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# The first reported generation of several induced pluripotent stem cell lines from homozygous and heterozygous Huntington's disease patients demonstrates mutation related enhanced lysosomal activity

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#### ABSTRACT

Neuronal disorders, like Huntington's disease (HD), are difficult to study, due to limited cell accessibility, late onset manifestations, and low availability of material. The establishment of an in vitro model that recapitulates features of the disease may help understanding the cellular and molecular events that trigger disease manifestations. Here, we describe the generation and characterization of a series of induced pluripotent stem (iPS) cells derived from patients with HD, including two rare homozygous genotypes and one heterozygous genotype. We used lentiviral technology to transfer key genes for inducing reprogramming. To confirm pluripotency and differentiation of iPS cells, we used PCR amplification and immunocytochemistry to measure the expression of marker genes in embryoid bodies and neurons. We also analyzed teratomas that formed in iPS cell-injected mice. We found that the length of the pathological CAG repeat did not increase during reprogramming, after long term growth in vitro, and after differentiation into neurons. In addition, we observed no differences between normal and mutant genotypes in reprogramming, growth rate, caspase activation or neuronal differentiation. However, we observed a significant increase in lysosomal activity in HD-iPS cells compared to control iPS cells, both during self-renewal and in iPS-derived neurons. In conclusion, we have established stable HD-iPS cell lines that can be used for investigating disease mechanisms that underlie HD. The CAG stability and lysosomal activity represent novel observations in HD-iPS cells. In the future, these cells may provide the basis for a powerful platform for drug screening and target identification in HD.

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#### Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by excessive expansion of a CAG trinucleotide repeat in the gene encoding the huntingtin protein (*htt*). This trinucleotide repeat results in the addition of a long stretch of glutamines (polyQ) near the N-terminus of the protein (HD Collaborative Research Group, 1993). The disease is characterized by movement, cognitive, and emotional disturbances that result from massive

*Abbreviations:* iPS, induced pluripotent stem; HD, Huntington's disease; WT, wild type; HTT, huntingtin; PH3, phospho-histone H3; MEF, mouse embryonic fibroblasts; EBs, embryoid bodies; ES, embryonic stem.

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neurodegeneration involving, at first, striatal medium spiny neurons and later, entire cortical structures (Reiner et al., 1988).

The disease occurs as a consequence of the expanded polyQ tract, whose length dictates the disease onset. However, recent evidence has suggested that the loss of physiological activity in the normal protein may also contribute to disease pathogenesis (Cattaneo et al., 2001, 2005; Zuccato et al., 2010). Consistent with this possibility, in a small collection of homozygous patients, disease progression appeared to precipitate faster than in heterozygous patients (Squitieri et al., 2003); this was also observed in a mouse model, although further confirmatory studies are needed (Lin et al., 2001).

To facilitate investigations of the disease mechanisms, an impressive series of HD models has been developed (Zuccato et al., 2010). Neuronal disorders are in fact probably among the most difficult to model due to limited cell accessibility, late onset manifestations, and low availability of material, which often reflects only the final phases of pathology. Cell lines that carry the HD mutation provide biochemically homogeneous material for testing specific hypotheses. For example, studies initially performed in rodent cell lines have led to the loss of HTT function hypothesis, evidence for a defect in BDNF, altered vesicular transport along microtubules in neurons (Colin et al., 2008; Gauhier et al., 2004) and data on cholesterol abnormalities in HD. Subsequently, those findings were verified in animal models and, where appropriate, in human post mortem patient samples (Zuccato et al., 2010). However, we lack a reliable, stable source of human cell lines with a central nervous system (CNS) character that correctly express the mutant gene during neuronal differentiation. Towards this goal, 8 human embryonic stem (hES) cell lines were derived from preimplantation genetic diagnosis embryos that carried the mutant gene. However, these cell lines remained poorly investigated (Bradley et al., 2011; Mateizel et al., 2006; Niclis et al., 2009; Verlinsky et al., 2005).

The discovery of human somatic cell reprogramming to generate iPS cells has captured scientific interest because it can facilitate the creation of patient-specific in vitro models of human disorders. Promising results have already been achieved with iPS cells derived from patients with Parkinson disease (PD), amyotrophic lateral sclerosis, spinal muscular atrophy, familial dysautonomia, X fragile syndrome, and Rett syndrome (Dimos et al., 2008; Ebert et al., 2009; Lee et al., 2009; Marchetto et al., 2010; Soldner et al., 2009; Urbach et al., 2010). However, this field is young, and only some studies have investigated and identified molecular alterations (and partial reversions) (Ebert et al., 2009; Lee et al., 2009; Marchetto et al., 2010). On the other hand, given that disease onset, as in HD or PD, occurs rather late in life, it remains unclear whether early pre-symptomatic changes are to be expected in pluripotent cells, in neural progenitors or in more differentiated neuronal cells. Furthermore, in diseases that develop in a noncell-autonomous manner, like PD, the search for a phenotype may be even more problematic. In HD, some molecular alterations have been found in cells that do not undergo degeneration, for example, glial cells (Lobsiger and Cleveland, 2007; Valenza and Cattaneo, 2011); however, the mutant gene is known to be preferentially toxic to neurons, particularly striatal and cortical neurons. For this reason, neuronal (and/or glial) cells obtained from HDiPS cell lines can potentially recapitulate at least a consistent part of the molecular underpinnings of the disease.

The first human HD-iPS cell line was generated by Park and collaborators in 2008, but no data about its characteristics were presented. The same HD-iPS cell line was later used to derive neuronal precursors and neurons, and these exhibited a mild increase in caspase activity (Zhang et al., 2010).

In the present study, we generated iPS cells from primary fibroblasts from three different patients with HD. These patients included two rare homozygous genotypes. We investigated the capacity of this unique disease-specific cellular system for analyzing possible molecular abnormalities and neurogenic potential of human HD stem cells derived from patients.

We demonstrated that the presence of one or two mutant HTT alleles did not impair the reprogramming process, iPS cell self-renewal and their conversion to neural progenitors as well as the yield of generated mature neurons. On the other hand, the HD mutant cells exhibited altered lysosomal content that was maintained during proliferation and in iPS-derived neurons.

