Pervasive chromatin remodeling at X-inactivation escape genes in schizophrenic males

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Abstract

Reanalyzing a large methylome dataset of 225 schizophrenic and 450 control samples derived from the prefrontal cortex revealed that 6 male patients have predominantly hypomethylated probes mostly on chromosome X, affecting the same genes in all six. Network analysis of the differentially methylated genes revealed a dense network of transcription factors, histone and chromatin remodeling proteins, with 15 of the X-located genes expressed at the synapse, including *NLGN4X*, *SYN1* and *MECP2*. Mapping a recent experimental dataset of G-quadruplexes (G4s) onto the differentially methylated probes revealed that the probes in the group of six overlapping with G4s on chromosome X are significantly more hypomethylated than non-overlapping and non-X probes whereas in the rest of the patients G4-overlapping probes are more methylated than non-overlapping ones, revealing a distinct pathology, involving chromatin remodeling for the six patients. Unexpectedly, the hypomethylated genes in them significantly overlapped with gene locations where X-inactivation escapism was observed in women.

Introduction

Despite a large body of work and identification of more than 2000 genes implicated in schizophrenia, the exact cause of the disease has not been identified (1). Several genome-wide studies of differentially methylated genes in schizophrenics identified gene sets that are hypomethylated in some patient cohorts but appear to be normal or hypermethylated in others. Wockner et al. (2) using a commercial chip identified two subsets of patients, each consisting of 6 individuals, whose prefrontal cortex regions are differentially methylated in the opposite direction when compared to each other, comprising more than 10 thousand genes. Several other methylome studies have been carried out in schizophrenics. Pidsley et al. (3) found that the most enriched/differentially methylated genes in schizophrenics are associated with developmentally important genes, thus indirectly confirming the long-suspected notion that schizophrenia-related changes may start *in utero*.

Interestingly, despite a significant number of genes on chromosome X related to cognition and brain development (4, 5) there is only limited information that connects schizophrenia to X chromosome genes (6). In this study we reanalyzed a large set of methylomes acquired from the prefrontal cortex of 225 patient samples and 450 controls (7). We clustered the patient sample profiles using the numbers of differentially methylated probes for each gene and identified a cluster of six male patients that have their differentially methylated probes predominantly on chromosome X, the majority of which have been hypomethylated. Comparing the differentially methylated probes to that of the controls identified a smaller set of genes that may account for the disease in these patients.

Our analysis also revealed that the predominantly hypomethylated probes in the X chromosome cluster patients significantly overlap with genomic (DNA-based) G-quadruplexes, identified by a recent genome-wide experimental study by Balasubramanian et al (8). Comparing them to the rest of the patients in the Jaffe study (7) revealed that in those patients the genomic G-quadruplexes overlap mostly with hypermethylated probes, revealing a distinct pathology mechanism involving chromatin remodeling for the cluster of six patients.

Comparing the 68 genes with significant hypomethylation on chromosome X in the six patients with a list of 114 genes that tend to escape X-inactivation in women (9) revealed a significant overlap of 14 genes between the two sets, with even more genomic regions shared, raising the possibility that chromosome X-related chromatin remodeling contributes substantially to schizophrenia in a susceptible subset of patients.

Results

In the first step we calculated the mean methylation values and their variance for each probe in the total of 287 adult control samples in the Jaffe study (7). We determined those probes in all the 225 patient samples that deviated from the control mean values by at least two standard deviations (Z-value >=2). We identified altogether 4883142 differentially methylated probes with an average number of 21703 differentially methylated probes per patient sample (all probe values deviating from the control means by at least 2 SDs are listed in **Supplementary Table 1**). Calculating the same for the controls resulted in 21822 probes per sample.

While the numbers are very similar for patients and controls, and the statistical significance for most of these probes does not reach genome-wide significance our goal at this step was the overall characterisation and clustering of patient profiles based on the similarities in methylation differences only, not the identification of driver genes with a clear-cut causative effect on schizophrenia.

