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Supplemental Data

Hypermethylation of the CpG Island

Near the G_4C_2 Repeat in ALS with a *C9orf72* Expansion

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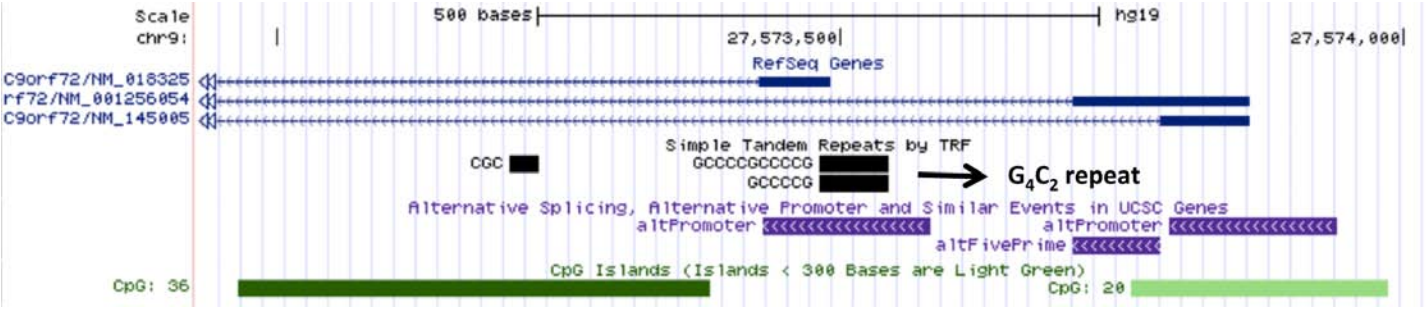

Figure S1. CpG islands flanking the G_4C_2-repeat. The publicly available UCSC database (http://genome.ucsc.edu) revealed two predicted CpG islands immediately flanking the G_4C_2-repeat. As *C9orf72* is reversely transcribed, the CpG island 5' of the repeat region is the light green block on the bottom right side. The G_4C_2-repeat is mapped to the promoter region of transcript NM_018325.3 and the CpG island 5' of the G_4C_2-repeat overlaps the promoter region of transcripts NM_001256054.1 and NM_145005.5.

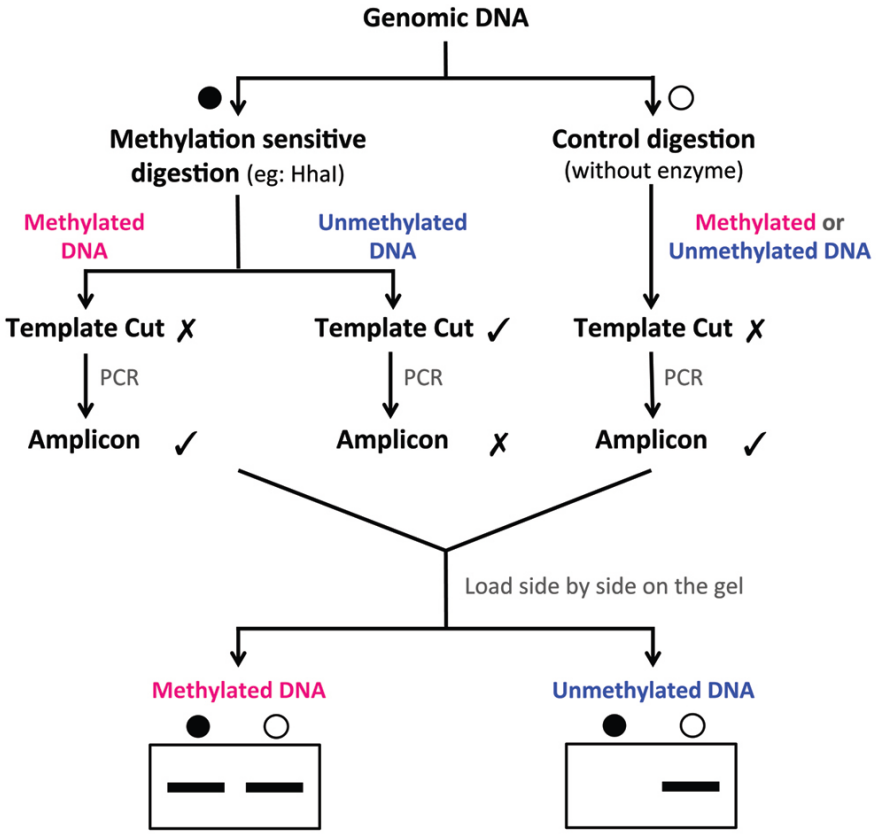

Figure S2. A flow chart showing the experimental principles of the methylation sensitive restriction enzyme assay.

