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

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SUMMARY

We established two human embryonic stem cell (hESC) lines with a GGGGCC expansion in the C9orf72 gene (C9), and compared them with haploidentical and unrelated C9 induced pluripotent stem cells (iPSCs). We found a marked difference in C9 methylation between the cells. hESCs and parental fibroblasts are entirely unmethylated while the iPSCs are hypermethylated. In addition, we show that the expansion alters promoter usage and interferes with the proper splicing of intron 1, eventually leading to the accumulation of repeat-containing mRNA following neural differentiation. These changes are attenuated in C9 iPSCs, presumably owing to hypermethylation. Altogether, this study highlights the importance of neural differentiation in the pathogenesis of disease and points to the potential role of hypermethylation as a neuroprotective mechanism against pathogenic mRNAs, envisaging a milder phenotype in C9 iPSCs.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS, OMIM #105400) is characterized by progressive muscle weakness and atrophy due to the degeneration of upper and lower motor neurons in the brain and spinal cord, while frontotemporal degeneration (FTD, OMIM #600274) affects behavior and cognition, and is caused by the preferential loss of neurons in the frontal and temporal lobe cortices. Most people who develop ALS/ALS-FTD are between the ages of 40 and 70 years, and die within 2–5 years from diagnosis. Currently there is no cure for ALS-FTD.

The leading known cause of ALS-FTD is a GGGGCC repeat expansion in the first intron of the C9orf72 gene (termed C9 mutation), between noncoding exons 1a and 1b (DeJesus-Hernandez et al., 2011; Dols-Icardo et al., 2014). This mutation can manifest as ALS, FTD, or a combined phenotype, and accounts for 20%–80% of familial and 5%–15% of sporadic ALS and FTD cases (reviewed by Cruts et al., 2013). While in most people the number of GGGGCC repeats is steady and varies between 2 and 19 units, in ALS-FTD it abnormally expands to more than 30 copies and becomes increasingly unstable (Dols-Icardo et al., 2014). The mechanism by which the C9 mutation leads to selective death of neurons is unknown, and the normal function of C9orf72 is just beginning to be defined. Multiple mechanisms for C9/ALS-FTD have been suggested, including haploinsufficiency, RNA toxicity, and abnormal translation of expanded repeat sequences by RAN translation (reviewed by Gendron et al., 2014). However, whether the C9 related neurodegeneration is initiated via a gain-of-function (toxic RNA and/or unconventional dipeptide translation) or a loss-of-function mechanism is still under investigation in animal and cellular models.

The GGGGCC repeat sequence is flanked by two CpG islands (CGIs) within a ~1-kb region that spans from the promoter sequence into intron 1 of C9orf72. Like many CGIs in the genome, this region typically remains free of DNA methylation in subjects with 2–90 repeats. Yet large expansions lead to the formation of one large CGI with abnormal CpG methylation at the repeats (Xi et al., 2015b), which could spread to the 5’ upstream CGI in up to 37% of C9 cases (Belzil et al., 2014; Gijselinck et al., 2015; Liu et al., 2014; Russ et al., 2015; Xi et al., 2013, 2014, 2015a, 2015b). Hypermethylation is suggested to be coupled with the local gain of repressive histone modifications (H3K9me3 and H3K27me3) (Belzil et al., 2013; Zeier et al., 2015). It is still unclear how, or whether, hypermethylation contributes to disease pathogenesis. While some reports demonstrate an overall decrease in C9orf72 transcription, others show a change in the relative distribution between the three different mRNA isoforms, favoring transcription from exon 1a (V1 and V3, NM_145005.5 and NM_001256054.1, respectively) over exon 1b (V2, NM_018325.3) (Donnelly...
et al., 2013; Haeusler et al., 2014; Lee et al., 2013). While previous reports failed to detect a correlation between hypermethylation and ALS versus FTD phenotype (Xi et al., 2015b), experimental evidence demonstrates that C9orf72 haploinsufficiency affects cell morphology and function of motor neurons in zebrafish (Ciura et al., 2013). On the other hand, hypermethylation protects against the accumulation of pathogenic RNA foci and dipeptides, caused by the repeat-containing mRNA variants 1 and 3 (Bauer, 2016; Day and Roberson, 2015; Liu et al., 2014). These conflicting results warrant further investigation regarding the contribution and timing of C9orf72 hypermethylation in ALS-FTD pathogenesis, and the discrepancies may be resolved by the use of in vitro derived neurons from C9/ALS-FTD pluripotent cells. Indeed, induced pluripotent stem cells (iPSCs) from C9/ALS patient fibroblasts have already been used to generate motor neurons in culture that recapitulate the key neuropathological features of FTD-ALS (Almeida et al., 2013; Cooper-Knock et al., 2014, 2015; Devlin et al., 2015; Donnelly et al., 2013; Li et al., 2015; Peters et al., 2015; Rossi et al., 2015; Sareen et al., 2013; Satoh et al., 2014; Wainger et al., 2014). Nevertheless, the epigenetic aspects of the disease have never been addressed using this model system. The aim of this study is to characterize the methylation state of the expanded region and explore its effect on C9orf72 variant transcription in C9/ALS human embryonic stem cells (hESCs), and compare them with that of their haploidentical (mother-to-child genetic identity) and unrelated C9 iPSCs before and after differentiation.