Chromosome-wide methylation values

After counting the differential methylation values for each sample and each chromosome (Supplementary table 2) we noticed that some of the patient samples (e.g. Sample250) have their highest number of hypomethylated probes on chromosome X. After comparing all patient sample profiles with one another we identified seven patient samples whose chromosome-wise counts of hypo- and hypermethylated probes correlated strongly within the group (Pearson correlation mean: 0.937) but much less between the group members and the rest of the samples (Pearson correlation mean: 0.578). One of the 7 samples, Sample596 was flagged by the original authors (7) as it failed a simple quality control, having been predicted male based on the sex chromosome probes, despite being derived from a female sample, therefore we ignored the sample in further analysis.

Chromosome-wide methylation values in controls

Carrying out the same analysis of the differentially methylated probes in the controls (only the 287 adult control samples as the fetal samples showed vastly different methylation values as noted also by the authors in (7)) we found that two control samples (Sample273 and Sample506) also have the highest number of their hypomethylated probes on chromosome X. The chromosome-wise distribution of hypo- and hypermethylated probes in the two control samples on average correlated well with the cluster of 6 patients (r=0.89) but again much less with the rest of the patient samples (r=0.476).

Exploring gene methylation profile similarities with Exploration Graph Analysis

To explore the methylation similarities gene-wise among the patients and controls we used a new method called EGA, Exploration Graph Analysis (10). EGA takes in an array of values for several samples and establishes the similarities among the samples by grouping them into a number of clusters.

Ranking both the patient and control samples for the hypomethylated probes on chromosome X, we selected the top 50 patient and top 30 control samples, containing both the cluster of six patient and the two control samples as outlined above. Using EGA for the hypomethylated probe counts of genes for these 80 samples resulted in seven groups (Fig 1). The green group consists of cluster 6 members and the two controls, clearly separated from the rest of the samples.

