

**Stem Cell Reports, Volume 7**

## **Supplemental Information**

### **Marked Differences in *C9orf72* Methylation Status and Isoform Expression between C9/ALS Human Embryonic and Induced Pluripotent Stem Cells**

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## SUPPLEMENTAL FIGURES AND LEGENDS

Fig S1. Characterization of ALS HESC lines, Related to section “Derivation and characterization of C9/HESC lines”. (A) Staining for OCT4, Tra 1-60 and alkaline phosphatase activity. Scale bars stand for 200µm. (B) Expression of *OCT4*, *NANOG*, *SOX2* and *REX1*, by RT-PCR. (C) Karyotype analysis of ALS HESC lines by Giemsa staining. (D) Teratoma sections stained by H&E derived from SZ-ALS1 and SZ-ALS3. Scale bars stand for 130 µm. (E) Southern blot analysis identified a ~270 repeat expansion in both C9-HESC lines.

Fig S2. Characterization of C9-iPSC clones, Related to sections “Analysis of C9orf72 methylation in C9 HESCs and their haplo-identical iPSCs” and “Methylation Analysis in C9 iPSCs derived from an unrelated symptomatic ALS patient”. (A) Staining for OCT4, Tra 1-60 and alkaline phosphatase activity. Scale bars stand for 200µm. (B) Expression of *OCT4*, *NANOG*, *SOX2* and *REX1*, by RT-PCR. (C) Karyotype analysis of C9 iPSC clones by Giemsa staining. (D) Southern blot analysis identified a ~700 and ~2,700 repeat expansions in C9 iPSCs derived from patients H and M, respectively.

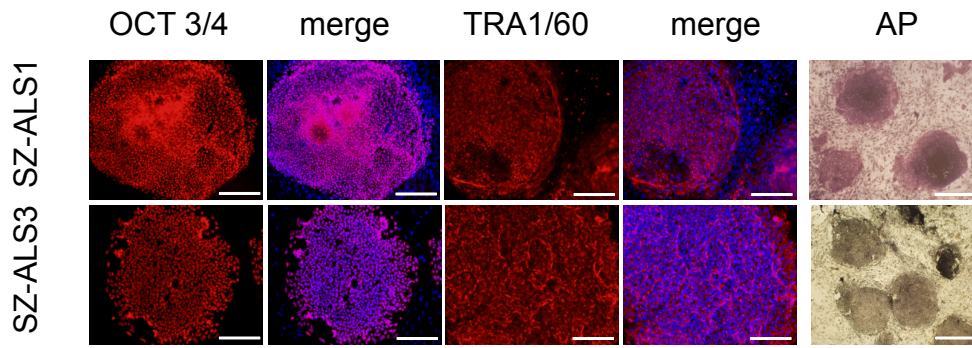
Fig S3. Methylation analysis at the promoter of *SIGLEC6* in primary fibroblasts, HESCs and iPSCs, Relates to Fig 1 and 3. Methylation levels in C9 primary fibroblasts (C9-fibroblasts H and M), iPSC clones derived from them (C9-iPSC H#8, H#10, M#1, M#9, M#10), and C9 HESCs (SZ-ALS1 and SZ-ALS3) by bisulfite DNA colony sequencing demonstrates *de novo* methylation of *SIGLEC6* exclusively in iPSCs.

Fig S4. Differentiation of HESCs and iPSCs into neural precursors (NPCs) and teratomas, Related to section “The effect of differentiation on the methylation status of C9orf72”. (A) Schematic illustration of differentiation protocol into NPCs using 2 inhibitors. (B) FACs analysis from NCAM1-positive cells in NPCs from wild type (WT) and C9 HESCs (SZ-ALS1 and SZ-ALS3), WT and C9 iPSC clones derived from patient H (C9-iPS#H8) and patient M (C9-iPS#M9). For each cell sample, unstained (left panel) and stained cells (right panel) are presented. (C) RT-PCR analysis for the expression of NPC-specific markers *SOX2*, *PAX6* and *Nestin*; the undifferentiated cell-specific marker *OCT4*; and a housekeeping gene *GAPDH* in undifferentiated and NPCs of WT, C9 HESCs (SZ-ALS1 and SZ-ALS3), and mutant iPSCs (C9-iPS#H8 and C9-iPS#M9). (D) Enrichment for mature neurons in teratomas from C9 HESCs and iPSCs. As determined by immunostaining for Tuj1-positive cells (red) and DAPI (blue) staining in teratoma sections from C9 HESCs (SZ-ALS1 and SZ-ALS3) and iPSCs (C9-iPS#H8 and C9-iPS#M9).