**RESULTS**

**Derivation and Characterization of C9/hESC Lines**

We established two hESC lines with a C9 mutation (SZ-ALS1 and SZ-ALS3) from embryos, which were obtained through preimplantation genetic diagnosis (PGD) and donated for cell line derivation by a family in which the mother was an expansion carrier (patient H, 30 years old, originally diagnosed as a carrier of an expansion with >40 repeats in blood by a repeat primed PCR (rp-PCR); data not shown). Our newly established C9 hESC lines display the key features of pluripotent cells, namely unrestricted growth in culture, expression of undifferentiated cell-specific markers, and potential to differentiate into a wide range of cell types by forming teratomas (Figure S1A, B, D). Chromosome analysis by Giemsa staining demonstrated a 46XX karyotype for SZ-ALS1 and a 45(XO) for SZ-ALS3 (Figure S1C). Southern blot analysis identified a GGGGCC expansion of at least ~270 repeats in both cell lines (Figure S1E).

**Analysis of C9orf72 Methylation in C9 hESCs and Their Haploidentical iPSCs**

Considering the accumulated data regarding hypermethylation in C9 carriers, we aimed to determine whether hypermethylation is already established in the undifferentiated state. Therefore we examined methylation levels, 200 bp upstream of the 5’ end of the GGGGCC repeats, by bisulfite DNA colony sequencing in the C9 hESCs (24 CpG sites). Interestingly, despite the presence of a large expansion, methylation was almost 0% in both cell lines (Figure 1A). To exclude the possibility that methylation had already begun, but failed to spread further upstream to the 5’ CGI, we searched for methylation at the 5’ end of the repeats by carrying out a qualitative (G4C2)n-methylation assay that is sensitive enough to detect repeat methylation in the mixture containing only 2%-5% highly methylated DNA (Xi et al., 2015b). Here again, methylation was undetectable in the mutant hESCs (Figure 1B). In contrast, the expanded allele in whole blood cells of the mother revealed methylation, although weak, in the expansion itself and the 5’ flanking region (estimated as 5% by bisulfite sequencing) (Figures 1A and 1B).

To generate a complement model system, we established haploidentical (genetically half-identical) iPSCs (more than ten different clones) from skin fibroblasts of the currently asymptomatic mother with a C9 expansion, who donated the embryos for hESC line derivation. The primary fibroblasts, which had an unmethylated ~700-repeat expansion (Figure S2D), were reprogrammed by standard protocol using Yamanaka’s four transcription factors (Takahashi et al., 2007). The newly established C9 iPSCs with typical ESC morphology demonstrated unrestricted self-renewal, expressed undifferentiated cell-specific markers, and presented a normal karyotype (Figure S2A–C). By comparing their methylation status with that of the C9 hESCs and parental fibroblasts, we found that the C9 iPSC clones were unusual in their methylation levels. Unlike hESCs and primary fibroblasts, methylation was exceptionally high in the iPSCs. Bisulfite DNA colony sequencing of the region upstream of the expansion identified 50% fully methylated DNA copies in all iPSC clones, suggesting complete methylation of the mutant allele (Figure 1C). The (G4C2)n-methylation assay revealed hypermethylation of the repeats at the 5’ end of the expansion exclusively in C9 iPSCs (Figure 1D). However, while the upstream flanking region of the mutant allele was completely methylated based on bisulfite DNA sequencing (50%), the GGGGCC repeats themselves were not methylated in all DNA molecules (evidenced by signals of repeats beyond the normal range in both the green and blue channels in the (G4C2)n-methylation assay). Importantly, differential methylation between the iPSCs and their parental fibroblasts could not be attributed to the increase in repeat
A

|           | WT HESC | SZ-ALS1 | SZ-ALS3 | C9-blood H |
|-----------|---------|---------|---------|------------|
| Methylation channel | | | | |
| Unmethylation channel | | | | |
| Exp methyl | | | | |
| Exp methyl (weak) | | | | |