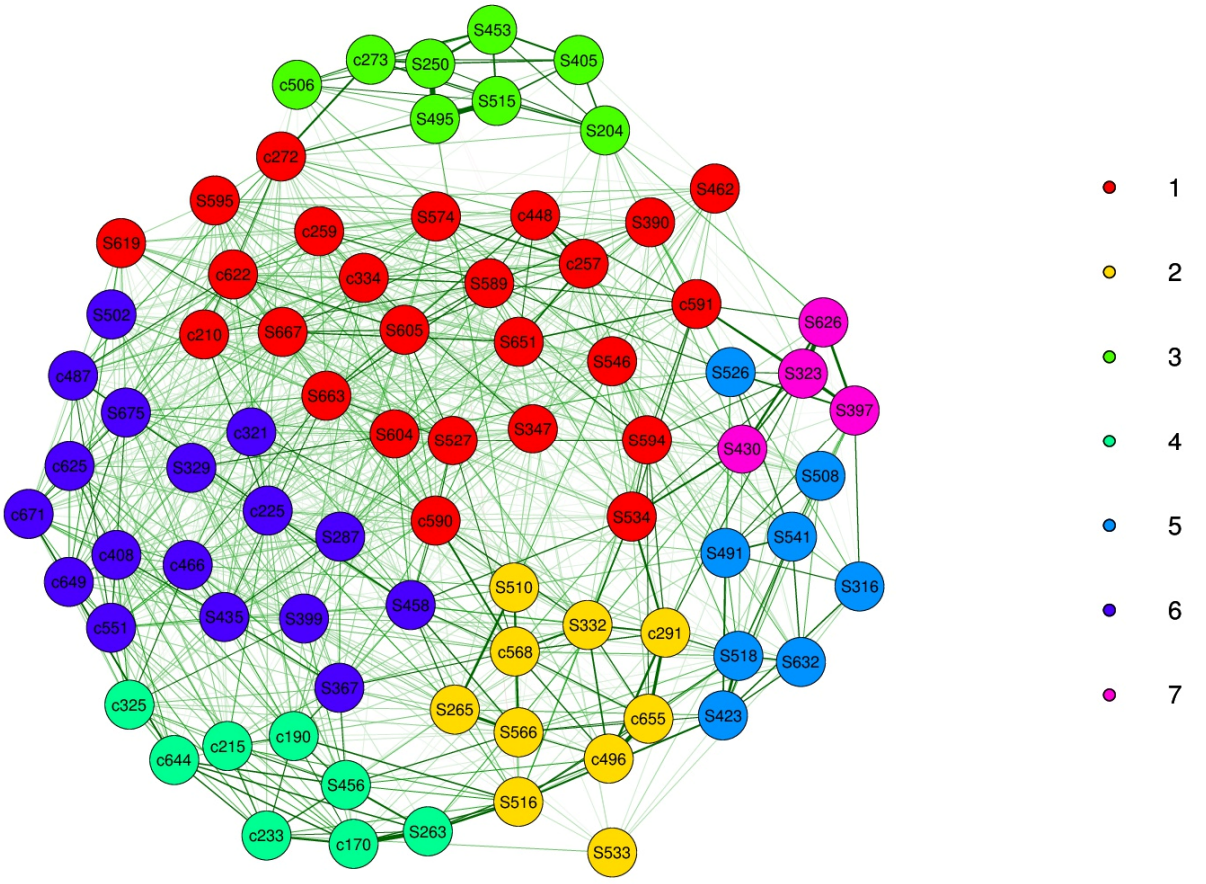

Figure 1. The 50 most hypomethylated patient samples and 30 most hypomethylated control samples clustered using EGA based on the counts of hypomethylated probes for all human genes in the Jaffe study (ref) deviating from control means by at least 3 SDs.

We also attempted to reconstruct the clusters of samples with EGA using only a reduced number of genes, using their counts of hypomethylated probes in the 50+30 (patient + control) samples. While using only chromosome X-coded genes did not recapture the same 6 patients +2 controls cluster, adding chromosome 1-coded genes (Supplementary Figure 1) resulted in the same grouping for the 6+2 cluster as the genome-wide probes in Figure 1.

G-quadruplexes affect methylation differently in the cluster of six

In the next step we analyzed the differentially methylated probes in the cluster of six patients and two controls with relation to their overlap with G-quadruplexes. We used the G-quadruplex definitions established by Balasubramanian et al (8) downloaded from the Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63874 and identified the probes on the Illumina chip that overlap with any of the G-quadruplexes in ref. (8) using the intersect function of bedtools. Figure 2 shows a boxplot statistics of the methylation of the CpG probes for G4-overlapping and G4-non-overlapping probes, separately for the six patients and the rest of them, and also for each group the chromosome X-located probes and the rest of the probes **(boxes A to H)**. Boxes **I to P** show the values for the controls, again separating the two controls (Sample273 and Sample506) with the hypomethylated X-chromosomes from the rest of the controls.

