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Supplemental Information

Marked Differences in C9orf72 Methylation Status and Isoform Expres-

sion between C9/ALS Human Embryonic and Induced Pluripotent Stem

Cells

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SUPPLEMETAL FIGURES AND LEGENDS

<u>Fig S1. Characterization of ALS HESC lines, Related to section "Derivation and characterization of</u> <u>C9/HESC lines"</u>. (A) Staining for OCT4, Tra 1-60 and alkaline phosphatase activity. Scale bars stand for 200 μ m. (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 μ m. (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, <u>Relates to Fig 1 and 3.</u> 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 <u>HESCs and iPSCs</u>, 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



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SZ-ALS1	N IS IS NO SO NO.
VLS3	
SZ-A	25 25 65 65 2 2 2





 $(G_4C_2)_n \ge 270$

 $(G_4C_2)_n \le 30$ (Normal range)

Fig S2



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differences	"	71				Total a	12	24
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68 66

Fig S3





C9-iPS M#9

SZ-ALS3

Fig S5

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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

В

	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



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609	NNNNNCCA	GC	T	G	TTTG					593
	11	1	1	1	1111					
1	~~~~~cctgata	ggagataaca	gattccacAT.	ATCTCCGGAG	CATTTGGATA	ATGTGACAGT	TGGAATGCAG	TGATGTCGACI	CTTTGCCCA	CCGCCA 94
		*	*	*	*	*	*	*	*	*
		*	*	*		*	*	*	*	*
592		-CARGACAGAG	AIIGUIIIAA	GIGGCAAAIC.	ACCITIATIA	GUAGUIAUII.	IIGCIIACIG	GGACAAIAIIC	, IIGGICCIA	GAGICA SU/
95	TETECAGETETTE		ATTCCTTTAA	GTCCCAAATC	11111111111 accerttatta	CCACCTACTT	TTGCTTACTG		TTGGTCCTN	CACTAA 194
50	*	*	*	*	*	*	*	*	*	*
	*	*	*	*	*	*	*	*	*	*
506	GGCACATTTGGGC	ICCAAAGACAG	GAACAGGTACT	TCTCAGTGAT	GGAGAAATAA	CTTTTCTTGC	CAACCACACT	CTAAATGGAGA	AATCCTTCG.	AAATGC 407
						11111111111				
195	GGCACATTTGGGC	ICCAAAGACA	GAACAGGTACT	TCTCAGTGAT	GGAGAAATAA	CTTTTCTTGC	CAACCACACT	CTAAATGGAGA	AATCCTTCG.	AAATGC 294
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406	ACACACTCCTCCT	- 	CTTTTTTCT	CTTGTCTGAA	A ACCCACTCA	TTATTCTTC	- 	- CATCCAAACT(CANTOCCO.	- TCCC&C 307
400										
295	AGAGAGTGGTGCT.	ATAGATGTAAJ	GTTTTTT-GT	CTTGTCTGAA.	AAGGGAGTGA	TTATTGTTTC	ATTAATCTTT(GATGGAAACTO	GAATGGGGA	TCGCAG 393
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306	CACATATGGACTA	ICAATTATAC:	TCCACAGACA	GAACTTAGTT	TCTACCTCCC	ACTTCATAGA	GTGTGTGTTG	ATAGATTAAC <i>I</i>	CATATAATC	CGGAAA 207
	111111111111111		1111111111		1111111111	1111111111				11111
394	CACATATGGACTA	ICAATTATAC:	TCCACAGACA	GAACTTAGTT	TCTACCTCCC	ACTTCATAGA	GTGTGTGTTG	ATAGATTAAC <i>i</i>	CATATAATC	CGGAAA 493
	*	*	*	*	*	*	*	*	*	*
	*	*	*	*	*	*	*	*	*	*
206	GGAAGAATATGGA	TGCATAAGGAJ	AGACAAGAAA	ATGTCCAGAA	GATTATCTTA	GAAGGCACAG	AGAGAATGGAJ	AGATCAGGGT	AGAGTATTA	TTCCAA 107
	11111111111111		1111111111		1111111111	1111111111				111111
494	GGAAGAATATGGA	IGCATAAGGA	AGACAAGAAA	ATGTCCAGAA	GATTATCTTA	GAAGGCACAG	AGAGAATGGAI	AGATCAGGGT	AGAGTATTA	TTCCAA 593
	*	*	*	*	*	*	*	*	*	*
	*	*	*	*	*	*	*	*	*	
106	TGCTTACTGGAGA.	AGTGATTCCT	TAATGGAACN	GCTTTCATCT.	ATGAAATCAC	ACAGTGT-CCI	NNAAGAAATA(GATANNGCNN	TNNANNNN	CA 12
504					11111111111					
594	TGCTIACIGGAGA.	*	*	*	AIGAAAICAC *	*	t GAAGAAATAI	* *	*	CAGIAC 585