B

|           | WT HESC | SZ-ALS1 | SZ-ALS3 | C9-blood H |
|-----------|---------|---------|---------|------------|
| Methylation channel | | | | |
| Unmethylation channel | | | | |
| Exp methyl | | | | |
| Exp methyl (weak) | | | | |

C

|           | WT IPS | C9-fibroblast H | C9-iPS H#8 | C9-iPS H#10 |
|-----------|--------|-----------------|-------------|-------------|
| Methylation channel | | | | |
| Unmethylation channel | | | | |
| Exp methyl | | | | |
| Exp methyl | | | | |

D

(legend on next page)
number, since expansion size remained steady following cell reprogramming, as determined by Southern blot analysis (Figure S2D). In addition, as expansion size in the C9 hESCs is well above the threshold necessary to elicit hypermethylaton in C9orf72 in any other cell type thus far examined, it is very unlikely that the striking differences in hypermethylation between the C9 iPSCs and their C9 hESCs counterparts stem from a difference in expansion size. We further confirmed the fundamental difference in the epigenetic state of the C9 mutation between both cell types by demonstrating significant enrichments for the repressive histone modification H3K9me3 by chromatin immuno precipitation (ChIP) analysis exclusively in mutant iPSCs (Figure 2A). No enrichments for H3K27me3 could be detected in wild-type (WT) or affected hESCs and iPSCs (Figure 2B). Taken together, these findings suggest that reprogramming alters the epigenetic state of this region as a consequence of the expansion in iPSCs, likely spreading from the 5’ border of the CGI toward the repeats.

**Methylation Analysis in C9 iPSCs Derived from an Unrelated Symptomatic ALS Patient**

To further corroborate our findings and examine whether methylation is affected by age or disease symptoms, we also generated iPSCs from a skin biopsy of a 65-year-old C9 ALS patient, 2 years following disease onset (patient M). Southern blot analysis demonstrated the presence of a ~2,700 repeat expansion in the primary fibroblasts of the patient (Figure S2D). Reprogramming of these fibroblasts led to the establishment of more than ten different C9 iPSC clones. The newly established C9 ALS patient-derived iPSCs had the typical characteristics of hESCs (Figure S2). By comparing expansion size and methylation levels between the iPSCs and their parental fibroblasts we found that, although expansion size remained the same, methylation levels at the 5’ CGI dramatically increased from 0% in parental fibroblasts to 50% in affected iPSCs, suggesting methylation levels of 100% on the expanded allele as determined by bisulfite colony sequencing (Figure 3A). The (G4C2)$_n$-methylation assay indicated methylation of the repeats at the 5’ end of the expansion, but not in the parental fibroblasts (Figure 3B). These results provide further evidence that somatic cell reprogramming excessively hypermethylates the 5’ UTR of the C9orf72 locus. In addition, we examined the methylation status of a different gene, SIGLEC6, as a reference locus for aberrant de novo methylation by transcription factor reprogramming, independent of the method used (integratable and nonintegratable vectors) (Huang et al., 2014). Here again, methylation was exclusively acquired in all iPSCs (WT and C9-iPSCs), but was completely absent in primary fibroblasts (patients H and M) or hESCs (SZ-ALS1 and SZ-ALS3) (Figure S3). However, unlike in C9orf72, hypermethylation in SIGLEC6 is not conditioned by a change in the DNA sequence.

