Add puromycin to the cultures 5 days after nucleofection, and keep in for 5 more days to ensure selection of correctly targeted clones.

11 Nucleofect puromycin-resistant cells with an excision-only piggyBac transposase (Transposagen), and a subsequent ganciclovir treatment selected clones in which the selection cassette had been correctly removed, leaving a minimal footprint (one extra nucleotide in the 5'UTR).

12 Pick and expand colonies derived from single cells for screening.

PCR amplification and Sanger sequencing identified correctly targeted clones, with no mutations in the CRISPR/Cas9 target sites or the piggyBac excision site. All positive clones were heterozygously targeted, which is a common finding in long genetic insertions via CRISPR/Cas9 mediated homologous recombination, and there appeared to be no preference as to which allele had been targeted.

13 Select two to three independent clones for each CAG length to serve as independent biological replicates.

14 Confirm normal HTT expression by western blot, and karyotype all lines by G-banding and aCGH analyses to assess their genomic integrity and CNV.

We found a variety of CNVs and loss of heterozygosity, within and outside of normal ranges (Fig. S2C, Table S2), but these did not correlate with any of the observed phenotypic differences and were not the result of gene editing per se, as they were also present in genetically unmodified clones.

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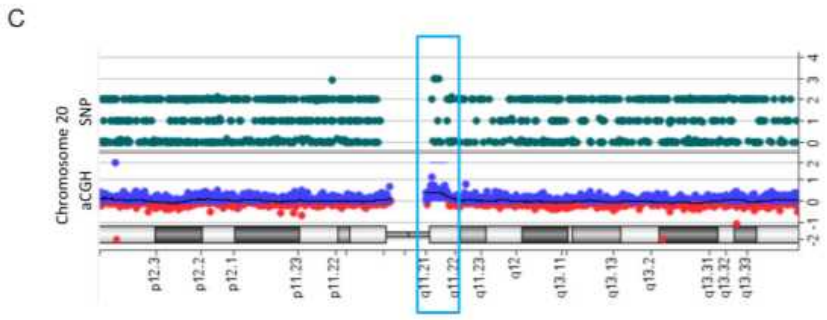

Figure S2. Karyotyping and pluripotency validation of the HD-hESC allelic series
(A) All hESC isogenic clones were karyotypically normal by G-banding. (B) The pluripotency status was not affected by the length of the polyQ tract or the HTT dosage. Immunostaining of micropatterned hESC cultures for pluripotency markers NANOG, SOX2 and OCT4. Scale bar 100µm. (C) Example of aCGH analysis result (amplification of 20q11). aCGH analyses revealed different CNVs present in different sub-clones.

Table S2. Summary of Arrayed Comparative Genomic Hybridization (aCGH) analysis to assess copy number variations in the RUES2-HD allelic series. We found a variety of copy number variations and loss-of-heterozygosity, within and outside of normal ranges, but these did not correlate with any of the observed phenotypic differences.

Color Legend	Cell line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
AMP	22Q-1		p12 - p11.2 q13 q34		p16.3						q23.32									q13.2-q13.31	q11.21			
DEL	22Q-2		p12 - p11.2 q13		p16.3	q21.3 - q22.1					q23.32				q32.33				q22.1 - q23	q13.2-q13.31				
LOH	22Q-3		p12 - p11.2 q13		p16.3	q21.3 - q22.1		q36.2			q23.32							q12 - q21.31		q13.2-q13.31	q11.21			
	45Q-1		p12 - p11.2 q13			q21.3 - q22.1					q23.32							q12 - q21.31		q13.2-q13.31				
	50Q-1		p12 - p11.2 q13 q14.2		p16.3	q21.3 - q22.1					q23.32				q32.33			q12 - q21.31						
	50Q-2		p12 - p11.2 q13			q21.3 - q22.1					q23.32				q32.33			q12 - q21.31						
	50Q-3		p12 - p11.2 q13		p16.3	q21.3 - q22.1					q23.32		q12.3							q13.2-q13.31				
	58Q-1		p12 - p11.2 q13		p16.3	q21.3 - q22.1					q23.32					q11.1 - q11.2								q22.1-q22.2
	58Q-2		p12 - p11.2 q13		p16.3	q21.3 - q22.1		q12.1-q11.2			q23.32				q32.33					q13.2-q13.31				
	58Q-3		p12 - p11.2 q13		p16.3	q21.3 - q22.1					q23.32				q32.33			q12 - q21.31		q13.2-q13.31				q22.1-q22.2
	67Q-1		p12 - p11.2 q13		p16.3	q21.3 - q22.1		q31.32			q23.32				q32.33					q13.2-q13.31				
	74Q-1		p12 - p11.2 q13		p16.3	q21.3 - q22.1					q23.32				q32.33					q13.2-q13.31	q11.21			
	74Q-2		p12 - p11.2 q13 q34		p16.3	q21.3 - q22.1	q22.31				q23.32				q32.33			q12 - q21.31		q13.2-q13.31	q11.21			
	HTT+/- 1		p12 - p11.2 q13		p16.3	q21.3 - q22.1					q23.32				q32.33			q12 - q21.31		q13.2-q13.31				q22.1-q22.2
	HTT+/- 2		p12 - p11.2 q13		p16.3	q21.3 - q22.1		q31.32			q23.32				q32.33			q12 - q21.31		q13.2-q13.31				q22.1-q22.2
	HTT+/- 3		p12 - p11.2 q13		p16.3	q21.3 - q22.1					q23.32										q11.21			
	HTT-/- 1		q13		p16.3	q21.3 - q22.1					q23.32				q32.33						q11.21			
	HTT-/- 2		p12 - p11.2 q13		p16.3	q21.3 - q22.1				q33.1	q23.32							q12 - q21.31			q11.21			
	HTT-/- 3		p12 - p11.2 q13		p16.3	q21.3 - q22.1					q23.32				q32.33			q12 - q21.31			q11.21			
	RUES2 parental stock 1		p12 - p11.2 q13		p16.3	q21.3 - q22.1					q23.32				q32.32			q12 - q21.31		q13.2-q13.31				
	RUES2 parental stock 2		p12 - p11.2 q13		p16.3	q21.3 - q22.1					q23.32				q32.33					q13.2-q13.31				
	RUES2 parental stock 3		p12 - p11.2 q13		p16.3	q21.3 - q22.1					q23.32							q12 - q21.31		q13.2-q13.31				