Taken together, these observations demonstrated that HD-iPS cells may be a relevant system for disease modeling while providing the basis for a powerful platform for drug screening and target identification in HD.

#### Materials and methods

#### Vector production

A reprogramming vector Stem Cell Cassette (STEMCCA) that included 4 factors (OCT4, SOX2, KLF4, and C-MYC) was a kind gift from G. Mostoslavsky. We also prepared a stock solution of a reprogramming vector that included 3 factors (OCT4, SOX2, KLF4), as previously described (Follenzi and Naldini, 2002). Briefly, 293T cells were co-transfected with 4 vectors by calcium phosphate precipitation; these vectors were the pCCLsin.PPT.pA.TK.mCMV.SFFV.OCT3/ 4\_FMDV 2A\_KLF4\_TaV 2A\_SOX2.Wpre.3'LTR\_loxP transfer vector plasmid (36 µg); the pMD.Lg/pRRE packaging plasmid (12.5 µg); the pMD2.VSV-G envelope-encoding plasmid (9 µg); and pCMV-Rev (6.25 µg). All four vectors were added to cells in a 15-cm dish, and 1 mM sodium butyrate was added to the collected medium. Vector particles were concentrated 250-fold by ultracentrifugation and measured with HIV-1 Gag p24 immunocapture assay (Perkin Elmer). Retroviruses were produced in Plat-E cells that were transfected with pBabe-based retroviral vectors for OCT4, SOX2, KLF4, in DMEM containing 10% FBS, using Fugene 6 (Roche).

#### Fibroblast culture and infection

Skin biopsies were obtained from patients coming for clinical follow-up visits at the Neurological Institute "C. Besta" in Milan. All subjects gave their written consent for the skin biopsy procedure and for the use of the sample material for research purposes. Fibroblasts were cultured in DMEM high glucose (Euroclone), 10% FBS (Euroclone), 2 mM L-Glutamine (Euroclone, Italy), and 1% penicillin/ streptomycin (Invitrogen). We plated  $1.5 \times 10^4$  cells in a 6-well dish and performed infection with viral construct that encoded the transcription factors OCT4, SOX2, and KLF4 (OSK, with or w/o C-MYC), in the presence of 4 µg/ml of polybrene. When reprogramming was performed using retroviral vectors, fibroblasts were previously transduced with SLC7A1 receptor. After 1 week, we replated the infected fibroblasts on a feeder layer of mouse embryonic fibroblasts (MEFs from strain CD1,  $3.5 \times 10^4$  cells/cm<sup>2</sup>) or on human neonatal Foreskin  $(3.5 \times 10^4 \text{ cells/cm}^2)$  mitotically inactivated by Mitomicin C treatment (Sigma Aldrich), in Knock-out-DMEM (Invitrogen) supplemented with 20% Knock-out serum replacement (Invitrogen), 2 mM L-Glutamine (Euroclone, Italy), 2 mM nonessential amino acids (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 0.1 mM  $\beta$ -mercaptoethanol (Invitrogen), with 10 ng/ml of bFGF (Invitrogen). Colonies started to appear 30 days later, and at around day 40, they were selected and transferred to a new feeder layer with the same culture conditions. Subsequently, iPS clones were passaged mechanically every 5-7 days and the presumed undifferentiated regions were transferred to a new feeder layer. In our experiments, we used only undifferentiated colonies.

#### Embryoid body differentiation

Entire colonies of hES, wild-type (WT), and HD-iPS cells were detached by incubation with collagenase IV (Invitrogen) for 40 min; next, cells were cultured in suspension in hES medium without adding bFGF. After 7 days, EBs had formed; these were plated on 0.1% gelatin (Sigma Aldrich) and the medium was changed every 3 days.

#### Teratoma assay

To test the pluripotency of the HD-iPS cells in vivo, cells were manually dispersed from the human fibroblast feeder layer and suspended in Matrigel™ (BD, Becton Dickinson). Then, 200 µl of Matrigel-suspended cells (~10<sup>6</sup> cells) were injected subcutaneously into four, 7-week-old, severe combined immune deficient (SCID), beige mice (C.B.-17/GbmsTac-scid-bgDF N7, M&B, Ry, Denmark). Four additional mice were injected with another batch of cells derived from the same cell line. A total of eight mice were injected. The mice were followed up, and teratoma growth was observed weekly. After 6 weeks, the mice were sacrificed, and the teratomas were excised, fixed in 4% buffered paraformaldehyde, prepared for histology, and embedded in paraffin. Morphology was analyzed in hematoxylin-eosin-stained, 4-µm sections. To confirm the presence of the yolk sac and other structures, we performed immunohistochemical staining using a Bond-Max automatic immunostainer (Leica Microsystems) with antibodies directed against Alphafetoprotein (AFP) (Novocastra), Glypican3 (GPC3) (Biomosaics) and OCT 3/4 (Novocastra).

#### Karyotyping

For HD-iPS<sup>hom</sup> 4F-1, HD-iPS<sup>hom</sup> 4F-3 cell lines, karyotype analyses was performed on twenty metaphase spreads, using the GTG-banding method. iPS cell lines were incubated in proliferation medium, supplemented with 0.2 mg/ml colcemid (Roche) at 37 °C for 20 min, and subsequently washed three times with 2 ml phosphate buffered saline (PBS) containing Ca<sup>2+</sup> and Mg<sup>2+</sup>. A minimum of fifteen colonies were mechanically detached from the feeder layer, collected in 2 ml  $1 \times$  trypsin-EDTA (Invitrogen) and incubated at 37 °C for 5 min. Subsequently the pellet was resuspended and incubated in 1 ml of pre-warmed potassium chloride solution (KCl, 0.075 mol/ L) for 10 min at 37 °C. Cells were then pre-fixed with 1 ml Carnoy fixative solution [methanol/acetic acid = 3/1] at -20 °C, and immediately spun at 1800 rpm for 10 min. Finally the pellet was resuspended again in Carnoy solution and prepared for analysis. For HD-iPS<sup>hom</sup> 3F-1 and WT-iPS 3F-1 cell lines, karyotype analysis was performed using QFQ-banding method, analyzing 51 metaphases.