**The Effect of Differentiation on the Methylation Status of C9orf72**

To explore the effect of differentiation on the methylation status of the C9 mutation, we induced the unmethylated mutant hESCs (SZ-ALS1 and SZ-ALS3) and C9 hypermethylated iPSC clones (H#8 and M#9) to differentiate into disease-relevant cell types, i.e., neural precursor cells (NPCs) and neural-enriched teratomas. For NPCs, we applied a commonly used and highly efficient differentiation protocol that relies on the generation of neural rosettes by the use of two inhibitors (dorsomorphin and SB431542) (Figure S4A) (Kim et al., 2010). Differentiation efficiency into NPCs was assessed by fluorescence-activated cell sorting (FACS) analysis for NCAM1-positive cells (above 90%, Figure S4B), and by monitoring for the expression of early neural differentiation markers SOX2, Nestin, and PAX6 by RT-PCR (Figure S4C). In addition we took advantage of the teratomas, which are highly enriched for mature neurons (assessed by histological examination [H&E staining, Figures S1] and the presence of Tuj1-expressing cells [Figure S4D]), to explore the effect of differentiation on the methylation levels of the mutation. Interestingly, methylation levels remained unchanged as determined by bisulfite colony sequencing. In the NPCs and teratomas generated from C9 hESCs, methylation remained at 0%, while in the NPCs and teratomas produced from C9 iPSCs
methyltransferase (HMT) (Figure 2A and 2D). These results were further validated by the (G4C2)n-methylation assay (Figures 2B and 2D).

The Effect of Hypermethylation on C9orf72 Expression

To explore whether the expansion alters C9orf72 gene expression and to examine whether it corresponds with hypermethylation, we determined the mRNA levels of the three isoforms (see schematic illustration in Figure 5A) in WT as well as C9 hESCs and iPSCs. Targeting transcript variants 1 (V1), 2 (V2), and 3 (V3) individually (amplicons spanning exon boundaries 1a-2 and 1b-2), and all together (amplicon spanning exon boundaries 2–3) with TaqMan probes, we show that while the general levels of C9orf72 (V1 + V2 + V3) as well as of V2 and V3 remain unchanged between WT and C9 cells in hESCs and iPSCs (Figure 5B), V1 alone is significantly higher in mutant versus WT undifferentiated cells (t test for equal variances, p < 0.05) (Figure 5B). Nevertheless, upregulation of V1 is at least 2.5-fold higher in C9 hESCs relative to C9 iPSCs (t test for equal variances, p < 0.05) (Figure 5B). Given the high abundance of V2 relative to the other variants (cycle threshold [CT] values of 26–27 versus 27–34), the change in V1, even though significant, reflects minute differences overlooked when measuring overall levels of C9orf72 mRNA. We conclude that the GGGGCC expansion alters the region leading to the increased expression of C9orf72 V1 transcripts in C9 iPSCs.

To explore whether neural differentiation contributes to the change in C9orf72 transcription, we also assayed the various isoforms upon differentiation into NPCs and teratomas. Interestingly, we found that differentiation of C9 hESCs into both cell types further enhanced the transcription of exon 1a-bearing transcripts by increasing the expression of both V3 and V1 (Figure 5C). This is in contrast to the upregulation of V2 (the most abundant transcript) in NPCs and teratomas of C9 iPSCs, contributing to the overall increase in C9orf72 mRNA levels (Figure 5C). Jointly, these findings demonstrate that the C9 mutation, together with neural differentiation, favors transcription from the upstream promoter. This effect is largely restricted in iPSCs, presumably due to hypermethylation.

Considering the central role of intron 1-retaining transcripts in the pathology of C9/ALS-FTD disease (Niblock et al., 2016), we aimed to search for the expression of repeat-containing mRNAs in C9 cells. First we generated rRNA-depleted RNA sequencing (RNA-seq) libraries from WT as well as C9-mutant undifferentiated hESCs (two cell lines) and iPSCs (five clones), and RNA deep-sequenced them utilizing next-generation sequencing (see Figures S5A and S5B for general coverage and amount of reads in intron 1 relative to exon 2 of C9orf72 and the RNA-seq data at GEO: GSE87273). As anticipated and in agreement with the report of Niblock et al. (2016), by zooming in to the 5’ part of C9orf72 we found preferential retention of intron 1 in both C9 hESCs and iPSCs (Figure 6A), indicating the propensity of the mutation to interfere with proper splicing of this region. However, quantifying this change between mutant and unaffected cells demonstrates a greater effect in hESCs (4.5-fold increase) relative to iPSCs (only 2-fold increase) (Figure 6B). Considering that C9 cells carry both the allele with expansion and a normal allele, these fold changes are predicted to reflect even greater differences in expression between WT and mutant alleles. We conclude that the shift in promoter usage is coupled with the general tendency to retain intron 1 by the expansion. Next, we aimed to explore whether this may promote accumulation of repeat-containing mRNA transcripts (exon...
1a-initiating transcripts that retain repeat expansion), which form the underlying mechanism for protein/RNA gain of function in C9/ALS-FTD. cDNA sequencing at the boundary between intron 1 and exon 5 confirmed the existence of such transcripts in our cells (Figure S5). Using primers located upstream of the repeats that specifically target repeat-containing mRNAs in undifferentiated (hESCs and iPSCs, Figure 6C) and differentiated (NPCs and teratomas, Figure 6D) cells, we monitored for the existence of these unusual mRNAs by real-time RT-PCR. Importantly, while no difference could be detected between C9 and WT undifferentiated cells (hESCs and iPSCs), their level became significantly higher upon differentiation into NPCs and teratomas exclusively in C9 hESCs (7.8- and 2.6-fold change, respectively) (Figures 6D and S5), and not in C9 iPSCs. Hence, our findings provide evidence that the C9 mutation interferes with proper splicing of intron 1, thereby enhancing the formation of potentially pathogenic repeat-containing mRNAs upon differentiation. Importantly, this effect is missing in C9 iPSCs, presumably due to hypermethylation.