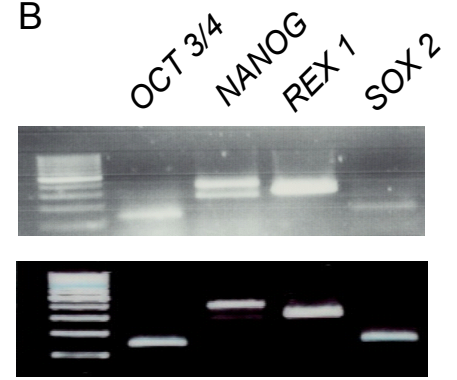
Fig S5. Expression of intron 1 retaining *C9orf72* transcripts in undifferentiated and differentiated HESCs and iPSCs, Related to Fig 6. (A) Description of the samples utilized to generate RNA-seq libraries. Samples 1-2 were derived from C9 HESCs (SZ-ALS1 and SZ-ALS3), sample 3 from wild type HESCs, samples 4-8 from C9 iPSCs clones (C9-iPS#H8, -iPS#H10, and -iPS#M1, -iPS#M9 and -iPS#H10), and sample 9 from wild type iPSCs. RNA extracted from these samples was DNase1 treated and we generated rRNA-depleted libraries utilizing a standard protocol. (B) Average coverage across intron 1 relative to exon 2 in all undifferentiated cells samples; wild type and affected HESCs and iPSCs. The data presented in fig 6B is intron/exon ratio. (C) Validation of intron 1 retaining transcripts in undifferentiated C9 mutant HESCs (SZ-ALS1 and SZ-ALS3) and iPSCs (C9-iPS#H8 and iPS#M9) and their differentiated cell counterparts; NPCs (NPC C9-iPS M#9 and H#8) and teratomas (teratoma C9-iPS M#9 and H#8) by RT-PCR. (D) Sanger sequencing of PCR products validated the existence of intron 1 retaining transcripts using primers that span over intron 1-exon 5.