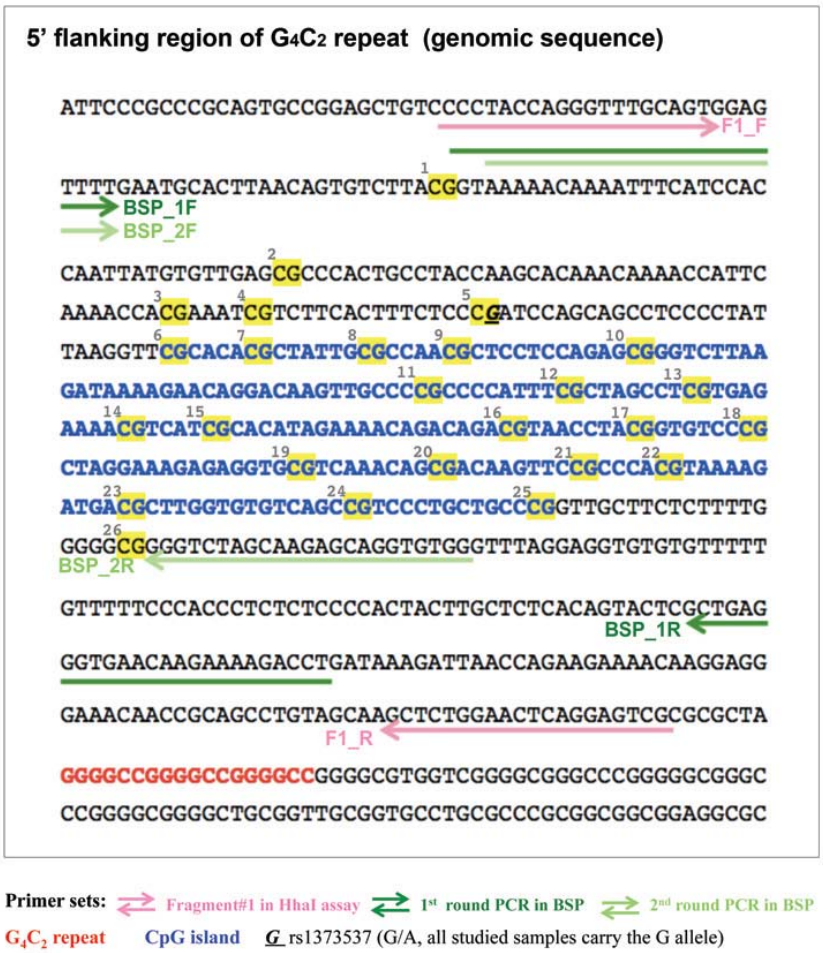

Figure S3. Genomic sequence 5' of the G_4C_2-repeat. G_4C_2-repeat is in red font, the CpG island is in blue font and the CpG dinucleotides is highlighted in yellow. The positions of primers for the bisulfite sequencing and HhaI assays are depicted by colored arrows. The investigated CpG sites are numbered at the upper left corner.