DISCUSSION

We report on the derivation and full characterization of two hESC lines (SZ-ALS1 and SZ-ALS3) with a GGGGCC expansion of approximately 270 repeats. Our C9 hESC lines were established from embryos obtained through PGD from a woman with a C9 mutation with >40 repeats in her peripheral blood. Interestingly, despite the sufficiently large expansion, both cell lines were completely

![Figure 3. Analysis of Methylation Levels in the Region Upstream of the G\textsubscript{4}C\textsubscript{2} Repeats and in the G\textsubscript{4}C\textsubscript{2} Repeat Itself in Primary Fibroblasts and iPSCs Derived from Patient M](image-url)
Figure 4. Analysis of Methylation Levels in Neural Precursors and Teratomas Derived from C9 and WT hESCs and iPSCs

(A) Methylation levels upstream to the repeats were determined by bisulfite sequencing in teratomas and neural precursor cells (NPC) derived from WT and C9 mutant hESCs (SZ-ALS1 and SZ-ALS3). Each line represents a single DNA molecule (upstream region of the repeats), with methylated and unmethylated CpGs designated by black and white circles, respectively.

(B) Results of the (G4C2)n-methylation assay in WT and affected (C9) teratomas and NPCs derived from hESCs. Left panel (blue channel) represents methylated alleles while the right panel (green channel) represents unmethylated alleles.

(C) Methylation levels upstream of the repeats were determined by bisulfite sequencing in teratomas and NPCs derived from WT and C9 iPSCs from patient H (C9-iPSC H#8, 700 repeats) and patient M (C9-iPSC M#9, 2,700 repeats). Each line represents a single DNA molecule (upstream region of the repeats), with methylated and unmethylated CpGs designated by black and white circles, respectively.

(D) Results of the (G4C2)n-methylation assay in WT and affected (C9/ALS) teratomas and NPCs derived from iPSCs. Left panel (blue channel) represents methylated alleles while right panel (green channel) represents unmethylated alleles.
unmethylated at the repeats (based on (G4C2)n-methylation assay [Xi et al., 2015a, 2015b]) and upstream of the repeats (based on bisulfite colony sequencing). In addition, we generated iPSCs clones that are haploidalentical to the mutant hESCs from skin fibroblasts of the asymptomatic C9-carrier mother (700 repeats, patient H), and from an unrelated 65-year-old ALS-manifesting patient (2,700 repeats, patient M). Unexpectedly, we found a striking difference in methylation levels at the 5’ UTR of C9orf72 between the C9 iPSCs and all other cell types examined. Unlike in the C9 hESCs and parental fibroblasts, methylation was detected at the expanded repeats, and reached almost 100% at the upstream CGI in all iPSC clones. Although the difference was generally fail to demethylate and reactivate the FMR1 gene by somatic cell reprogramming when producing iPSCs from cells of fragile X-affected patients (Avitzour et al., 2014; Sheridan et al., 2011; Urbach et al., 2010). It should be noted that our results contradict the report of Esanov et al. (2016), who showed demethylation (rather than de novo methylation) of the C9 mutation in C9 iPSCs. The discrepancy could result from the different cell states employed. We used primary skin fibroblasts from two unrelated patients, whereas they used an immortalized cell line from blood cells of a single patient.

