Supplementary Materials and methods

Cell culture & Media

hESC (H9; purchased from WiCell, Madison, WI, passages 36-65), ZiCs (ZiC1, ZiC2 and ZiC3) passages 12–40, pZiCs (pZiC1, pZiC2, pZiC3) passages 3-5 and OSK-induced hiPSC were cultured on mitomycin treated mouse embryonic fibroblasts (Globalstem, Rockville, MD). Cells were grown in DMEM/F12 (Invitrogen, Carlsbad, CA), 20% knockout serum replacement (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St Louis, MO), 6 ng/ml FGF-2 (Invitrogen), NEAA (Invitrogen), Sodium Pyruvate (Invitrogen) and Pen-strep (Invitrogen). Cells were passaged using 2mg/ml of collagenase IV (Invitrogen), washed and replated at a dilution of 1:4 to 1:10 every 3-4 days. OSK-induced hiPSC were generated by transduction of BJ1 fibroblasts with OCT4, SOX2 and KLF4 retroviral vectors (Addgene, Cambridge, MA) and characterized by RT-qPCR for endogenous pluripotency gene expression and transgene expression, immunostaining for SSEA4, TRA160, OCT4 and NANOG, EB formation, teratoma formation, microarray analysis and targeted differentiation (data not shown). All studies using primary human fibroblasts as well as human ESC were approved by the Ethic Committee for Use of Humans in medical research, at KU Leuven.

Alkaline Phosphatase staining

Cells were washed twice with PBS, fixed and stained with alkaline phosphatase substrate as per manufacturer’s instruction (Stemgent® Alkaline Phosphatase Staining Kit, Cambridge, MA, USA). After incubation with substrate for 15 min, colonies were stained purple and the image was captured using a digital camera (Canon, Tokyo, Japan).

Plasmid construction, retroviral transduction
The coding region of the mouse Zic3 gene was amplified by RT-PCR with primers listed in Supplementary Table 1, and was cloned in pMIG-IRES-GFP plasmid (Addgene). The pMXs plasmids encoding for OCT4, SOX2 and KLF4 were purchased from Addgene. pMXs and pMIG-based retroviral vectors were transfected individually along with the viral packaging genes gag-pol (Addgene) and VSVG (Addgene) into 293T cells (ATCC; Washington, DC) using Fugene HD reagent (Roche, Basel, Switzerland) according to the manufacturer’s directions. Generation of reprogrammed cells was performed as previously described (Okita et al., 2007) with some modifications. Briefly, human BJ1 cells (Lonza BioWhittaker, Basel, Switzerland) or human primary fibroblasts were seeded at 1.5 × 10^5 cells/well in 6-well plates (Sigma-Aldrich,) without feeders. Cells were transduced with Zic3, KLF4, OCT4 and SOX2 retroviral vector containing supernatants mixed equally. To achieve maximum transduction efficiency, cells were transduced twice. Four days after transduction, the cells were reseeded at 1.5 × 10^5 cells per 100-mm dish containing MEF and cultured in hESC medium. ZiCs were picked 15-20 days and pZiCs 23-26 days post transduction, and expanded further.

ZiC-GFP cells were generated by transduction of ZiCs with pLVX-GFP lentiviral vector using the viral packaging plasmids pMD2.G and psPAX2 (Addgene).

**Genomic DNA PCR**

Genomic DNA was isolated as per the manufacturers’s instructions (Qiagen DNeasy kit, Qiagen, Hilden, Germany). Using transgene specific primers (Supplementary Table 2), the target genes were amplified and loaded on a 1% Agarose (Sigma-Aldrich) gel and were visualized to detect the presence of transgene integration. Primers used are listed in supplementary table 2.
**Real time Quantitative PCR**

RNA was obtained from cells using the RNeasy microkit (Qiagen). 1 mg of DNase treated RNA was reverse transcribed using a superscript III first strand cDNA synthesis kit (Invitrogen). cDNA was further diluted to 100 μl and 2 μl of cDNA was used for quantitative PCR using the Sybergreen PCR kit (Invitrogen). All primers are listed in Supplementary Table 2.

**Short Hairpin RNA (shRNA) experiments**

Two independent Zic3 shRNA sequences (V3THS_304236, V3THS_304235) obtained from Open Biosystems (Huntsville, AL) were cloned in pTRIPZ doxycycline inducible lentiviral vector (Open Biosystems) by digesting with EcoRI and XhoI (Roche). A universal non-silencing construct (Open Biosystems) was used as control. Constructs were sequenced and transfected into 293T cells using the viral packaging plasmids pMD2.G and psPAX2 (Addgene). Supernatants were collected after 48h and 72h, and used to transduce ZiCs. To select for the transduced cells, puromycin (1 mg/ml) (Sigma) selection was performed 1d after transduction. Expression of Zic3 specific shRNA was induced by adding doxycycline (1 mg/ml) (Sigma) to the media.