15 Perform whole-genome sequencing at NY Genome Center (NYGC) using the TruSeq DNA PCR-Free Kit for Library Preparation and run in an Illumina HiSeqX sequencer.

16 Identify around 1000 unique single-nucleotide polymorphisms in each line, probably caused by a founder effect from a genetically variable parental RUES2 cell line.

17 Validate the pluripotency status and absence of differentiation of the clones through immunofluorescence staining of cells seeded and cultured on micropatterned coverslips (CYTOO).

Generation of isogenic $HTT^{+/-}$ and $HTT^{-/-}$ hESC lines 2d

18 Use CRISPR/Cas9 technology to generate a set of isogenic hESC lines with different HTT gene dosages ($HTT^{+/+}$, $HTT^{+/-}$, $HTT^{-/-}$).

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19 Use RUES2 hESC (NIH hESC-09-0013) as the parental line.

20 To create HTT-/- and HTT+/- lines, use CRISPR/Cas9 to delete the first exon of HTT, ensuring that no protein fragment would be produced in the targeted ('null') allele.

An optimized strategy that combined two sgRNAs that flanked the first exon proved to be efficient in generating the desired deletion: hHTT_sgRNA22 and hHTT_sgRNA14 (Table S5).

Table S5. sgRNAs

sgRNA	Protospacer + PAM sequence
hHTT_sgRNA25	GGTAAAAGCAGAACCTGAG CGG
hHTT_sgRNA22	CTGCTGCTGGAAGGACTTGA GGG
hHTT_sgRNA14	GCTGCACCGACCGTGAGTTT GGG

21 Clone these two sgRNAs into an SpCas9 expression vector (pX330 from the Feng Zhang lab), modify to coexpress a puromycin cassette to facilitate selection of transfected cells, thus maximizing the percentage of targeted colonies.

22 Nucleofect both CRISPR plasmids into RUES2 cells using a Nucleofector II instrument and Cell Line Nucleofector Kit L (Lonza).

23 2d

Add puromycin to the cultures **24:00:00** after nucleofection, and keep in for a further **24:00:00** to select for transfected cells.

24 Pick and expand colonies derived from the puromycin-resistant cells for screening.

PCR amplification and Sanger sequencing identified correctly targeted clones with the desired exon 1 deletion. Two to three clones per genotype are identified and further validated as above.

Neural differentiation 2d

25 Subject isogenic lines to a default neural induction protocol adapted from Shi et al. (2012).

26 In short, seed cultures in suspension, or in confluent adherent culture, at 0.5-1×106 cells/ml and feed every other day with N2B27 serum-free medium with ROCK inhibitor (**[M]20 Micromolar (µM)** , Abcam) for the first 2 days, TGFβ inhibition (**[M]10 Micromolar (µM)** SB-431542, Sigma and **[M]0.2 Micromolar (µM)** LDN-193189, StemGent) for the first 10 days, treated with **[M]5 ng/ml** FGF8 (R&D Systems) from day 12-22 to maximize the frequency of CTIP2

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(BCL11B)-positive cells, dissociate and seed on adherent substrate (polyornithine/laminin, Ibidi) at day 14, fix and analyze at day 19 or continue in culture with added BDNF (R&D Systems) and IGF1 (R&D Systems, [M]**10 ng/ml**), cAMP ([M]**1 Micromolar (µM)** , R&D Systems) and ascorbic acid ([M]**200 Micromolar (µM)** , Sigma), until dissociation and reseeding on substrate at day 40, and fixation and analysis at day 45.