To explore the effect of differentiation on the methylation status of the C9 mutation, we induced the unmethylated mutant hESCs (SZ-ALS1 and SZ-ALS3) and C9 hypermethylated iPSC clones (from C9 individuals H and M) to differentiate into disease-relevant cell types (NPCs and teratomas), which are generally enriched in neural cells. Interestingly, methylation levels remained unchanged at the repeats and upstream of them in both hESCs and iPSCs, following in vitro and in vivo differentiation (0% and 50%, respectively). Perhaps extending differentiation length to fully matured neurons, or to specific subtypes of neurons, would have elicited C9 methylation in the hESCs. Finally, to associate hypermethylation with disease pathogenesis, we analyzed the expression of transcript variants 1, 2, and 3 individually. We show that the GGGGCC expansion alters the region to allow the enhancement of V1 transcripts, albeit with much lower levels in iPSCs relative to hESCs. In addition, we demonstrate that the C9 mutation, together with neural differentiation, favors transcription from an upstream promoter (exon 1a-initiating transcripts, V1 and V3) over a downstream promoter (exon 1b-initiating transcript, V2), and that this effect is largely restricted in iPSCs. Furthermore, we found preferential retention of

Figure 5. Analysis of C9orf72 Variant Expression Levels in Undifferentiated and Differentiated Derivatives of hESCs and iPSCs by qRT-PCR
(A) Schematic illustration of the three C9orf72 mRNA isoform variants (V1, V2, and V3).
(B and C) Mean value of qRT-PCR for C9orf72 transcription in both C9 hESC lines (SZ-ALS1 and SZ-ALS3), C9 iPSC clones derived from patients H (H8, H10) and M (M1, M9, M10), and their WT controls from the TaqMan gene expression assay for C9orf72 transcript variants 1, 2, and 3. mRNA transcription levels were determined in (B) undifferentiated (hESCs and iPSCs) and (C) differentiated cell derivatives (NPCs and teratomas) of C9 mutation carrying cells and appropriate controls (WT hESCs and WT iPSCs). Using TaqMan probes targeting transcript variants 1, 2, and 3 individually (V1, V2, V3) as well as altogether (V1 + V2 + V3), we determined the relative abundance of each transcript variant. The expression level in each cell type represents an average of three to six independent experiments. Cycle threshold (Ct) values were normalized to the corresponding Ct value of GUS. WT hESC line is SZ-13. Error bars represent SE (t test for equal variances, *p < 0.05, **p < 0.01, ***p < 0.001).
intron 1 in both C9 hESCs and iPSCs by RNA deep sequencing, illustrating the propensity of the mutation to interfere with the proper splicing of this region in both exon 1a- and 1b-initiating transcripts. However, this change between WT and C9 cells demonstrates a greater effect in hESCs (4.5-fold increase) relative to iPSCs (only a 2-fold increase). More importantly, by monitoring for the expression of potentially pathogenic mRNA transcripts (i.e., exon 1a-initiating transcripts that retain intron 1), we find no difference between mutant and WT undifferentiated cells (hESCs and iPSCs). However, their level becomes significantly higher upon differentiation into disease-relevant cell types (NPCs and teratomas) exclusively in C9 hESCs, and not in C9 iPSCs. We propose that methylation counteracts the effect of the expansion by downregulating exon 1a-initiating mRNA species. In line with this idea are previous reports by Liu et al. (2014) and others (Bauer, 2016; Day and Roberson, 2015), which point to a mechanistic link between hypermethylation and reduced accumulation of RNA foci and dipeptide inclusions in patient cell lines, brain samples, and HEK293T transgenic cell lines.

Our data related to C9orf72 mRNA levels conflict with the reports of others, who demonstrate a general reduction in C9orf72 in diseased cells (Ciura et al., 2013; Cooper-Knock et al., 2013; Donnelly et al., 2013; Waite et al., 2014; Xi et al., 2013). These discrepancies may stem from the different types of samples (single versus mixed type of cells) employed. Nevertheless, even if our mutant NPCs do not
reflect the physiological levels observed in fully mature disease-relevant cells, this model system could help to interpret the role of methylation in disease pathogenesis because it features both an extreme C9 hypermethylation state and an extreme C9 hypomethylation state.

To summarize, this study clearly demonstrates how reprogramming excessively hypermethylates the C9 expanded locus, and how the C9 mutation alters C9orf72 variant transcription and processing. In addition, the current work highlights the importance of neural differentiation in the pathogenesis of ALS-FTD and points to the potential role of C9 hypermethylation as a neuroprotective mechanism that attenuates the accumulation of potentially toxic repeat-containing mRNAs in C9 neurons.