**Embryoid body formation**

hESC and ZiCs were plated on low adherent plates (Elscolab, Kruibeke, Belgium) in embryoid body (EB) medium (IMDM medium, 15% FBS, 2 mM L-glut, 1% NEAA, 1 mM Sodium pyruvate, 100 U penicillin/streptomycin, 200 μg/ml Iron-saturated-transferrin, 10 μM β-mercaptoethanol, 50 μg/ml ascorbic acid (Sigma)) for 8 days. Subsequently, the cells were
cultured for another 8 days on plates coated with 0.1% gelatin (Chemicon, Freiburg, Germany) in EB medium.

**Maintenance of ZiCs as neurospheres**

ZiCs were cultured and maintained as neurospheres on low adherent plates (Elscolab, Kruibeke, Belgium) in NSC maintenance medium consisting of DMEM/F12 (Invitrogen), N2 supplement 1:100 (R&D Systems, Minneapolis, MN), Glucose 0.16% (Sigma), B27 supplement 1μl/ml (R & D Systems), Insulin 20 μg/ml (Sigma), EGF 10ng /ml (R & D Systems), bFGF 10ng/ml (R & D Systems).

**Generation of neurospheres from hESC**

hESC cultures were disaggregated using accutase for 10 min and plated on Matrigel-coated dishes in mTESR medium. hESC were allowed to expand for 3d or until they were nearly confluent. The differentiation towards neural progenitors was performed in hESC media lacking bFGF with 10 μM TGF-β inhibitor (Tocris, Bristol, UK) and 500 ng/ml of Noggin (R&D). Upon day 5 of differentiation, the TGF-β inhibitor was withdrawn while maintaining 500 ng/ml of Noggin and increasing amounts of N2 media (25%, 50%, 75%) were added for an additional 6 days with media changes every 2d.

**In vitro differentiation studies**

Astrocyte differentiation: cells were cultured on gelatin-coated plates in neural stem cell medium (DMEM F12 (Invitrogen), N2 Supplement (R&D), MEM NEAA, Heparin 2 mg/ml; (Invitrogen) with 1% FCS (Invitrogen) for 14 days (Hong et al., 2008). Oligodendrocyte differentiation: ZiCs were cultured in neural differentiation medium in the presence of 20ng/ml platelet-derived...
growth factor (R&D, Minneapolis, MN), 20 ng/ml bFGF (Invitrogen) and 10ng/ml EGF (Sigma) for 7 days, after which cells were maintained for an additional 14 days in medium alone (Hong et al., 2008).

Motor neuron differentiation: ZiCs were differentiated towards the motor neuron lineage using previously described protocols (Chambers et al., 2009; Hu et al., 2009) but without the initial SMAD inhibition step. ZiCs were seeded on Poly-L-ornithine (Sigma) and Laminin (Sigma) coated plates with neural differentiation medium (DMEM F12 (Invitrogen), N2 Supplement (1X) (R&D), MEM NEAA 1% (Invitrogen), Heparin 1 mg/ml(Sigma) containing SHH (200ng/ml) (R&D) and RA (0.1uM) (Sigma). On day 4, the medium was changed to medium containing BDNF (10ng/ml), GDNF (10ng/ml), IGF1 (10ng/ml) (R & D Systems), cAMP (1uM) and Ascorbic acid (200ng/ml) (Sigma), for another 10 days. Primers and antibodies used are listed in supplementary table 2&3.

In vivo tumor formation assay

10⁶ ZiCs maintained on MEFs were harvested, resuspended in 50% hESC-qualified matrigel (VWR, Radnor, PA) and injected subcutaneously in 6-8 week old Rag2-/- γc-/- mice. After 4-5 weeks, mice were sacrificed, the tumors were removed, fixed with formalin and embedded in paraffin. Paraffin blocks were sectioned, and sections stained with H&E or with antibodies against beta-tubulin-3 and synaptophysin. All experiments with mice were approved by the institutional review board of KU Leuven.

In vivo differentiation assay

a) Stereotactic surgery
10-12 week old Rag2--/γc-/- mice were anesthetized with 75mg/kg ketamine (Ketamine 1000, CEVA, Sante Animale) and 1mg/kg medetomidin (Domitor, Orion Pharma, Espoo, Finland) intraperitoneally and positioned in a stereotactic head frame (Stoelting, Illinois, U.S.A) for stereotactic injection in the caudate putamen (striatum) using bregma as a reference point. Stereotactic coordinates starting from the dura were the following: anteroposterior= 0.5mm; mediolateral= -1.7mm; dorsoventral= 2.5-1.5mm. Using a 26S Hamilton syringe (VWR international, Pennsylvania, USA) 3μl of ZiCs, transduced with GFP and cultured as neurospheres, at 33×10³ cells/μl suspended in PBS were injected at a rate of 0.5μl/min. After injection the needle was left in place for an additional 4 min and anesthesia was reversed with 0.5mg/kg atipamezole (Antisedan, Orion Pharma, Espoo, Finland) i.p.