Fig S1

A



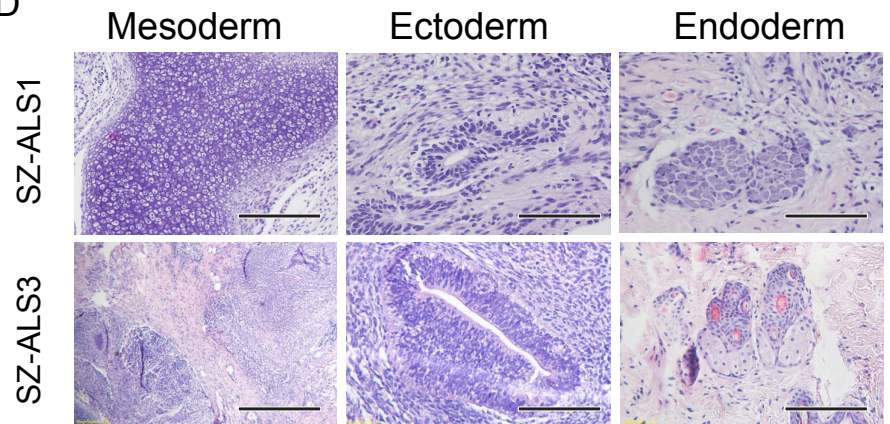
B



C



D



E

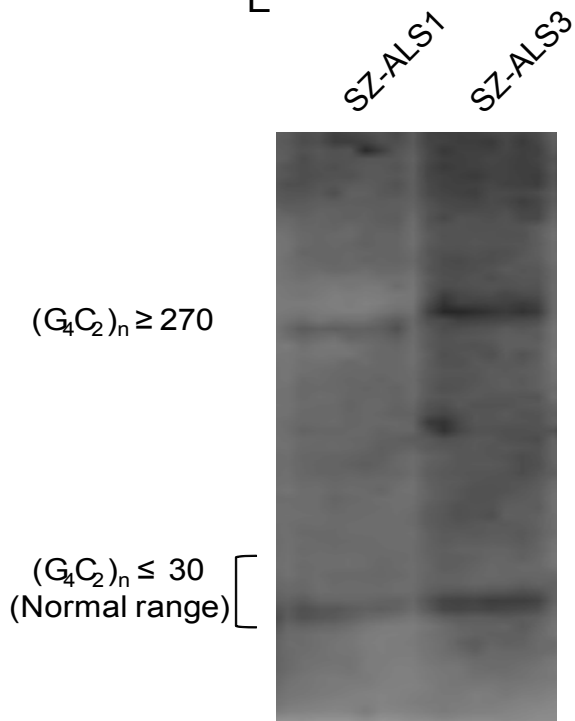
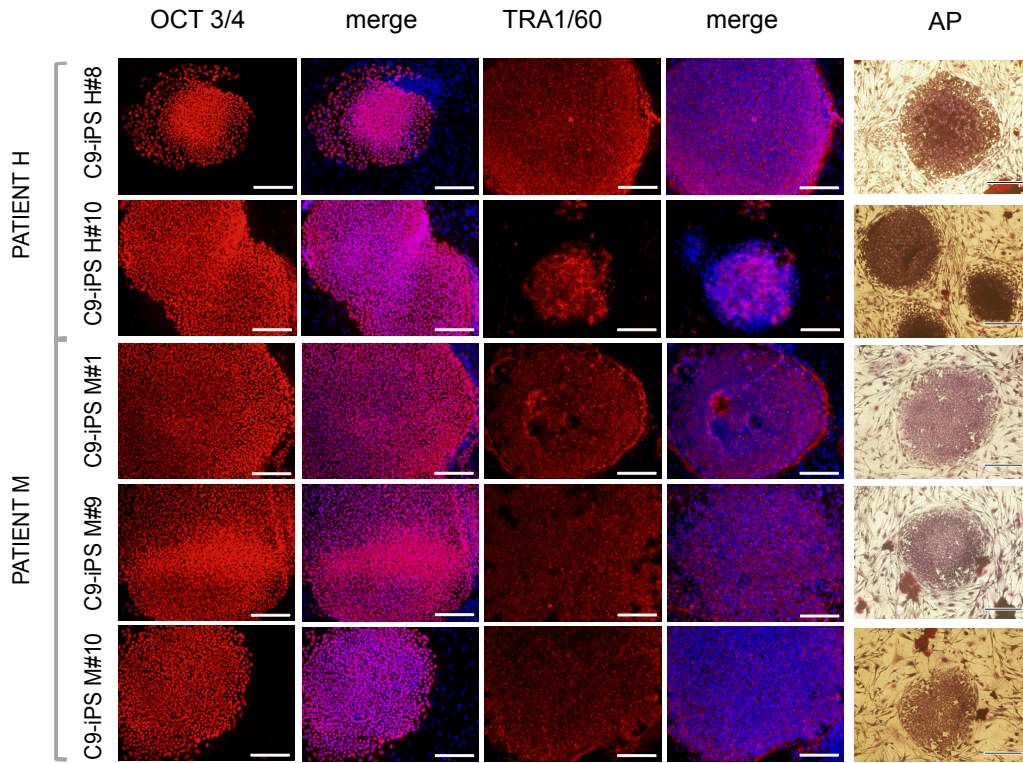
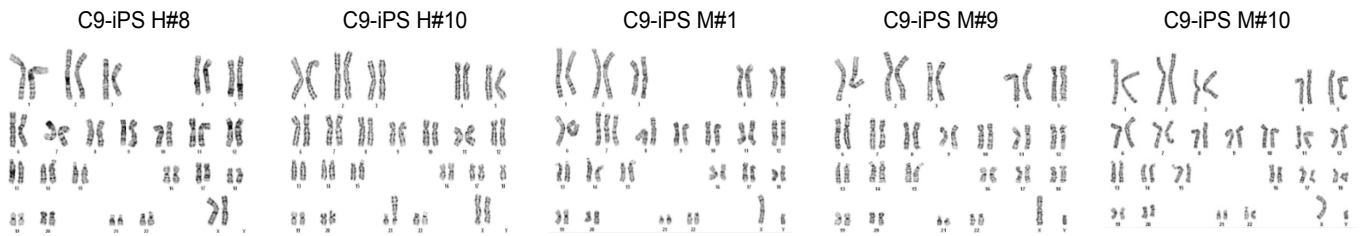