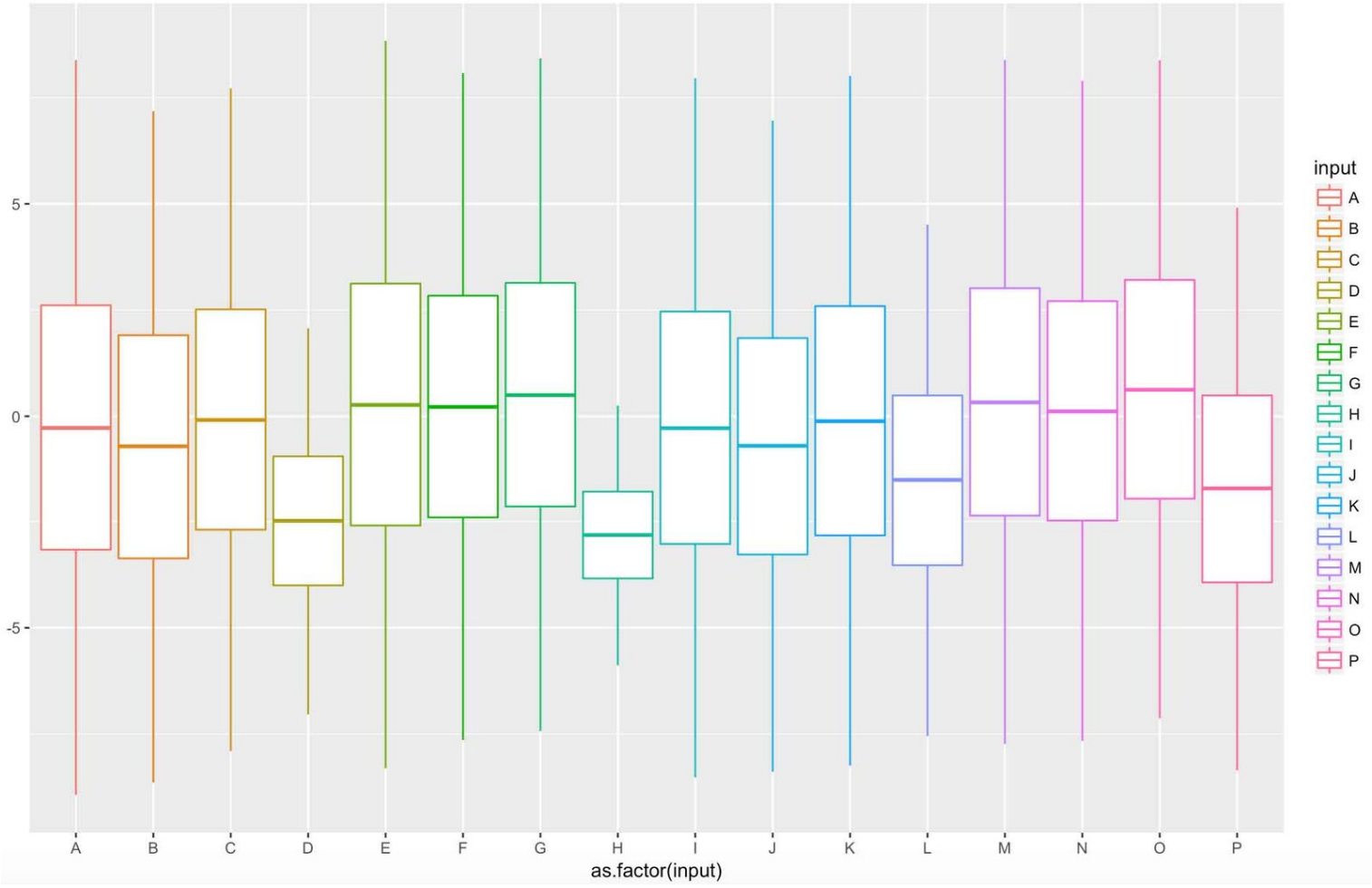

Figure 2. Boxplot statistics of mean methylation values for G4-overlapping (columns E to H and M to P) and non-overlapping CpG probes for patients (columns A to H) and controls (columns I to P). Patient columns: A & B: G4-non-overlapping, non-cluster 6, non-chromosome X and chromosome X probes, respectively; C & D: G4-non-overlapping, cluster 6, non-chrX and chrX probes, respectively; E & F: G4-overlapping, non-cluster 6, non-chrX and chrX probes, respectively; G & H: G4-overlapping, cluster 6, non-chrX and chrX probes, respectively. Columns I to P designations are the same as for A to H, but for the control samples. For the controls the cluster 6 is replaced with the two outlier controls, Samples 273 and 506.

We used Student's t test to determine if any two sets of probes are statistically different. Chromosome X probes in the cluster 6 patients are apparently the most hypomethylated, the G4-overlapping probes being more hypomethylated (**box H**, mean: -2.815) than the non-G4-overlapping ones (**box D**, mean: -2.482, p-value=1.9e-12). This is in contrast with the rest of the patients where the G4-overlapping chromosome X-located probes (**box F,** mean: 0.219) are significantly more methylated than the non-overlapping probes (**box B**, mean: -0.728, p-value =0). The G4 non-overlapping probes in cluster 6 are also significantly more hypomethylated on chromosome X (**box D**, mean: -2.482) than the non-overlapping non-X probes (**box C**, mean: -0.087, p-value=0). The most striking

difference is for the G4-overlapping probes in cluster 6 between chromosome X (**box H**, mean: -2.815) and non-X probes (**box G**, mean: 0.498, p-value=0). This is in stark contrast with the rest of the samples where there is no difference between the G4-overlapping X-probes (**box E**, mean: 0.266) and G4-overlapping non-X probes (**box F**, mean: 0.219). Finally, it is notable that for the cluster 6 patients the G4-overlapping non-X probes (**box G**, mean: 0.498) are significantly more methylated than the non-overlapping non-X probes (**box C**, mean: -0.087, p-value=2e-06), similarly to the non-cluster 6 patients (G4-overlapping, non-X: **box E**, mean: 0.266 vs. non-overlapping, non-X: **box A**, mean: -0.274, p-value=0.00037).