b) Tissue processing and immunohistochemistry

Animals were sacrificed 4-5 weeks following stereotactic surgery with an i.p. overdose of pentobarbital (Nembutal, CEVA, Sante Animale) and transcardially perfused with 4% (w/v) PFA in PBS. Brains were removed and postfixed overnight in 4% PFA at 4°C. Serial 50μm coronal sections were made using a vibratome. Free floating sections were washed three times for 5 min in PBS/Triton 0.1% X-100 (v/v) and immunostaining for GFP together with primary antibodies against MAP2, NeuN, OTX2, 200kDNF, O4 and GFAP was performed (antibodies used are listed in supplementary table 3). A number of brains were paraffin embedded and 5μm coronal sections were made. Following deparaffinisation and rehydration, antigen retrieval was performed by boiling the sections in Dako target retrieval solution (Dako, Glostrup, Denmark) for 20 min. using a pressure cooker. After cooling down for 20 min to RT the sections were rinsed in water for 5 min and washed twice in PBS/Triton 0.1% for 5 min. Further staining
was performed using the same protocol as described for processing vibratome sections followed by dehydration and mounting in DPX before microscopy analysis.

**Immunostaining**

Paraffin sections (5 μm thick) were rehydratated using standard procedures or cells were fixed using 10% Neutral Buffered Formalin (NBF) for 15 minutes at room temperature, rinsed twice in PBS (Invitrogen). Permeabilization was performed for 15 minutes using PBS containing 0.2% Triton X-100 (PBST) (Acros Organics, New Jersey). Nonspecific blocking was carried out with 10% serum corresponding to the animal source of secondary antibody (Dako) for 30 minutes. Primary antibodies at the dilutions described in supplementary Table 3 were diluted with Dako antibody diluent and the cells were incubated overnight at 4°C followed by incubation with secondary antibodies conjugated with Alexa dyes for 1 hr at room temperature (Invitrogen). For nuclear staining, 1 ug/ml Hoechst 33258 (Sigma-Aldrich) was added along with secondary antibody incubation.

**Microarray analysis**

RNA was extracted from hESC, ZiCs, and hESC-derived neurospheres. RNA concentration and purity were determined spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE) and RNA integrity was assessed using a Bioanalyser 2100 (Agilent, Santa Clara, CA). 100 ng total RNA spiked with bacterial poly-A RNA positive controls (Affymetrix, Santa Clara, CA) was converted to double stranded cDNA in a reverse transcription reaction using the Ambion WT Expression Kit (Life Technologies, Carlsbad, CA). The sample was converted and amplified to antisense cRNA in an *in vitro* transcription reaction, which was subsequently converted to single stranded sense cDNA. Finally, samples were
fragmented and labeled with biotin in a terminal labeling reaction according to the Affymetrix WT Terminal Labeling Kit. A mixture of fragmented biotinylated cDNA and hybridisation controls (Affymetrix) was hybridised on Affymetrix GeneChip Human Gene 1.1 ST Arrays followed by staining and washing in the GeneTitan® Instrument (Affymetrix) according to the manufacturer’s procedures. To assess the raw probe signal intensities, chips were scanned using the GeneTitan® HT Array Plate Scanner (Affymetrix). Analysis of the microarray data was performed in the R programming environment, in conjunction with the packages developed within the Bioconductor project (http://www.bioconductor.org; Gentleman et al., 2004). The analysis was based on the Robust Multichip Average (RMA) expression levels of the probe sets that had at least once a present detection above background (DABG) detection call. Differential expression was assessed via the moderated t-statistic [24]. To control the false discovery rate, multiple testing correction was performed and probes with a corrected p-value below 0.05 were selected. The microarray data are deposited in the NCBI’s Gene Expression Omnibus and are accessible through GEO series accession number GSE29770.

**Statistics**

Where appropriate, results were expressed as means ± SEM. Statistical analysis was performed by unpaired Student’s t test, where P < 0.05 was considered significant.
Supplementary Figure S1 Zic3 transduced colonies are not reprogrammed to an ESC fate.

(A) Morphology of a ZiC colony by light microscopy.

(B) Transcript levels for the endogenous pluripotency TF in hESC, hiPSC, ZiC1 and ZiC2 as determined by RT-qPCR. Data are shown as mean ΔCT values ± SEM relative to GAPDH from triplicate experiments in representative cell lines.