Altogether, this implies that the difference between C9 hESCs and iPSCs may be crucial for investigating the neural phenotype of the C9/ALS-FTD disease, given that mutant hESCs are likely to present a more accurate and more severe phenotype than comparable iPSCs. Recognizing that C9 hypermethylation limits the increase in intron 1-retaining transcripts, it may be possible in the future (with the advent of gene editing) to modify the C9 expanded locus by specifically targeting chromatin silencing factors/DNMTs to the region. This would affect epigenetic inactivation of exon 1a-initiating variants so as to reverse/minimize the adverse effect of the C9 mutation in disease-affected tissues.

**EXPERIMENTAL PROCEDURES**

**hESC Cell Line Derivation and Characterization**
The use of embryos carrying the C9 mutation, derived from PGD treatment, for hESC derivation was performed in compliance with protocols approved by the National Ethics Committee. All cell lines were established at the Shaare Zedek Medical Center (87/07). Cell line derivation was carried out as previously described (Eiges et al., 2007). All hESC lines were examined for all typical characteristics of hESCs (for primers and conditions see Supplemental Experimental Procedures).

**C9 iPSC Derivation**
For transcription reprogramming, Yamanaka’s four retroviral vectors expressing OCT3/4, SOX2, KLF4, and c-MYC were individually packaged in 293T cells. Infectious viruses were collected 24 and 48 hr post transfection and immediately added to primary fibroblasts (88/11). Four days following infection the cells were placed on mitomycin C-treated mouse embryonic fibroblasts (MEFs) and maintained in hESC medium. Manual isolation of single clones was carried out approximately 30 days post transfection, resulting in stable cell lines with hESC-like morphology.

**Teratoma Induction**
Cells (2.5–5 × 10⁶) were harvested, diluted 1:1 in medium/Matri- gel, and injected subcutaneously to both sides of the back of NOD-SCID IL2Rγ−/− mice. Six to eight weeks later the mice were euthanized and tumors were isolated, sectioned, and assessed for differentiation by H&E staining.

**Neuronal Precursor Differentiation of HESCs and iPSCs**
The cells were grown on MEFs to similarly sized, defined colonies using hESC medium. Induced embryoid body (EB) formation was achieved by detaching colonies with collagenase IV for 30 min. The cells were centrifuged and transferred as colonies to a Petri dish containing hESC medium without basic fibroblast growth factor (bFGF) + 5 μM dorsomorphin (catalog #11967, Cayman) and 5 μM SB431542 (SB #13031, Cayman). The EBs were cultured for 4–5 days with medium replacement every other day. For the NPC expansion, the cells were plated on Matrigel-coated plates (without dissociation) and cultured with KO DMEM medium + 1XN2 supplement (stock X100) and 20 ng/mL bFGF for 8–10 days with medium replacement every other day.

**Chromosome Analysis**
Karyotype analysis was carried out by Giemsa staining according to standard protocol.

**Southern Blot Analysis**
Genomic DNAs (10–25 μg) were digested with EcoRI and HindIII (Ferments) restriction endonucleases, separated on 0.8% agarose gels, blotted onto Hybond N+ membranes (Amersham), and hybridized with a PCR Dig-labeled 576-bp probe (primer F: TTG CGA TGA CTT and primer R: CAG CGA GTA CTG TGA GAG). The use of embryos carrying the C9 mutation, derived from PGD treatment, for hESC derivation was performed in compliance with protocols approved by the National Ethics Committee. All cell lines were established at the Shaare Zedek Medical Center (87/07). Cell line derivation was carried out as previously described (Eiges et al., 2007). All hESC lines were examined for all typical characteristics of hESCs (for primers and conditions see Supplemental Experimental Procedures).

**Expression of Undifferentiated Cell-Specific Markers**
Undifferentiated cell cultures were examined for the expression of undifferentiated cell-specific markers by immunostaining using monoclonal mouse OCT3/4 (Santa Cruz Biotechnology #sc-5279, 1:50 dilution) or TRA-1-60 (Santa Cruz #sc-21705, 1:50 dilution), together with Cy3-conjugated goat anti-mouse polyclonal antibodies (Jackson Immunostaining #115-035-062, 1:100 dilution). Nuclear staining was performed with Hoechst 33258 (Sigma #861405). Staining for alkaline phosphatase was carried out using an Alkaline Phosphatase Kit (Sigma Diagnostics #86R-1KT) according to the manufacturer’s protocol.