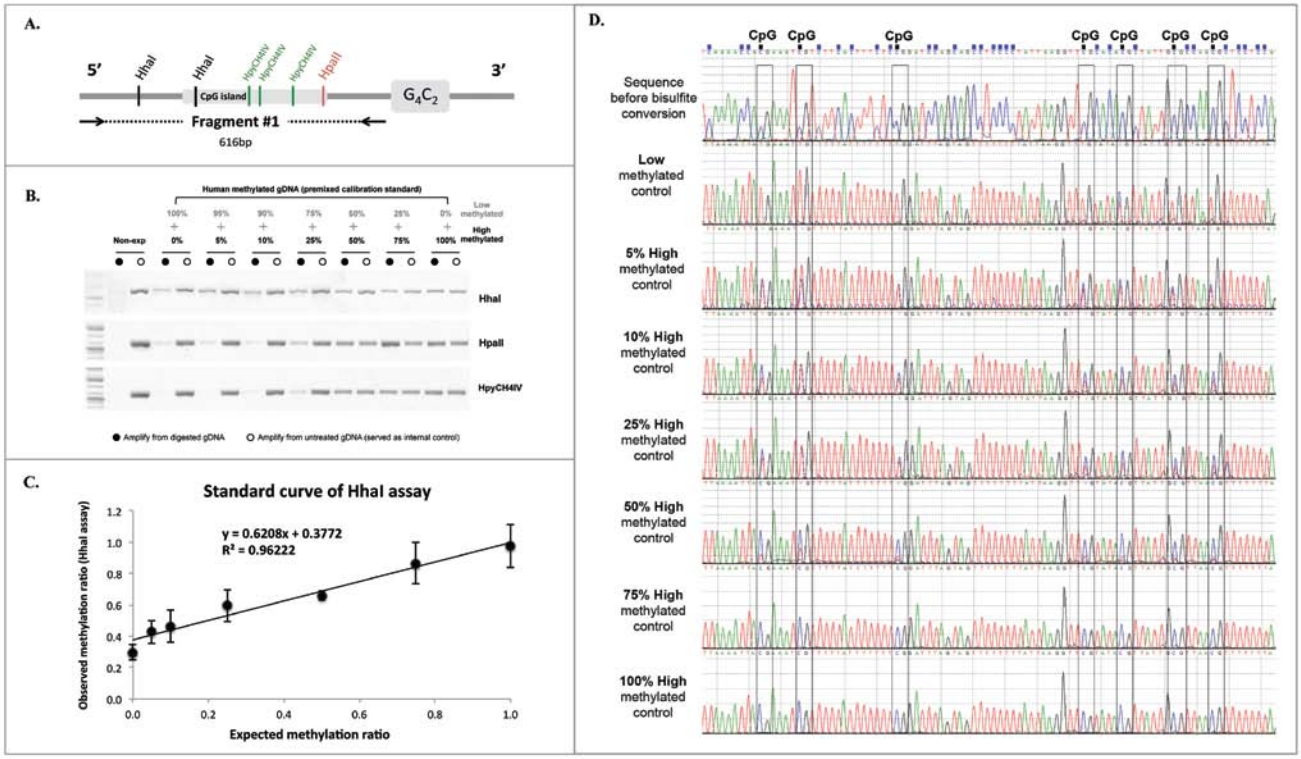

Figure S4. Development of the methylation dependent restriction assay and bisulfite sequencing assay. **(A)** A schematic representation of the region 5' of the G_4C_2-repeat. Fragment #1 covers 2 HhaI sites, 1 HpaII site and 3 HpyCH4IV sites. **(B)** Black/white inverted gel images of the PCR results for fragment #1 from the assays of the three enzymes were compared using a pre-mixed calibration standard DNA (EpigenDx), as well as a randomly selected non-expansion carrier. The HhaI-assay was the most sensitive since it was able to detect methylation in a mixture containing only 5% high methylated DNA, while the other two enzymes detected methylation from 25%-50%. **(C)** By measuring band intensity (4 experimental replicates), a standard curve for the HhaI-assay was obtained. Linear regression indicates (R^2=0.96) reliable quantification ability. Error bars represent standard deviation of the four experimental replicates. **(D)** The same pre-mixed calibration standards were used to test the bisulfite-sequencing assay. Sequence chromatograms for the calibration standard of a region containing seven CpG were aligned to the original sequence (prior to bisulfite treatment). All non-CpG C (indicated by blue squares at the top of the sequence diagram) were successfully converted to T. This assay is efficient enough to detect all methylated CpG cytosine (indicated by black squares) in a mixture containing as little as 5% high methylated DNA.