(C) Gene expression profile for the transgenes and endogenous ZIC3 in hESC, hiPSC, ZiC1 and ZiC2 as determined by RT-qPCR. Data are shown as ΔCT values relative to GAPDH (n=3).
Supplementary Figure S2 Transduction of Zic3 together with OCT4, SOX2 and KLF4 in primary human fibroblasts yields similar colonies as Zic3-transduced BJ1 fibroblasts. These colonies are referred to as pZiC1, pZiC2 and pZiC3.

(A) Quantification of the number of colonies (white bar) and 14 (black bar) days after retroviral transduction of $0.15 \times 10^6$ primary human fibroblasts with different combinations of retroviral vectors encoding for OCT4 (O), SOX2 (S), KLF4 (K) and Zic3 (Z).

(B) Transcript levels for the endogenous pluripotency TF in hESC, hiPSC and three independent pZiC (pZiC1, pZiC2 and pZiC3) colonies were determined by RT-qPCR. Data are shown as $\Delta CT$ values relative to GAPDH.

(C) Transcript levels of endodermal (FOXA2, SOX17), mesodermal (BRACHYURY, GSC) and ectodermal (PAX6) genes in hESC, hiPSC, three independent pZiC colonies and fibroblast controls determined by RT-qPCR. Data are shown as $\Delta CT$ relative to GAPDH.
Supplementary Figure S3 Gene expression analysis of ZiCs

(A) Transcript levels of endodermal (FOXA2, SOX17), mesodermal (BRACHYURY, GSC) and ectodermal (PAX6) genes in hESC, hiPSC, ZiC1 and ZiC2 determined by RT-qPCR. Data are shown as ΔCT values relative to GAPDH (n=3).

(B) The global gene expression profile of ZiCs and hESC-derived NSC were compared with that of hESC by microarray analysis. Venn diagram of genes significantly higher expressed in NSC derived from hESC and/or ZiCs compared to hESC. Significance was assigned with a corrected p-value<0.05.

(C) Functional annotation of gene categories significantly higher expressed in ZiCs and ESC-NSC, than in hESC. In the upper panel of the table, gene categories higher expressed in both ZiCs and ESC-NSC; in bottom part, gene categories more highly expressed in either ZiCs or ESC-NSC, as analysed by gene ontology analysis (http://david.abcc.ncifcrf.gov/).
Supplementary Fig. 4

Supplementary Figure S4 Immunofluorescence analysis of neural progenitor genes

Immunostaining for the neural progenitor proteins FOXG1, OTX1 OLIG2 and PAX6 in ZiCs (Representative example of n=3). Merge represents positive staining merged with nuclear DAPI staining. (Scale bar 50 μM)
**Supplementary Figure S5** Expression of neural genes is dependent on Zic3

The expression of Zic3 was silenced in ZiCs by two different shRNA constructs and the expression of neural genes analyzed by RT-qPCR. Data are shown as relative expression levels compared to the non-silencing control (fold induction) (n=2).
Supplementary Figure S6  Assessment of the differentiation potential of ZiCs by embryoid body and teratoma formation.

A) ZiCs and hESC were cultured in EB conditions and transcript levels of ectodermal (*PAX6*, *SOX1*), endodermal (*AFP*) and mesodermal (*FLK1*) genes analyzed by RT-qPCR. Data are shown as ΔCT values relative to *GAPDH*. (n=2)

B) Tumors obtained after subcutaneous injection of one million ZiC1 cells dissolved in 50% matrigel into Rag2<sup>-/-</sup> γc<sup>-/-</sup> mice were analyzed by HE staining and immunostaining for β3-tubulin and synaptophysin. (10X, Axioimager, Zeiss) (Representative example of n=3)
Supplementary Figure S7 *In vitro* differentiation of ZiCs towards an astrocyte, oligodendrocyte and motor neuron fate.

(A) ZiC1 cells were cultured in neural differentiation medium supplemented with 1% FCS to induce astrocyte differentiation. Astrocyte-specific transcripts in ZiCs and ZiCs differentiated towards the astrocyte lineage. Data are shown as ΔCT values relative to GAPDH (n=3).

(B) Progeny was immunostained for S100β (I) and GFAP (II). (Scale bar=50 µm) (Representative example of n=3)

(C) ZiC1 cells were cultured in neural differentiation medium with PDGF, FGF and EGF for 7 days, followed by culture in neural differentiation medium alone for 14 days to induce oligodendrocyte differentiation. Oligodendrocyte-specific transcripts in ZiCs and ZiCs differentiated towards the oligodendrocyte lineage. Data are shown as ΔCT values relative to GAPDH
(D) Progeny was immunostained for the oligodendrocyte protein O4. (Scale bar=50 µm)

(Representative example of n=3)

Supplementary Figure S8 ZiCs can be efficiently genetically manipulated

(A) Neurospheres transduced with GFP and cultured in NSC maintenance media on low adherent plate.

(B) ZiCs transduced with lentiviral vectors harboring GFP

(C) hESC transduced with lentiviral vectors harboring GFP