Fig S2

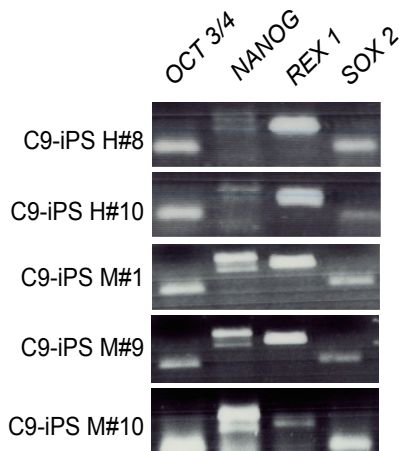
A



B



C



D

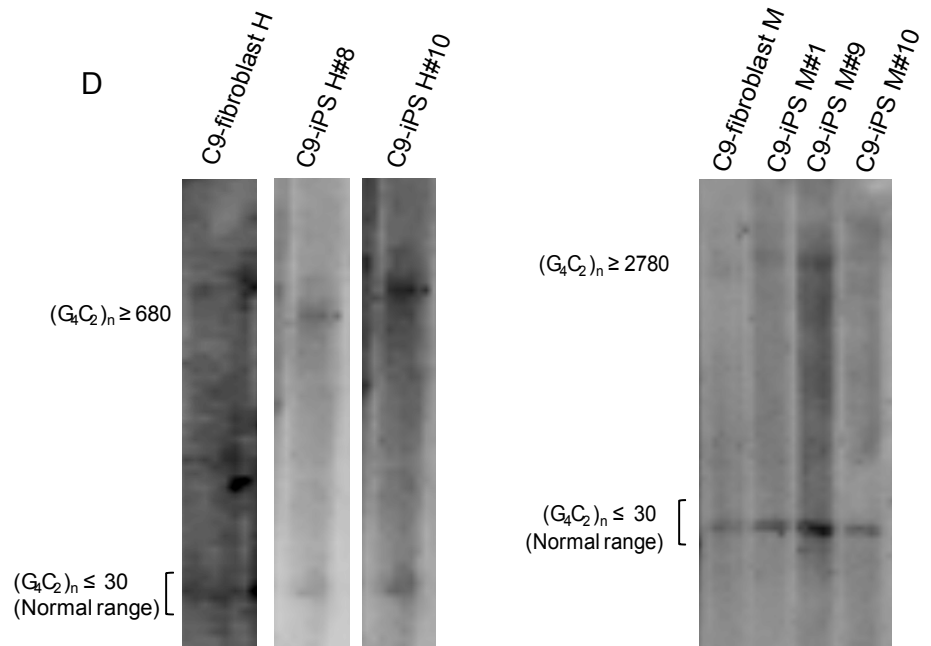


Fig S3