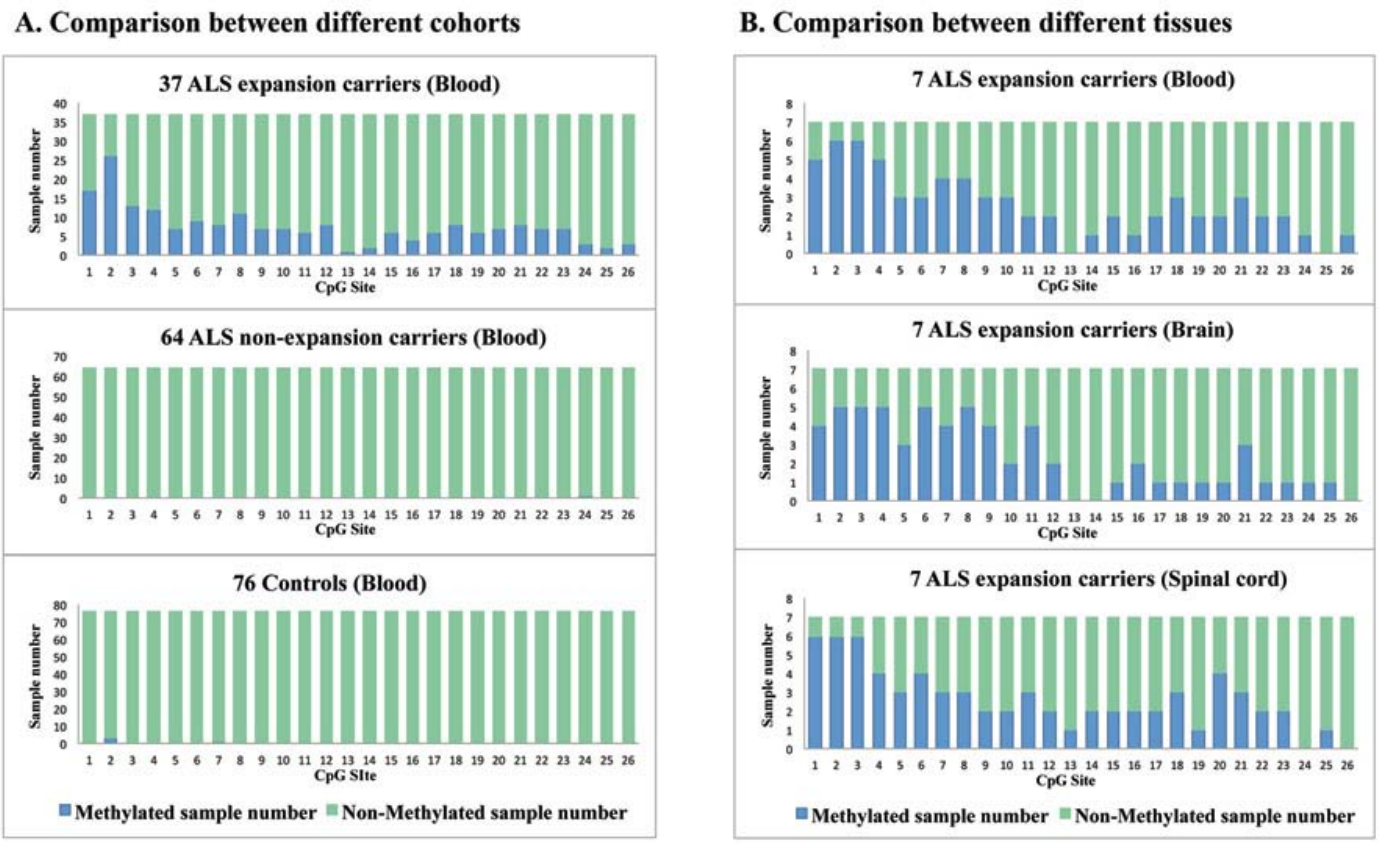

Figure S5. A stacked column plot of the number of methylated and unmethylated samples for each CpG site. (A) A comparison of the different cohorts revealed a difference in methylation level between expansion carriers and non-expansion carriers. **(B)** A comparison of the blood, brain and spinal cord of 7 ALS expansion carriers revealed a similar methylation profile between the different tissues.