**Real-Time TaqMan PCR**
TaqMan gene expression assays for C9orf72 were carried out according to Belzil et al. (2013). qRT-PCR experiments were conducted in triplicate using custom-made TaqMan-based expression assays for transcript variants 1 (NM_145005.5), 2 (NM_018325.3), and 3 (NM_001256054.1) individually, and altogether (adopted
5 mL of PBS-FCS and centrifuged at 150 for 1 hr. After staining the cells were washed two times by adding mary anti-NCAM1 (R&D #af2408, 1:150) and incubated on ice antibody only. The cells were washed twice and assayed by FACS.

Expression of Intron 1-Retaining Transcripts

Upstream of the Repeats by qRT-PCR
Total RNA was isolated from cells by TRI reagent extraction. RNA (1 μg) was reverse transcribed (Multi Scribe RT, ABI) with random hexamer primers. Real-time PCR was performed using Power SYBR Green Master Mix (ABI) on an ABI 7900HT instrument. Primers are listed in Supplemental Experimental Procedures.

FACS Analysis for NCAM1-Positive Cells
NPCs were washed with PBS and dissociated to single cells using preheated (to 37°C) TrypLE Selected (Life Technologies #12563-011) for a minimal amount of time. After cell dissociation, the cells were resuspended with cold sterile PBS-FACS medium (Commercial PBS without Ca²⁺ Mg²⁺ to prevent adhesion with FCS 10%), and filtered through a mesh tube (#352235). From this point onward everything was carried out on ice or under cold centrifugation (4°C). After centrifugation 1 × 10⁶ cells were resuspended into 200 μL of PBS-FCS. The cells were stained directly with primary anti-NCAM1 (R&D #af2408, 1:150) and incubated on ice for 1 hr. After staining the cells were washed two times by adding 5 mL of PBS-FCS and centrifuged at 150 × g for 5 min. The cells were then resuspended into the initial volume and incubated with a secondary antibody (1:200, fluorescein isothiocyanate-conjugated donkey anti-goat immunoglobulin G [IgG], #705095147) for an additional 1 hr. Control cells were stained for secondary treatment. Cells were harvested and then fixed, quenched, and washed in 50-ml tubes. Sonication was carried out using a Vibra Cell VCX130 with a 3-mm microtip and 30% amplitude, in five cycles of 10 s and 30 s rest on ice. Immunoprecipitation was performed using an anti-H3K27me3 (Abcam #6002) and anti-H3K9me3 (Abcam #8898) antibody. Immunoprecipitation efficiency was evaluated by HOX49 (enriched in H3K9me3 and H3K27me3 in pluripotent stem cells). Real-time PCR was carried out on an ABI 7900HT instrument (primers are listed in Supplemental Experimental Procedures). ΔΔCt values were normalized according to a negative control (APRT) to account for histone modification enrichment.

RNA Deep Sequencing
RNA was extracted using TRI Reagent (Sigma). Depletion of rRNA was performed using the Ribominus kit (Invitrogen). RNase-R treatments were performed by adding 3 U of RNase-R (Epicenter Biotechnologies) per milligram of RNA and 15-min incubation at 37°C. cDNA libraries were generated using the TruSeq RNA sample preparation kit and protocol (Illumina), and stranded, ligation-based libraries were sequenced as previously described (Engreitz et al., 2013). RNA-seq reads were aligned to the genome (hg19) using STAR. Coverage of Chr17q72 second exon (chr17:27566674-27567162) and first intron (chr19:27567163-27573426) were calculated using the samtools depth tool. We normalized the total coverage values to the length of the exon/intron to obtain average coverage per base.

ACCESSION NUMBERS
The accession number for the RNA-seq data reported in this paper is GEO: GSE87273.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.09.011.

AUTHOR CONTRIBUTIONS
Y.-C.-H., M.Z., E.R., and S.E.-L. contributed to the conception and design of the study, the collection and assembly of data, data analysis and interpretation, and manuscript writing. G.A., T.E.-G.,
E.L.-L., M.G., O.B., R.A.-F., and S.K. contributed to the collection of data. R.E. contributed to the conception and design of the study, financial support, data analysis and interpretation, and manuscript writing.

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