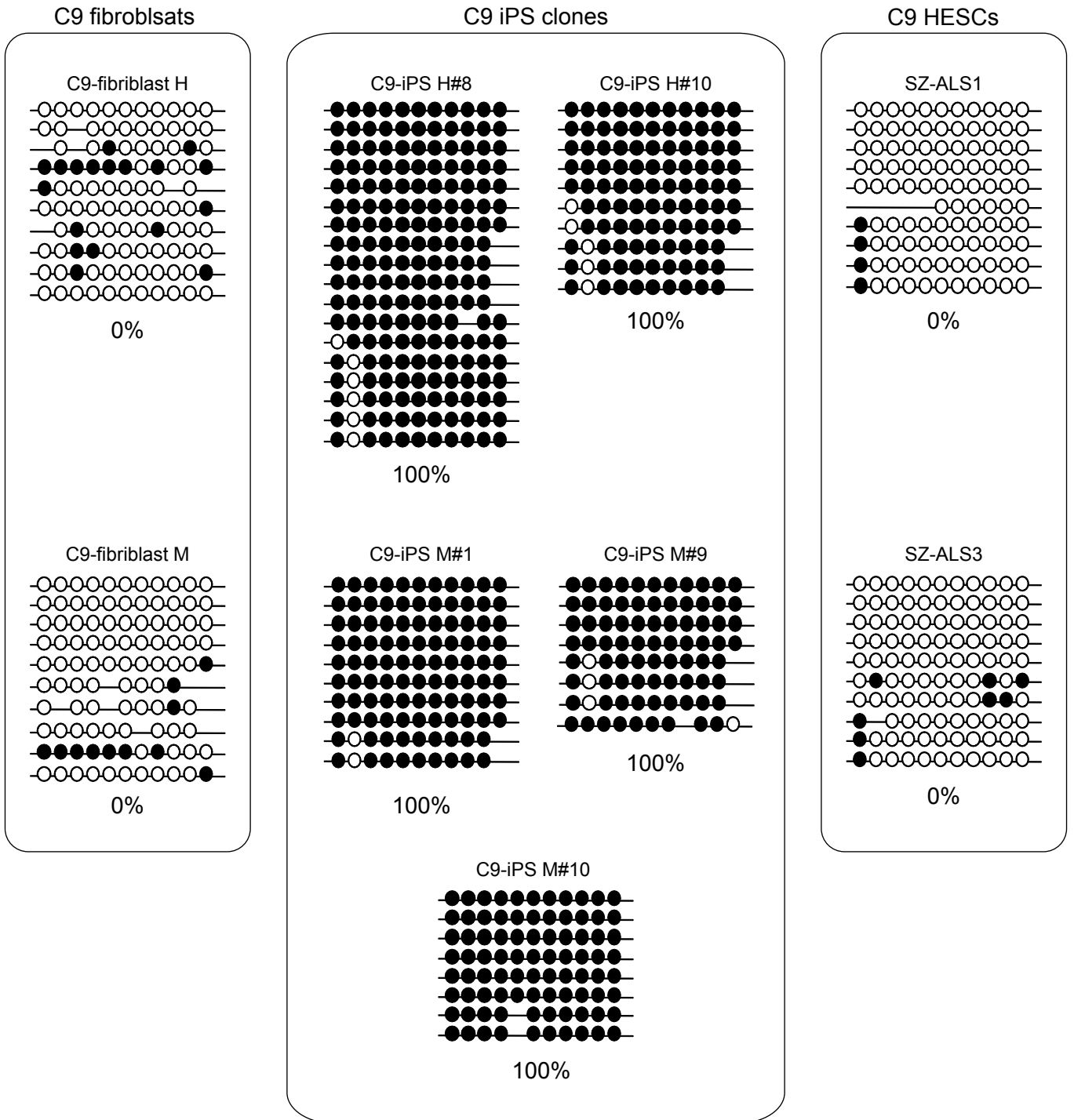
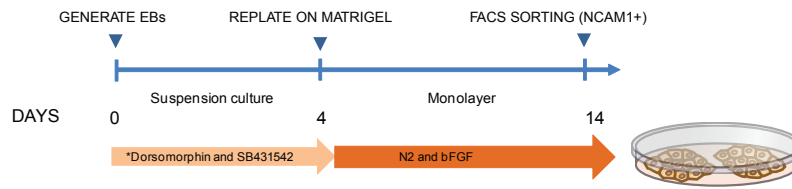
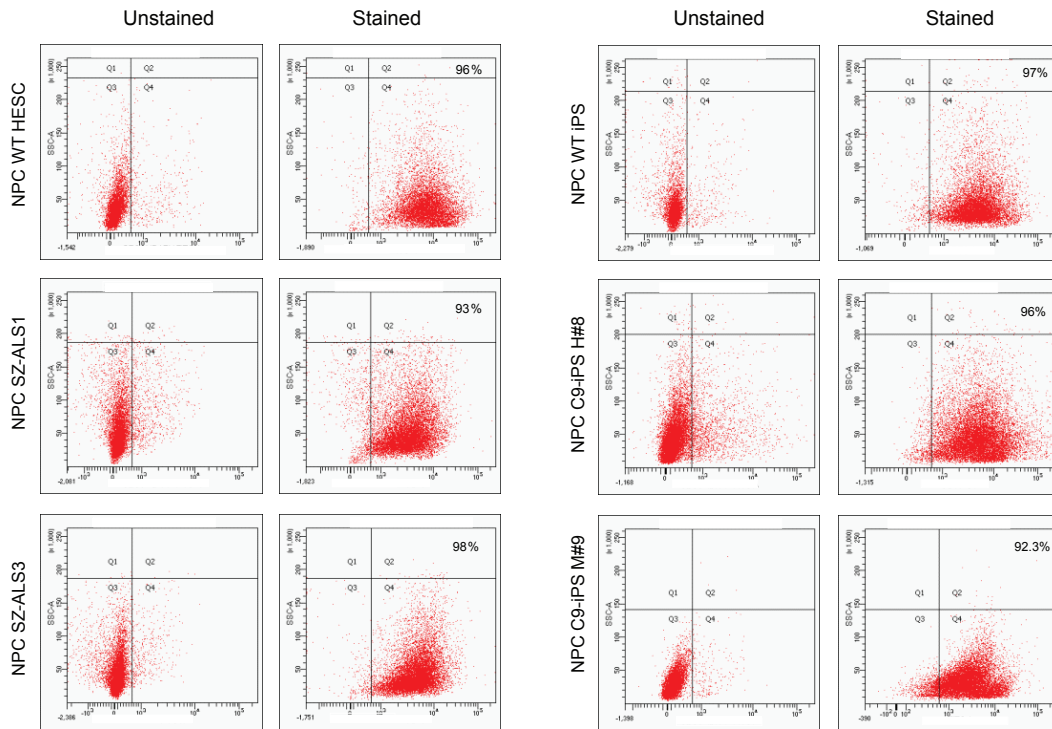