#### Neural differentiation

The cells were differentiated according to the neural differentiation protocol described by Chambers et al. (2009). Briefly, dissociated WT-iPS and HD-iPS were plated on Matrigel<sup>TM</sup> (BD, Becton Dickinson) at a density of  $3.5 \times 10^4$  cells/cm<sup>2</sup> and maintained in hES medium with 10 ng/ml of bFGF2 for 3 days, until they reached 90% confluence. Then, we exposed the cells to 500 ng/ml of Noggin (R&D) and 5  $\mu$ M of SB431542 (Tocris) for 12 days. Then, we replated the cells on 3  $\mu$ g/ml of laminin (Invitrogen) and maintained them in N2 medium (composed of DMEM/F12, N2 100×) (Invitrogen), supplemented with 30 ng/ml BDNF (Peprotech) and  $50 \times B27$  (Invitrogen).

#### RNA isolation

After differentiation, total RNA was prepared from WT-iPS and HD-iPS cells with Trizol (Invitrogen), according to the manufacturer's

instructions; human fetal brain RNA was used as a positive control (Clontech). Before reverse transcription, the RNA was treated with DNase I (Qiagen) and purified with the RNeasy kit (Qiagen). After DNase treatment, 1  $\mu$ g of total RNA was reverse-transcribed to produce single-stranded cDNA with SuperScript III reverse transcriptase and random primers in a total volume of 20  $\mu$ l, according to the manufacturer's instructions (Invitrogen).

#### **RT-PCR** analysis

After reverse transcription, PCR was performed in a total volume of 25  $\mu$ l containing 25 ng cDNA, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.4  $\mu$ M of each primer, and 1U Taq polymerase (Invitrogen). RT-PCR was performed in 30 cycles, as follows: denaturing for 30 s at 94 °C, annealing for 30 s, and extension for 45 s at 72 °C. PCR products were resolved in a 2% agarose gel. Primer sequences, annealing temperatures, and PCR product sizes are listed in Table S1.

#### Gene expression analyses

We analyzed 36 ng of cDNA in duplicate in a Viia7 Real-time PCR thermal cycler with the Hs03005111\_91 TaqMan® Gene Expression Assay (Applied Biosystems) for OCT4, and the following primers and probe were used to amplify vector-derived transcripts (to detect the 3'UTR of the vector): Forward primer: 5'-GGCTGTTGGGCACTGA-CAAT-3' 900 nM, Reverse primer: 5'-ACGTCCCGCGCAGAATC-3' 900 nM, Probe: 5'-6-FAM-TTTCCATGGCTGCTCGCCGT-MGB-3' 250 nM. Standard TaqMan amplification conditions were used. The Viia 7 RUO Software 1.0 was used to extract raw data (Ct and raw fluorescence). The relative expression level of each gene was calculated by the  $\Delta\Delta$ Ct method (Pfaffl, 2001), normalized to *HPRT* expression (housekeeping gene control). Results are represented as the fold change relative to fibroblasts 10 days after transduction with the reprogramming vector (calibrator).

#### Vector copy number analysis

Quantitative PCR analysis was performed as previously described (Lombardo et al., 2007). Briefly, 200 ng of genomic DNA was analyzed with primers and probes complementary to the vector backbone sequence ( $\psi$ -PBS) and to the human *TERT* gene; the latter was used for normalization. A standard curve for  $\psi$ -PBS was generated by serial dilutions of DNA from human cell lines containing a known number of vector integrations.

#### DNA extraction

iPS colonies were isolated mechanically from at least one 3.5 cm dish. Cells were collected and DNA was extracted with the Nucleospin Kit (Macherey Nagel) according to the manufacturer's instructions.

#### Analysis of CAG expansion

Amplification of PCR fragments that encompassed the CAG repeat was performed with the Taq Gold Polymerase at standard operative conditions in the presence of 1% DMSO and 1.5 mM MgCl<sub>2</sub> for 30 cycles. Each cycle consisted of a denaturation step at 94 °C for 1 min, then an annealing step at 65 °C for 1 min; and a polymerization step at 72 °C for 2 min. PCR fragment lengths were determined by capillary electrophoresis with the ABI PRISM 3130 XL Genetic Analyzer.

#### Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 15 min at room temperature (RT) and washed  $3 \times$  with PBS. The cells were then

permeabilized with 0.5% Triton (SIGMA) and blocked with 5% FBS (Euroclone) for 1 h at RT. Fixed, permeabilized cells were incubated overnight at 4 °C with the following primary antibodies and dilutions: anti-Nanog, 1:500 (Novus Biological); anti-OCT4, 1:100 (Santa Cruz); anti-TRA-1-81, 1:50 (gift from ESTOOLS consortium); anti-SOX2, 1:200 (Millipore); anti- $\beta$ 3-Tubulin, 1:1000 (Sigma); anti- $\alpha$  smooth muscle actin ( $\alpha$ -SMA), 1:800 (Sigma), anti-GATA4, 1:200 (Santa Cruz); anti-Nestin, 1:200 (Millipore); anti-MAP2, 1:500 (BD Bioscience); anti-Calbindin, 1:200 (SWANT); anti-Phospho Histone H3 (PH3), 1:100 (Cell Signaling); and anti Caspase-3, 1:150 (Cell Signaling). After incubation with the primary antibody, the cells were washed  $3 \times$  with PBS, and we added the appropriate secondary antibodies conjugated to Alexa fluorophores 488 or 568 (Molecular Probes, Invitrogen). After incubation for 1 h at RT, cells were washed  $3 \times$  with PBS and nuclei were stained with Hoechst 33258 (5 µg/ml; Molecular Probes, Invitrogen). Images were acquired with a Leica DMI 6000B microscope (4×, 10×, and 20× objectives), analyzed with LAS-AF imaging software (Rasband, W.S., Imagel, U.S. National Institutes of Health, Bethesda) and then processed using Adobe Photoshop and ImageJ.

#### LysoTracker staining

The cells were incubated for 30 min at 37 °C in proliferation medium containing 50 nM LysoTracker® Red DND-99 (Molecular Probes, Invitrogen). The cells were then incubated for 15 min at 37 °C with Hoechst 33258 (5  $\mu$ g/ml, Molecular Probes, Invitrogen).