SUPPLEMETAL EXPERIMENTAL PROCEDURES

	5' Primer (sequence 5'-3')	3' Primer (sequence 5'-3')	Annealing Temp ⁰C	Product Size (bp)					
Primer Sets for RT-PCR for endogenous pluripotent genes									
OCT4	GACAGGGGGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	CTTCCCTCCAACCAGTTGCCCCAAAC	60	144					
NANOG	CAGCCCCGATTCTTCCACCAGTCCC	CGGAGATTCCCAGTCGGGTTCACC	55	342, 390					
REX1	CAGATCCTAAACAGCTCGCAGAAT	GCGTACGCAAATTAAAGTCCAGA	60	306					
SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG	55	151					
GAPDH	CCACTCCTCCACCTTTGAC	ACCCTGTTGCTGTAGCCA	62	102					
Primer Sets for early neuron	al cell markers		•						
РАХб	GCGGAAGCTGCAAAGAAATA	TTTGGCTGCTAGTCTTTCTCG	58	118					
Nestin	TGCGGGCTACTGAAAAGTTC	AGGCTGAGGGACATCTTGAG	60	130					
Primer Sets for ChIP Analysis	s								
НОХА9	CTCAGGAGCCTCGTGTCTTT	GTGACCAGGTGGAGGTGTGT	60	82					
APRT	GCCTTGACTCGCACTTTTGT	TAGGCGCCATCGATTTTAAG	60	85					
C9ORF72	AGGAAAGAGAGGTGCGTCAA	CAGGTGTGGGTTTAGGAGGT	60	138					
Primer Sets for Southern blo	ot Analysis								
Probe	TTGCGATGACTTTGCAGGGGACC	CAGCGAGTACTGTGAGAG	60	576					
Primer Sets for Bisulfite Ana	lysis								
BSP 1	TTTATTAGGGTTTGTAGTGGAGTTTT	AAATCTTTTCTTATTCACCCTCAAC	58	554					
BSP 2	TATTAGGGTTTGTAGTGGAGTTTT	CCACACCTACTCTTACTAAACCC	58	504					
SIGELC6	TTGTGTAGAGGGAGTGGAGTT	ТССТАААССААААССССТАТАА	60	284					
Primer Sets for methylation assay									
Methyl-specific primers	FAM-Methyl-F	FAM- <u>TGTAAAACGACGGCCAGT</u> AG <u>TT</u> TGGAA <u>T</u> T <u>A</u> GGAGTCGC							
	Methyl-R1	CAGGAAACAGCTATGACCGAACCCGCCCCGACCACGCCCCGACCCCGACCCCGACCCCG							
	Methyl-R2c	CAGGAAACAGCTATGACCGAACCCGCCCCGACCACGCCCCGACCCCG							
Unmethyl-specific primers	HEX-Unmethyl-F	HEX- <u>TGTAAAACGACGGCCAGT</u> AG <u>T</u> AAG <u>T</u> TTGGAATTTAGGAGTTGTG							
	Unmethyl-R1 CAGGAAACAGCTATGACCAAACCCCAACCAACCACACCCAACCAA		A <u>A</u> CCCCA <u>A</u> CC	CCCCA <u>A</u> CCCCA <u>A</u>					
	Unmethyl-R2c	CAGGAAACAGCTATGACCAAACCCAACCAACCACACCCCAA							
Anchor primer		CAGGAAACAGCTATGACC							
Taqman primers and probes									
	primer F	CGGTGGCGAGTGGATATCTC							
Variant 2	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)								
GUS (NM_000181.3)	Hs99999908 (Applied Biosystems)								
Real Time Primer Sets for In	tron 1 retaining product	1							
Exon 1a-Intron 1 Real time	GGTGCGTCAAACAGCGACAAGTTC	GGAAACAACCGCAGCCTGTAGC							
GUS	CTCATTTGGAATTTTGCCGATT	CCGAGTGAAGATCCCCTTTTA							
Intron 1- Exon 5	CCTGATAGGAGATAACAGGATTCCAC	GGTGACAGCTGTCATGAAGGC							