Fig S4

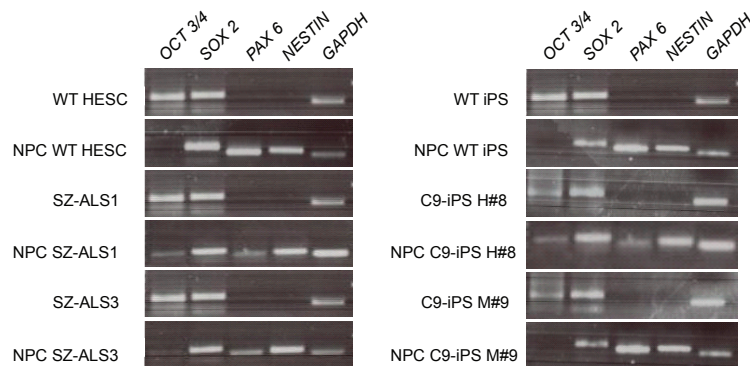
A



B



C



D

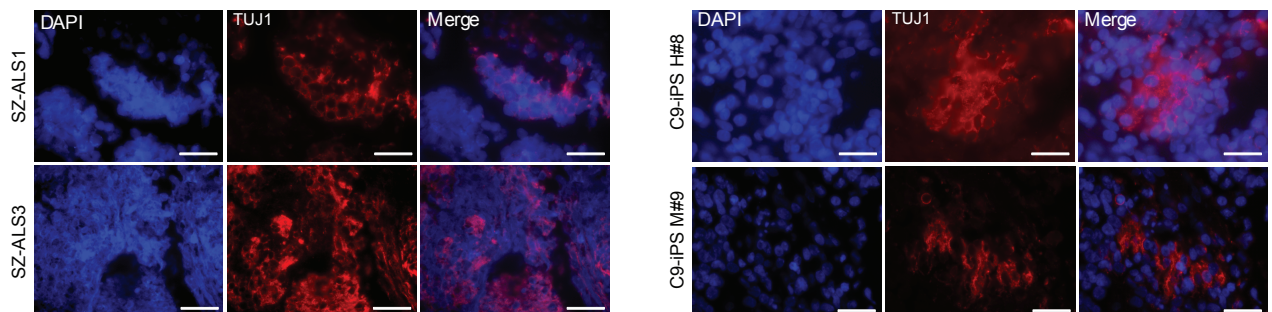


Fig S5

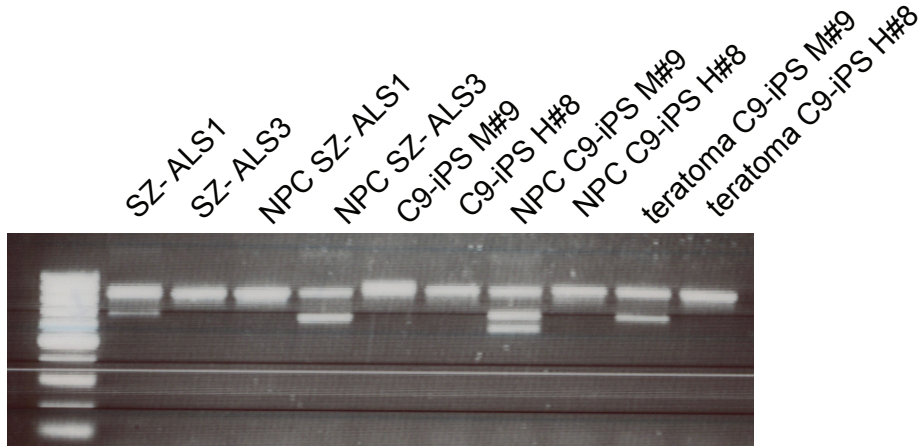
A

| CELL TYPE      | NUMBER OF NON-rRNA READS |
|----------------|--------------------------|
| SZ-ALS 1 HESCs | 27,855,214               |
| SZ-ALS 3 HESCs | 26,362,223               |
| WT HESCs       | 60,412,437               |
| C9-iPSCs M#1   | 21,860,174               |
| C9- iPSCs M#9  | 26,015,064               |
| C9-iPSCs M#10  | 44,822,844               |
| C9-iPSCs H#8   | 28,726,995               |
| C9-iPSCs H#10  | 28,038,769               |
| WT iPSCs       | 60,412,437               |