Flow cytometry measurements were carried out on live cells exposed to 200 nM Lysotracker® Dye with a FACS Canto II (BD Bioscience). Data were analyzed with BD FACSDiva v6.1.3 software.

#### Western blot

Cells were homogenized in RIPA buffer (tris HCl pH8 50 mM, NaCl 150 mM, SDS 0,1%, NP40 1%, Sodium deoxycholate 0,5%) with PMSF 1 mM, DTT 0.5 mM and protease inhibitor cocktail (Sigma). Proteins were quantified using the BCA kit. 50 µg of protein was loaded per track onto a 11% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes that were blocked in TBS-T with 5% not fat dry milk and immunoprobed with LC3 (Cell Signaling, 1:2000) and  $\alpha$ -tubulin (Sigma, 1:5000) at 4 °C overnight. Washes were in TBS-T. Secondary antibody probing and detection was by use of the ECL kit (Pierce).

#### Statistical analysis

The Student's *t*-test or one-way ANOVA and Dunnet post hoc test were used to compare samples. P values, SEM, and means were calculated with Graph Pad Prism version 4.0.

#### Results

#### Production of iPS cells from Huntington's disease fibroblasts

We derived human iPS cell lines from primary HD fibroblast cell lines generated from three different patients with HD, designated HD509, HD832, and HD1657. The former two were rare homozygous individuals carrying, respectively, 42/44 CAG and 39/42 CAG. The latter carried 17/45 CAG. We also derived two different human iPS cell lines from WT adult fibroblasts with 15/17 CAG and WT newborn fibroblasts carrying 15/18 CAG (Table 1).

We reprogrammed HD cells by infection with two different polycistronic lentiviruses. In a first round of infection, fibroblasts were transduced with a single polycistronic lentiviral vector encoding the four reprogramming human genes *OCT4*, *SOX2*, *KLF4*, and *C-MYC* (4F). In a second round of infection, we used an improved polycistronic lentivirus

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Reprogrammed fibroblast cell lines and nomenclature of generated clones.

Fibroblast lines	Haplotype <sup>a</sup>	Age	Reprogramming factors	iPS lines
HD509 HD832 HD1657 Dermal fibroblasts Foreskin fibroblasts	42/44 39/43 17/45 15/17 15/18	59 44 36 36 Newborn	OKSM <sup>b</sup> (lentivirus) OKSM (lentivirus) OKS (lentivirus) OKSM (lentivirus) OKSM (lentivirus) OKS (retrovirus) OKSM (lentivirus)	HD-iPS <sup>hom</sup> 4F-1 HD-iPS <sup>hom</sup> 4F-2 HD-iPS <sup>hom</sup> 3F-1 HD-iPS <sup>hom</sup> 3F-2 HD-iPS <sup>hom</sup> 4F-3 HD-iPS <sup>het</sup> 3F-1 WT-iPS 3F-1 WT-iPS 4F-1

<sup>a</sup> Haplotype indicates the number of CAG repeats on each allele.

<sup>b</sup> OKSM: OCT4 (O), KLF4 (K), SOX2 (S), and C-MYC (M) genes.

encoding only three reprogramming factors, *OCT4*, *SOX2*, and *KLF4* (3F). We used a multiplicity of infection (MOI) of 1.66, because this provided an optimal compromise between transgene expression, cellular viability, and genomic preservation. At 5 days post infection, exogenous OCT4 and SOX2 proteins were detected in almost all transduced fibroblasts (Suppl. Fig. 1).

The infection with 4F had a reprogramming efficiency of 0.011% (17 colonies obtained from infecting  $15 \times 10^4$  fibroblasts). At about 35 days post infection, ES-like colonies were selected amplified, and propagated, either on human neonatal foreskin fibroblasts or mouse embryonic fibroblasts (Fig. 1A and Suppl. Fig. 2A). In comparison, infections with the lentivector lacking *C-MYC* showed greatly reduced reprogramming efficiency; only rare colonies (3–5) were observed, and they took 3 weeks longer to appear. We concluded that the HD mutation or the lack of normal HTT in homozygous cells, did not affect the reprogramming process. From each HD fibroblast line, we expanded 3 independent colonies that grew without any detectable differences.

For further evaluations, we randomly selected three clones from the 4F reprogrammed HD509 fibroblasts and HD832 fibroblasts (HD-iPS<sup>hom</sup> 4F-1, HD-iPS<sup>hom</sup> 4F-2 and HD-iPS<sup>hom</sup> 4F-3) and two clones from 3F reprogrammed HD509 fibroblasts (HD-iPS<sup>hom</sup> 3F-1 and HD-iPS<sup>hom</sup> 3F-2; Table 1). We also included one clone from the reprogrammed heterozygous HD1657 iPS cells (HD-iPS<sup>het</sup> 3F-1) and two from the reprogrammed wild-type fibroblast cell lines (WT-iPS 4F-1 and WT-iPS 3F-1). All clones were positive for pluripotency stem cell markers, Nanog and TRA1-81 (Fig. 1A and Suppl Fig. 2A).

Karyotype analyses in four iPS cells show occasional alterations both on WT than HD lines, such as additional chromatin on chromosome 2, a small deletion on chromosome 11 and a chromosome 12 trisomy (Suppl. Fig. 3). Notably HD-iPS<sup>hom</sup> 4F-1 and HD-iPS<sup>hom</sup> 3F-1 which were derived from the same HD509 fibroblasts, carry the same mutation (Suppl. Fig. 3).

Quantitative PCR analysis revealed that the reprogramming vector was successfully silenced after iPS selection. In two representative iPS clones (HD-iPS<sup>hom</sup> 3F-1 and HD-iPS<sup>hom</sup> 3F-2), the transgenic expression of vector (OKS)-derived transcripts was reduced to undetectable levels compared to transduced fibroblasts collected 10 days after infection, when the lentiviral promoter was highly active (Fig. 1B). In particular, while the reprogramming genes were nearly completely silenced (up to 250 fold), the endogenous *OCT4* was highly expressed; this confirmed a strong reactivation of the pluripotency program.