Immunostaining and imaging 2d

27

Fix cultures with 4% paraformaldehyde (PFA), rinse twice with PBS.

28 1h

Permeabilize and quench cultures with wash buffer (PBS+0.1% Triton X-100) containing [M]**100 Milimolar (mM)** glycine and 0.1% sodium azide, block (wash buffer, 10% normal donkey serum, 0.1% sodium azide) for **00:30:00** , incubate with primary antibodies **Overnight** at **4 °C** (for primary antibodies and dilutions, see Table S6).

Table S6. Antibodies

Name	Supplier	Catalog number	Dilution Factor
PAX6	Covance	PRB-278	200
PAX6	BD Biosciences	561462	200
SOX1	R&D	AF3369	1000
NES	EMD Millipore	ABD69	500
FOXG1	Abcam	ab18259	200
HTT (D7F7)	Cell Signaling	5656S	100
N-cadherin	BioLegend	350802	100
Acetylated tubulin	Sigma-Aldrich	T-6793	200
Pericentrin	Abcam	ab28144	200
SOX2	R&D	AF2018	1000
CTIP2	Abcam	ab18465	200
MAP2	Abcam	ab5392	5000
Aurora b kinase	Abcam	ab2254	100
Alpha-tubulin	Abcam	ab89984	500
CREST	Antibodies Incorporated	15-235-0001	100

29 1h 5m

Wash three times in wash buffer for **00:05:00** each, incubate with secondary antibodies (Alexa 488, 555, 594, 647, Molecular Probes; 1:500) and DAPI nuclear counterstain for **01:00:00** at **Room temperature** , and then wash as above.

30 18h

For mitotic synchronization and microtubule visualization, synchronize cultures at G-to-M transition with nocadazole ([M]**0.2 Micromolar (µM)** ; Abcam) for **18:00:00** .

31 2h 35m

Rinse and release for **02:00:00** , fix for **00:05:00** at **Room temperature** in culture medium with 2% PFA, then incubate for **00:30:00** at **37 °C** in microtubule stabilization buffer [0.1 M PIPES pH 6.9, [M]**5 Milimolar (mM)** MgCl2, [M]**2.5 Milimolar (mM)** EGTA, 2% formaldehyde, 0.1% Triton X-100, [M]**1 Micromolar (µM)** paclitaxel (Sigma), **10 U/ml** aprotinin (Sigma) and 50% deuterium oxide (Messinger and

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Albertini, 1991)] before quench and blocking as above.

32

Acquire Z-stack images on a Leica SP8 inverted confocal microscope at 12 bits in 1024 pixels ×1024 pixels using an HCX PL APO CS ×20/0.75 NA airimmersion objective, an HC PL APO CS2×40/1.10 NA water-immersion objective, or an HC PL APO ×100/1.4 oil-immersion objective (Leica) at Nyquist spacings.

33 Deconvolve images with a 3D blind algorithm (ten iterations) using AutoDeblur X software (Autoquant) and rendered in Imaris (Bitplane).

34 Subject 3D rendering channels to uniform non-linear adjustment across the entire volume to highlight salient details; adjustments are identical across genotypes.

Analysis of neural rosette spatial distribution

35 Identify rosettes by training a classifier on N-cadherin antibody stains using Ilastik (Sommer et al., 2011).

36 Threshold N-cadherin-positive areas in size (15 μm^2) to eliminate small N-cadherin spots that are not fully developed rosettes.

The quantification results are not dependent on the specific value chosen for the threshold.

37 Obtain the nearest-neighbor distributions using the k-d tree implementation in scikit-learn (http://scikit-learn.org) and are compare with the random distribution using the one-sample Kolmogorov–Smirnov distance between them.

The cumulative nearest-neighbor distribution F for a random set of points in two dimensions can easily be calculated as

$F(d) = 1 - e^{-\lambda \pi d2}$

where λ is the 2D density of foci, and d is the distance between two neighbors. We rescaled the data for the individual lines separately according to their λ.

Time-lapse imaging 2d

38 Dissociate neural cultures to single cells at day 25, and reseed at 0.5×106/cm2 on polyornithine laminin-coated 35 mm imaging dishes (Ibidi).

39 One day later, tranduce cultures with GFP and RFP nucleus-labeling baculoviruses (CellLight BacMam 2.0, ThermoFisher Scientific) for **24:00:00** . 1d

1d

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40

Two days later, when the nuclear signal was clearly observable, carry out a time-lapse imaging acquisition for **24:00:00** using a CellVoyager CV1000 spinning-disk confocal microscope (Yokogawa).

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