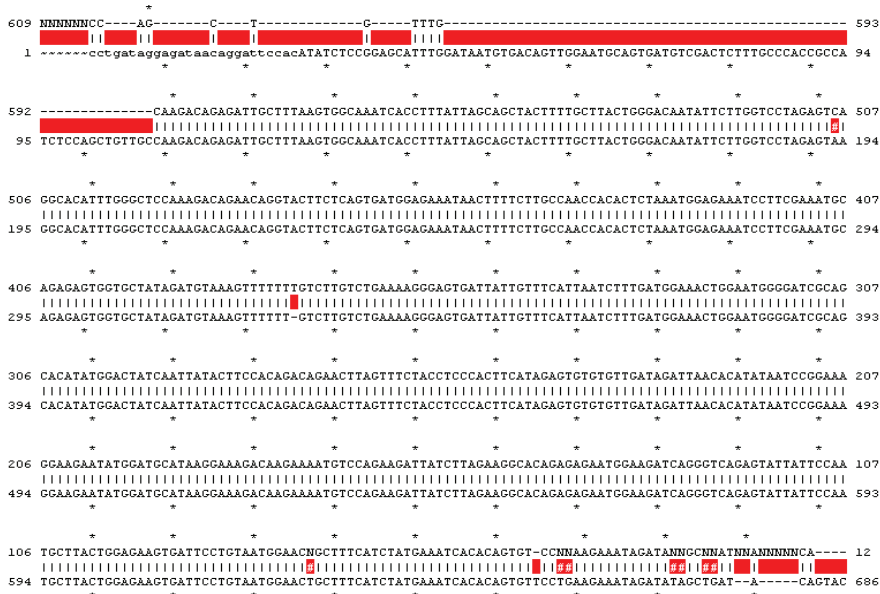
B

|                 | SZ-ALS1 HESCs | SZ-ALS3 HESCs | WT HESCs    | C9-iPSCs -M | C9 iPSCs -H | WT iPSCs    |
|-----------------|---------------|---------------|-------------|-------------|-------------|-------------|
| INTRON 1        | 3.928         | 3.470         | 1.284       | 1.697       | 0.685       | 1.404       |
| EXON 2          | 12.367        | 8.385         | 15.285      | 8.926       | 3.944       | 16.797      |
| INTRON 1/EXON 2 | 0.317584964   | 0.413791435   | 0.083997598 | 0.169390848 | 0.163897482 | 0.083583307 |