Embryoid bodies (EBs) were next generated from WT-iPS, HD-iPS<sup>het</sup> 3F-1, and HD-iPS<sup>hom</sup> lines to assess their ability to generate cells of the three germ layers. After 15 days in vitro, EBs from all cell lines gave rise to cells immunopositive for the three germ layer markers,  $\beta$ 3-Tubulin,  $\alpha$ -SMA, and GATA 4. Furthermore, teratoma-like structures were found in each mouse injected with iPS cells (Fig. 1D and Suppl. Fig. 2C). These teratomas contained tissue components of the three germ layers, ectoderm, mesoderm, and endoderm



**Fig. 1.** Characterization of self-renewal condition by detection of pluripotency markers. (A) Phase contrast images display typical ES-like morphology; immunofluorescence images show expression of pluripotency markers, Nanog and TRA1-81. Scale bar, 100 μm. (B) Silencing of the reprogramming vector upon iPS derivation from fibroblasts of patients with HD. Histogram shows the fold changes in the levels of expression of the *OCT4* and vector (OKS)-derived transcripts in two representative iPS clones (red bars), relative to transduced fibroblasts (blue bars). *HPRT* expression was used for normalization. Note the nearly complete silencing of the reprogramming vector (up to 250 fold) and the high level of expression of the endogenous *OCT4* in iPS clones. (C) HD-iPS<sup>hom</sup> 4F-1, HD-iPS<sup>hom</sup> 3F-1, and WT-iPS 3F-1 were subjected to EBs formation in order to test their pluripotency. After 15 days, all EBs expressed markers of mesoderm (α-SMA), ectoderm (β3-Tubulin), and endoderm (GATA4). Scale bar, 100 μm. (D) To demonstrate the pluripotency of the iPS clones in vivo, cells were injected subcutaneously into four 7-week-old SCID beige mice. Teratoma-like tumors were found in each mouse injected with HD-iPS<sup>hom</sup> 3F-1 and HD-iPS<sup>hom</sup> 3F-2 cells. These teratomas contain prominent yolk sac-like structures. In the upper left image is present hepatoid differentiation (\*). In the upper right corner tubules lined by a cylindrical epithelium is seen (\*). The latter structures reveal strong nuclear reactivity with antibodies directed against OCT3/4. Cell with a hepatoid differentiation stain positive for OCT3/4 are negative for GPC3 and vice-versa. Scale bar, 100 μm. (E) CAG stability during self-renewal was analyzed before (top) and after 20 (P20) and 40 (P40) passages. The capillary spectra of fragment lengths indicate prevalent peaks at 162 and 168 base pairs, which correspond to 42 and 44 CAG repeats, respectively.

(Suppl. Fig. 2C). In addition, we found very prominent yolk sac-like tissue components, similar to those observed in human germ cell tumors and teratomas. The yolk sac-like nature of these tumors was demonstrated with immunohistochemical staining for AFP and GPC3 (Zynger et al., 2010) (Fig. 1D). Furthermore, cell aggregates resembling embryoid structures expressed OCT 3/4 indicating retention

of a pluripotent cell compartment. Hence, these pluripotent iPS cells also showed the capacity to form primitive endoderm that developed into extraembryonic yolk sac tissue. This was not described previously for human pluripotent stem cell injections into SCID mice.

Furthermore, because lentiviruses are expected to give rise to only one or a few integrations, we analyzed the vector copy number per cell (VCN) in two representative cell lines generated with the 3F vector. As shown in Suppl. Fig. 3, these clones carried only one or two copies of the integrated virus per cell. This result was also consistent with the MOI used for the infection.

## CAG repeat length during HD-iPS cell reprogramming, long-term growth, and differentiation

The CAG repeat in *htt* is reported to undergo tissue-specific somatic instability in transgenic mice models and in HD patient brains, predominantly in the striatum (Dragileva et al., 2009; Gonitel et al., 2008; Kennedy et al., 2003). Accordingly, it was suggested that brain-specific changes in CAG length could modify the disease process (Kennedy et al., 2003). Moreover, in a human ES cell line carrying the HD gene, a (limited) expansion of five CAG repetitions after neural differentiation was reported (Niclis et al., 2009), although this was not confirmed in other hES cell lines (Seriola et al., 2011).

We therefore analyzed our HD-iPS cell lines during proliferation and after neuronal differentiation to determine whether the native CAG length had changed after the reprogramming process and during passaging. We employed capillary electrophoresis with an ABI PRISM 3130 XL Genetic Analyzer. We found (Table 2) that all HD-iPS cells retained the same CAG lengths that they exhibited in the original fibroblasts from which they were derived. Moreover, DNA analyses of clone HD-iPS<sup>hom</sup> 4F-1 showed that the CAG repeats remained stable for at least 40 passages in vitro (Fig. 1E and Table 2). Furthermore, 30 days after the HD-iPS<sup>hom</sup> lines were differentiated into neurons (see below), the DNA showed no expansion in CAG repeats. On the contrary, we occasionally detected a contraction of CAG repeats; e.g., one haplotype with 42/44 CAG had lost two CAG triplets (see Fig. 2A showing the unique prevalent peak at 162 base pairs, corresponding to 42 CAG repeats). These data indicated that the CAG repeats in the human gene were stable during passaging and remained unaltered after neuronal differentiation.

## Neural differentiation of HD-iPS was variable, but not impaired by CAG expansion

To assess the neuralization capacity of our human HD-iPS cell lines, we applied a recently published monolayer protocol for differentiation (Chambers et al., 2009). Briefly, dissociated pluripotent cells were plated on Matrigel<sup>™</sup> and exposed for 15 days to dual SMAD pathway inhibitors, Noggin and SB431542. This treatment potently suppressed endoderm, mesoderm, and trophoectoderm differentiation and enhanced conversion to neural ectoderm (Chambers et al., 2009). After 15 days, we monitored the appearance of typical neural markers. As shown in Fig. 2B and in Suppl. Fig. 4, both WT and HD-iPS lines were immunopositive for neuroepithelial markers, Nestin and PAX6. The cells were organized in radial structures that resembled neural rosettes; the human ES cell line, H9 was used as an internal control to verify the efficacy of the protocol (Suppl. Fig. 4). We found that the different pluripotent lines responded to the neural induction protocol similarly as approximately 15-20% of cells in all lines showed expression of PAX6 (Fig. 2B and Suppl. Fig 4B).