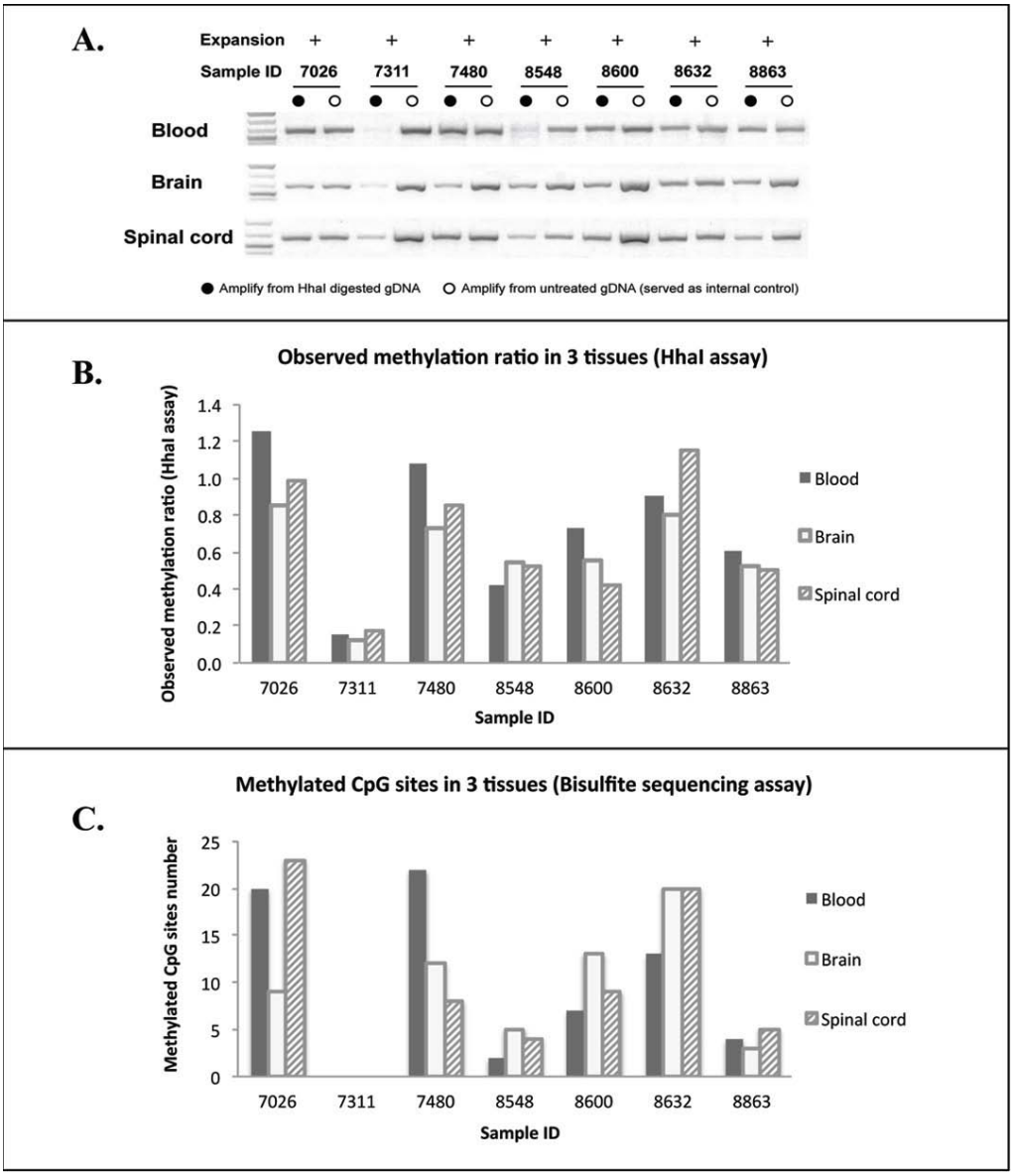

Figure S6. DNA from blood, frontal cortex and spinal cord from 7 ALS expansion carriers showed a similar trend of methylation pattern. (A) Black/white inverted gel image from the HhaI-assay of fragment #1. **(B)** Column plot of the observed methylation ratios (HhaI-assay). **(C)** Column plot of the number of methylated CpG sites (bisulfite sequencing assay).

Table S1. Primer list.

Experiment	Primer Name	Sequence (5'-3')
Methylation sensitive restriction enzyme assay	F1_F	CCCTACCAGGGTTTGCAGT
	F1_R	CGACTCCTGAGTTCCAGAGC
	F2_F	TGCGGTTGCGGTGCCTGC
	F2_R	GGGCGGGAAAGCAAGGAAGAGG
	F3_R	GAATGGGGAGCACACCGACTTGC
Bisulfite sequencing	BSP_1F	TTTATTAGGGTTTGTAGTGGAGTTTT
	BSP_1R	AAATCTTTTCTTATTCACCCTCAAC
	BSP_2F	TATTAGGGTTTGTAGTGGAGTTTT
	BSP_2R	CCACACCTACTCTTACTAAACCC

Table S2. Methylation level of brain DNA from ALS expansion carriers and controls.

Number of methylated CpG	ALS exp		Control	
	N	**Freq**	**N**	**Freq**
0	1	0.14	8	0.57
1-3	1	0.14	6	0.43
4-26	5	0.71	0	0.00
Total	**7**		**14**	
p[a]			**0.003**	

a. 2-sided Pearson $\chi 2$ test was used, df=2. Fisher's exact test was used (when expected value <5)

Table S3. Correlation analysis of methylation level between different tissues.

HhaI-assay		**Brain (n=7)**	**Spinal Cord (n=7)**
Blood (n=7)	Correlation Coefficient[a]	0.901	0.75
	P value	**0.01**	**0.05**
Brain (n=7)	Correlation Coefficient[a]	-	0.901
	P value	-	**0.01**
Bisulfite sequencing assay		**Brain (n=7)**	**Spinal Cord (n=7)**
Blood (n=7)	Correlation Coefficient[a]	0.679	0.786
	P value	0.09	**0.04**
Brain (n-7)	Correlation Coefficient[a]	-	0.75
	P value	-	**0.05**

a. Spearman correlation analysis was used.