C



D



## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

|  | 5' Primer (sequence 5'-3')      | 3' Primer (sequence 5'-3')   | Annealing Temp °C | Product Size (bp) |
|--|---------------------------------|--|-------------------|-------------------|
| <b>Primer Sets for RT-PCR for endogenous pluripotent genes</b> |                                 |  |                   |                   |
| <i>OCT4</i>  | GACAGGGGGAGGGGAGGAGCTAGG        | CTTCCCTCAACCAGTTGCCCAAAC   | 60                | 144               |
| <i>NANOG</i>   | CAGCCCCGATTCTCCACCAGTCCC        | CGGAGATCCAGTCGGGTTCAAC   | 55                | 342, 390          |
| <i>REX1</i>  | CAGATCTAAACAGCTCGAGAAT          | GCGTACGCAAATTAAGTCCAGA   | 60                | 306               |
| <i>SOX2</i>  | GGGAAATGGGAGGGGTGAAAAGAGG       | TTGCGTGAGTGTGGATGGGATTGGTG   | 55                | 151               |
| <i>GAPDH</i>   | CCACTCTCCACCTTTGAC              | ACCCTGTTGCTGTAGCCA   | 62                | 102               |
| <b>Primer Sets for early neuronal cell markers</b>             |                                 |  |                   |                   |
| <i>PAX6</i>  | GCGGAAGCTGCAAAGAAATA            | TTTGGCTGCTAGTCTTTCTCG  | 58                | 118               |
| <i>Nestin</i>  | TGCGGGCTACTGAAAAGTTC            | AGGCTGAGGGACATCTTGAG   | 60                | 130               |
| <b>Primer Sets for ChIP Analysis</b>                           |                                 |  |                   |                   |
| <i>HOXA9</i>   | CTCAGGAGCCTCGTGTCTTT            | GTGACCAGGTGGAGGTGTGT   | 60                | 82                |
| <i>APRT</i>  | GCCTTGACTCGCACTTTTGT            | TAGGCGCCATCGATTTAAG  | 60                | 85                |
| <i>C9ORF72</i>   | AGGAAAGAGAGGTGCGTCAA            | CAGGTGTGGGTTTAGGAGGT   | 60                | 138               |
| <b>Primer Sets for Southern blot Analysis</b>                  |                                 |  |                   |                   |
| Probe  | TTGCGATGACTTTGCAGGGGACC         | CAGCGAGTACTGTGAGAG   | 60                | 576               |
| <b>Primer Sets for Bisulfite Analysis</b>                      |                                 |  |                   |                   |
| BSP 1  | TTTATTAGGGTTTGTAGTGGAGTTTT      | AAATCTTTCTTATTCACCCTCAAC   | 58                | 554               |
| BSP 2  | TATTAGGGTTTGTAGTGGAGTTTT        | CCACACTACTCTACTAAACCC  | 58                | 504               |
| <i>SIGELC6</i>   | TTGTGTAGAGGGAGTGGAGTT           | TCCTAAACCAAACCCCTATAA  | 60                | 284               |
| <b>Primer Sets for methylation assay</b>                       |                                 |  |                   |                   |
| Methyl-specific primers  | FAM-Methyl-F                    | FAM- <u>TGTAAAACGACGGCCAGTAGT</u> <u>TTTGGAAATT</u> AGGAGTCGC          |                   |                   |
|  | Methyl-R1                       | <u>CAGGAAACAGCTATGACCGAA</u> CCCGCCCCGACCACGCCCCG <u>ACCCCG</u> ACCCCG |                   |                   |
|  | Methyl-R2c                      | <u>CAGGAAACAGCTATGACCGAA</u> CCCGCCCCGACCACGCCCCG <u>ACCCCG</u>        |                   |                   |
| Unmethyl-specific primers                                      | HEX-Unmethyl-F                  | HEX- <u>TGTAAAACGACGGCCAGTAG</u> <u>TAAGTTT</u> GGAAATTTAGGAGTTGTG     |                   |                   |
|  | Unmethyl-R1                     | <u>CAGGAAACAGCTATGACCAA</u> CCCAACCCCAACCCCAACCCCA <u>ACCCCA</u>       |                   |                   |
|  | Unmethyl-R2c                    | <u>CAGGAAACAGCTATGACCAA</u> CCCAACCCCAACCCCAACCCCA <u>ACCCCA</u>       |                   |                   |
| Anchor primer  |                                 | <u>CAGGAAACAGCTATGACC</u>  |                   |                   |
| <b>Taqman primers and probes</b>                               |                                 |  |                   |                   |
| Variant 2  | primer F                        | CGGTGGCGAGTGGATATCTC   |                   |                   |
|  | primer R                        | TGGGCAAAGAGTCGACATCA   |                   |                   |
|  | Probe                           | TAATGTGACAGTTGGAATGC   |                   |                   |
| Variant 1 (NM_145005.5)  | Hs00331877 (Applied Biosystems) |  |                   |                   |
| Variant 3 (NM_001256054.1)                                     | Hs00948764 (Applied Biosystems) |  |                   |                   |
| Variants (1+2+3)   | Hs00376619 (Applied Biosystems) |  |                   |                   |
| <i>GUS</i> (NM_000181.3)                                       | Hs99999908 (Applied Biosystems) |  |                   |                   |
| <b>Real Time Primer Sets for Intron 1 retaining product</b>    |                                 |  |                   |                   |
| Exon 1a-Intron 1 Real time                                     | GGTGCGTCAAACAGCGACAAGTTC        | GGAAACAACCGCAGCCTGTAGC   |                   |                   |
| <i>GUS</i>   | CTCATTTGGAATTTTGCCGATT          | CCGAGTGAAGATCCCCTTTTAA   |                   |                   |
| Intron 1- Exon 5   | CCTGATAGGAGATAACAGGATCCAC       | GGTGACAGCTGTATGAAGGC   |                   |                   |