Table 2			
Analysis of CAG lengths after	reprogramming and	during passages	in vitro.

Fibroblast lines	iPS lines	Cell passage	Haplotype
HD509	HD-iPShom 4F-1	20	42/44
	HD-iPShom 4F-1	40	42/44
	HD-iPShom 4F-2	12	42/44
	HD-iPShom 3F-1	13	42/44
	HD-iPShom 3F-2	13	42/44
HD832	HD-iPShom 4F-3	46	39/43

We next investigated the capacity of WT and HD-iPS lines to give rise to mature neurons. The neural progenitors generated during the neural conversion phase were replated on laminin to stimulate differentiation. After 20 days in a serum free-medium supplemented with N2, B27, and BDNF, neuronal cells started to appear. At day 30, they began to project thin, branched neurites from compact clusters of cellular bodies. Again, no major differences were observed between WT and HD iPS cells in the timing of  $\beta$ 3-Tubulin and MAP2 expression (Figs. 2B, C and Suppl. Fig. 4); however, the percentage of immunopositive  $\beta$ 3-Tubulin cells was intrinsically variable among the HD-iPS lines, ranging from 12% ± 1.20 to 34% ± 2.40 (Suppl. Fig. 4C). This was not unexpected, because differentiation capacities were previously reported to be intrinsically different in different ES and iPS lines (Hu et al., 2010; Kim et al., 2010, 2011; Osafune et al., 2008).

Furthermore, after 50 days of differentiation both WT- and HDiPS show equivalent proportions of GABA immunopositive cells (Fig. 2C), revealing also neurotransmitter maturation.

HD primarily affects adult striatal and cortical neurons (Reiner et al., 1988). However, developmental abnormalities may occur early during neuronal specification. For example, it was reported that the striatal anlage of HdhQ111 mice expressed Nanog which, together with SOX2 and Stat3, may cause developmental abnormalities that might subsequently render neurons more vulnerable to HD (Molero et al., 2009). To assess whether gross alterations in developmental programs were present in maturing HD-iPS cells, we exposed WT-iPS 3F-1 and HD-iPS<sup>hom</sup> 4F-1 lines to a differentiation protocol that lasted > 50 days. At days 0, 10, and 20, we analyzed the mRNA expression levels of OCT4, Nanog and anterior specification genes, SIX3 and FOXG1. An RT-PCR analysis (Fig. 2D) showed that, as expected, Nanog mRNA became undetectable during differentiation of WT and HD-iPS lines. In contrast, both cell lines showed active expression of SIX3 and FOXG1 after 20 days. Furthermore, at the end of differentiation (day 50), we assessed mRNA expression levels for typical GABA-ergic and striatal markers, including GAD65/67, ARPP21, and DARPP32 (Fig. 2E). We found no significant differences between lines that could be attributed to CAG expansion. We concluded that the HD mutation did not appear to affect specification of the anterior fate and neuronal subtype differentiation programs, at least in the available lines and for parameters/protocols tested.

#### Phenotypic assays in HD-iPS cells

To identify an HD-related phenotype in our iPS cells, we analyzed specific molecular readouts that have been implicated in disease pathogenesis in animal and cell HD models. In a preliminary investigation, we considered the main cellular processes of proliferation, death, and lysosomal expansion. We analyzed cell proliferation and apoptotic activity in self-renewal conditions by immunofluorescence. In particular, phospho histone H3 (PH3) identifies cells in mitosis, and activated caspase-3 indicates apoptosis. Fig. 3A shows the absence of noticeable differences in proliferative rates, assessed by the percentage of PH3 positive cells in WT- and HD-iPS lines. In particular, the numbers of PH3 positive cells showed that about 5% of the control population was in the M-phase of the cell cycle, and this prevalence was not altered by the HD mutation (Fig. 3B). The same was observed in all HD-iPS lines tested (not shown). We then measured apoptosis by counting the number of cells immunoreactive for activated caspase-3; we found no differences between genotypes, which suggested that the presence of the mutation was not sufficient to activate a cell death program (fluorescence signal intensities: WTiPS 3F-1: 30.4 ± 4.1; HD-iPS<sup>hom</sup> 4F-1: 21 ± 4.1; and HD-iPS<sup>hom</sup> 4F-2:  $20.7 \pm 1.8$ ) (Fig. 3A).

Aggregates of mutant HTT protein have been reported in HD human brain and several HD mouse models (Zuccato et al., 2010). In mammalian cell culture systems, a strong correlation was described between HTT aggregates formation and susceptibility to cell



**Fig. 2.** Characterization of HD-iPS<sup>hom</sup> 4F-1, HD-iPS<sup>hom</sup> 3F-1, and WT-iPS 3F-1 cell lines after differentiation. (A) CAG stability after 30 days of differentiation. Capillary spectra of fragment lengths indicate a prevalent peak at 162 base pairs, which corresponds to 42 CAG repeats. (B, top) After 15 days of differentiation, immunofluorescence shows expression of PAX6 (green) and Nestin (red); (bottom) after 30 days of differentiation, immunofluorescence shows expression of  $\beta$ -3-Tubulin (green) and MAP2 (red). Scale bar, 100 µm. (C) After differentiation for over 50 days in a medium with neuronal supplements and neurotrophins, WT-iPS 3F-1 and HD-iPS<sup>hom</sup> 4F-1 were immunopositive for GABA (bottom). Scale bar, 100 µm. (D) PCR analysis performed during neuronal induction show, after 0, 10, and 20 days, changes in mRNA expression of *OCT4*, *Nanog*, *SIX3*, and *FOXG1*; equivalent levels of *GAPDH* (control) show equivalent loading. The reaction run without reverse transcriptase (-RT) and with standard RNA templates (+CTRL) served as negative and positive controls, respectively. (E) After 55 days of differentiation, terminally differentiated neurons show mRNA expression of GABA-ergic and striatal genes, *GAD65/67*, *ARPP21*, and *DARPP32*.

death (Zuccato et al., 2010). Alternatively, an independent set of data argued that aggregates in HD were neuroprotective; they acted by stimulating the autophagic process and enhanced clearance of mutant HTT (Ravikumar and Rubinsztein, 2006). Two major protein clearance pathways exist in mammalian cells; the ubiquitin–proteasome pathway and the autophagy–lysosome pathway. An increased number of autophagosome-like structures was described both in human HD postmortem material (Davies et al., 1997; Sapp et al., 1997) and in HD cell models (Ravikumar and Rubinsztein, 2004).

Based on those observations, we analyzed the lysosomal content in HD-iPS cells during proliferation. We used LysoTracker® Red DND-99, a red-fluorescent dye that stains acidic compartments, like lysosomes, in live cells. After exposure of WT-iPS 3F-1, HD-iPS<sup>hom</sup> 4F-1, and HD-iPS<sup>hom</sup> 4F-2 to the dye for 30 min, cells were fixed, and images were analyzed with ImageJ software to measure the fluorescence signal intensity for positive cells. As shown in Fig. 3C, the fluorescent signal was very bright in a percentage of HD-iPS cells. In particular, we found that HD-iPS cell lines had very high



**Fig. 3.** Immunofluorescence visualization of PH3 and activated caspase-3 expression in WT-iPS 3F-1, HD-iPS<sup>hom</sup> 4F-1, and HD-iPS<sup>hom</sup> 4F-2 cells during self-renewal. (A) No evident differences were detectable between WT and HD-iPS lines. Scale bar, 100  $\mu$ m. (B) Percentage of PH3 positive cells in control and HD-iPS cells. Four fields per clone were analyzed from two independent experiments. Error bars show SEMs, assessed by one-way ANOVA followed by Dunnet post hoc test. (C) Staining with LysoTracker Red DND-99, a red-fluorescent dye that stains acidic compartments in live cells. Three representative colonies are shown for each cell line. The cropped images show a higher magnification of the central portion of colonies. Scale bar, 100  $\mu$ m. (D) LysoTracker red mages were analyzed in HD lines with ImageJ software to quantify the numbers of labeled dots per field. Three fields per clone were counted from two independent experiments. Error bars show SEMs, assessed by one-way ANOVA followed by One-way ANOVA followed by Dunnet post hoc test; \* p < 0.05; \*\* p < 0.01. (E) Cytofluorimetry analysis after staining with LysoTracker and adding 100 mM sucrose for 48 h; we analyzed  $2 \times 10^4$  cells/line; a threshold was set on the isotypic intensity signal.

signal accumulation; i.e., 4 times higher than that observed in WT-iPS 3F-1. The number of stained dots/field was  $16.25 \pm 2.83$  in WT-iPS 3F-1;  $45.01 \pm 5.56$  in HD-iPS<sup>hom</sup> 4F-1; and  $60.33 \pm 9.68$  in HD-iPS<sup>hom</sup> 4F-2. These results suggested that iPS lines harbored an increased number of lysosomes compared to controls (Fig. 3D).

To more quantitatively confirm this observation, we performed flow cytofluorimetry on live cells after staining with LysoTracker dye. To enhance the lysosomal responses in different lines, we induced lysosome engulfment by exposing HD-iPS<sup>hom</sup> clones to 100 mM sucrose for 48 h (Sardiello et al., 2009). The analysis was performed with a threshold set on the isotypic intensity signal. This showed that the proportion of labeled HD-iPS cells was twice the proportion of labeled WT-iPS cells (Fig. 3E and Suppl. Fig. 5). This suggested that HD-iPS lines were highly susceptible to stressors.

We performed the same experiment in WT-iPS 3F-1 and HD-iPS<sup>hom</sup> 4F-1 cells that were entering the first steps of neuronal differentiation. On day 5 after exposure to the monolayer differentiation protocol (Figs. 4A, B), the proportion of labeled HD-iPS cells was twice  $(46.79 \pm 4.09)$  the proportion of labeled WT-iPS cells (22.62  $\pm$  3.21). This reflected elevated lysosomal activation. We next extended the same analysis to all WT and HD-iPS cell lines after 25 days of differentiation to assess lysosomal activity in more mature neuronal cells. We found that the absolute value of fluorescence signal intensity after sucrose treatment was higher in all differentiated

cells compared to self-renewing cells. Hence, we established a second intensity threshold, set on a fluorescence intensity of  $10^4$ . Again, HD-iPS cell lines exhibited a higher percentage of positive cells compared to WT-iPS (Figs. 4C, D). We confirmed the association of increased lysosomal content with neuronal cells by co-staining of LysoTracker dye with Nestin and  $\beta$ 3-Tubulin (Suppl. Fig. 7).

Furthermore, the differentiated neurons derived from the heterozygous iPS cell line HD-iPS<sup>het</sup> 3F-1 showed results similar to those from homozygous HD-iPS<sup>hom</sup> lines (Figs. 4C, D). This suggested that lysosomal activation was similar between neurons derived from HD homozygous and heterozygous iPS cells. The same phenotype was observed in a mouse HD-iPS cellular model (Castiglioni et al., in press). To further verify this evidence, we next performed a Western blot analysis on day 30-differentiated WT-iPS 3F-1, HD-iPShom 4F-1 and HD-iPS<sup>het</sup> 3F-1 cell lines, to investigate the presence of the autophagic marker LC3 (Fig. 4E). Macroautophagy is a lysosomaldependent process that mediates the turnover of organelles and misfolded proteins that are too large to be degradeted by ubiquitin proteasomal system, such us huntingtin aggregates (Kegel et al., 2000). Upon synthesis, LC3 is cleaved into the cytosolic protein LC3 BI. Activation of macroautophagy leads to conversion of LC3 BI into LC3 BII, a form that associates with autophagosomes. As a consequence, the amount of LC3 BII correlates closely with the number of autophagosomes. In Fig. 4E the LC3 BII/LC3 BI ratio has been used as a measure



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**Fig. 4.** LysoTracker assay in WT-iPS 3F-1 and HD-iPS<sup>hom</sup> 4F-1 cells entering the first steps of neuronal monolayer differentiation. (A) On day 5 of neural induction, HD-iPS cells showed a higher signal intensity compared to WT-iPS cells. Scale bar, 100 µm. (B) Quantification of LysoTracker labeled dots. Five fields/cell line from two independent experiments were counted with ImageJ software; the intensity of the signal was normalized to the total number of cells. Error bars show SEMs, assessed by the Student's *t*-test followed by Dunnet post hoc test; \*\*p<0.01. (C–D) Cytofluorimetry analysis on iPS clones after 25 days of neuronal differentiation and exposure to 100 mM sucrose stress for 48 h. Blue bars indicate the proportion of cells with intensities greater than the threshold of 10<sup>4</sup> fluorescence intensity. 2×10<sup>4</sup> cells/line were analyzed. (E) Western blot analysis for LC3 marker on WT-iPS 3F-1, HD-iPS<sup>hom</sup> 4F-1 and HD-iPS<sup>het</sup> 3F-1 after 30 days of neuronal differentiation. (F) Densitometric analysis of western blot for LC3 marker performed on WT-iPS 3F-1, HD-iPS<sup>hom</sup> 4F-1 after 30 days of neuronal differentiation.

of autophagosome formation (Fox et al., 2010; Kabeya et al., 2000). In agreement with the LysoTracker data, LC3 BII form is more abundant in HD-iPS lines when compared to WT-iPS. This data reinforces the evidence of an increased lysosome content and specifically of autophagosome in HD-iPS cells and it is in full accordance with data from an in vitro HD model of rat primary neurons, transfected with mutant HTT (Liang et al., 2011),

#### Discussion

In this study, we generated HD-iPS cells from three patients that included two rare homozygous HD haplotypes. We showed that both homozygous and heterozygous HD iPS lines could be derived, expanded, and differentiated into cells of the three germ layers or into neurons. Hence, these data showed that two mutant alleles did not constrain efficient neuronal differentiation.

In this work, we generated HD-iPS lines from fibroblasts, both in the presence (4F) or absence of C-MYC (3F). These somatic cells underwent reprogramming under both conditions; molecular and cellular analyses showed no differences between HD-iPS<sup>hom</sup> 3F and HD-iPS<sup>hom</sup> 4F cells in pluripotency markers expression, colony morphology, or growth rate. Neuronal differentiation was also comparable between the 3F- and 4F-iPS cells.

Teratoma formation was achieved with reprogramming. Teratomas, teratocarcinomas, and yolk sac tumors are not uncommon among humans; they occur often in children and young individuals. They often contain yolk sac structures (Almstrup et al., 2007; Pierce et al., 1970; Prevedello et al., 2007; Thomas et al., 2010). Ectopic human embryos also form yolk sac tissues (Sathananthan et al., 2011). This was the first study to show that the teratomas formed by iPS cells included yolk sac tissue components. It is quite interesting that yolk sac tissues can be formed by reprogramming skin cells from older individuals.

CAG instability is a well-documented phenomenon in HD gametes. It may cause genetic anticipation, with earlier HD onset in the next generation (Pearson et al., 2005; Yoon et al., 2003). CAG instability was also shown in lymphoblasts when expansion repeats exceeded 60 CAG (Cannella et al., 2009), in somatic cells, specifically in postmitotic mouse neurons (Dragileva et al., 2009; Gonitel et al., 2008), and in human brain neurons (Kennedy et al., 2003). Although, no CAG instability has been detected in HD fetal tissues (Benitez et al., 1995). We followed CAG expansion during reprogramming and confirmed that conversion from fibroblasts to iPS cells did not affect its length (Park et al., 2008; Zhang et al., 2010). The CAG repeat also remained stable for over 40 passages in vitro. This result was in agreement with that observed in human ES cell models for HD (Niclis et al., 2009; Seriola et al., 2011). We also tested CAG size in neuronally differentiated HD-iPS cells. Thirty days after differentiation, approximately 15-30% of the cells had a neuronal character, however we did not detect any major change in CAG repeats; in contrast, a deletion of two triplets on one allele occurred in all four HD-iPS<sup>hom</sup> cell lines analyzed (not shown). Seriola and collaborators saw no difference in CAG expansion in heterozygous HD-ES (Seriola et al., 2011); however, another study reported low-level instability in neurospheres derived from two human HD-ES lines, with an expansion of five CAG repeats (Niclis et al., 2009).

We also noted that neural differentiation efficiency was quite variable in the different lines. An analysis of the behavior of all HD-iPS cell lines, including four that were derived from the same fibroblasts, indicated that this variability was intrinsic to the line, or even to the clone (e.g., Suppl. Fig. 4C), and did not reflect a role of the CAG mutation. This variability among cell lines was consistent with recent observations on the innate differentiation propensity of hES and iPS cell lines (Hu et al., 2010; Kim et al., 2010; Osafune et al., 2008). This unpredictability has attracted much attention, because it may mask subtle molecular differences in the original

fibroblasts or iPS cells. A recent study identified miR-371-3 as a predictive marker of neural differentiation potential in pluripotent lines (Kim et al., 2011).

The similarities between WT and HD-iPS, despite intrinsic line variability, were further reinforced in the RT-PCR analysis of genes diagnostic for telencephalic specification (*SIX3* and *FOXG1*) and striatal differentiation (*GAD65/67*, *ARPP21* and *DARPP32*). These analyses did not reveal major differences between WT and HD cell lines. Thus, our results suggested that cells with mutations in HTT were perfectly capable of reaching maturation. This result was consistent with the evidence that homozygous carriers of HD are born asymptomatic (Squitieri et al., 2003).

Increased autophagy was observed in HD mouse brains. This was shown to cause a long-term failure in toxic protein clearance (Martinez-Vicente et al., 2010). In this study, we found higher lysosomal activation in HD-iPS lines than controls during self-renewal and differentiation conditions. We also found an increased number of subcellular acidic compartments compared to controls. This suggested highly active protein degradation. This process may contribute to preserving neurons in vitro.

Collectively, our observations suggested that our human HD-iPS cells could replicate some of the molecular and biological phenotypes typically observed in HD and remained fully capable of generating neurons. In future studies, this revolutionary technology may be used to establish a powerful platform for target discovery and drug screening approaches.

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