We repeated the calculations for the control samples, again handling the two controls with cluster 6-like behavior separately from the rest (values shown in **boxes I to P** in Figure 2). The overall values are similar in most cases, however there is a significant difference between cluster 6 of patients (columns 4, mean: -2.482) and the two outlier controls for the chromosome X probes (column 12, mean: -1.518) between the non-overlapping probes (p-val=0.002) but no significant difference for the G4-overlapping probes for chromosome X (columns 8 & 16, mean values: -2.815 & -1.719, p-value=0.14).

Specific genes distinguishing the 6 patients from the controls

To pinpoint specific genes that distinguish the six patients from the controls we took into account only those probes that differ by at least 3 standard deviations (3 SDs) from the mean values derived from the controls.

After counting the differentially methylated probes using the 3 SD threshold for each gene and each sample we ran a t-test for each gene between the individual counts for the 6 patients and the controls (after removing the two outlier controls). We also calculated the mean person-wise counts for the cluster of six and the controls. Table 1 shows the results, listing the 82 genes with p-value<0.05 for the t-test and the relative person-wise probe counts >5, i.e. in cluster 6 the gene in question has at least 5 times more probes per person on average than in the controls.

Table 1. 82 genes significantly hypomethylated (p-value<0.05) in the cluster 6 patients with relative person-wise counts of probes min. 5 times higher (in cluster 6) than in the control samples (except in the two outlier controls).

Gene	p-val(ttest)	SZsc	CRsc	Syn	Esc	Chr	5' end	Description
ANKRD58	0.004616	0	0	0	0	chrX	119758613	Ankyrin Repeat Domain-Containing Protein 58
APOO	5.66E-05	0	0	0	1	chrX	23851464	apolipoprotein O
AR	0.011244	0.58	3.64	1	0	chrX	66788682	androgen receptor
ARHGAP4	0.000959	0.58	0	1	1	chrX	153172829	Rho GTPase activating protein 4
ARMCX4	1.62E-12	0	0	0	0	chrX	100743030	armadillo repeat containing, X-linked 4
ARMCX5	0.013519	0	0	0	0	chrX	101854425	armadillo repeat containing, X-linked 5
ARX	0.000161	0	0	0	0	chrX	25021812	aristaless related homeobox
BEND2	0	0	0	0	0	chrX	18189056	BEN domain containing 2
CCDC22	1.62E-12	0	0	0	1	chrX	49091926	coiled-coil domain containing 22
CTPS2	0	0	0	0	1	chrX	16606121	CTP synthase 2
TXLNGY	0	0	0	0	0	chrY	19567313	taxilin gamma pseudogene, Y-linked
DACH2	0	0	0	1	0	chrX	85517970	dachshund family transcription factor 2
DDX3X	0.026757	0	1.23	1	1	chrX	41193414	DEAD (Asp-Glu-Ala-Asp) box helicase 3, X-linked
DLG3	0.015022	10.9	0	1	0	chrX	69674945	discs, large homolog 3 (Drosophila)
DOCK11	0	0	0	0	1	chrX	117629871	dedicator of cytokinesis 11
DUSP9	0.014525	0	0	0	0	chrX	152912656	dual specificity phosphatase 9
DYNLT3	0	0	0	1	0	chrX	37698088	dynein, light chain, Tctex-type 3
EFHC2	0	0	0	0	0	chrX	44007127	EF-hand domain (C-terminal) containing 2
ELF4	0	0	0	0	1	chrX	129198894	E74-like factor 4 (ets domain transcription factor)
ERAS	0	0	0	0	0	chrX	48684922	ES cell expressed Ras

FAM151B	0.004601	0	0	0	0	chr5	79783799	family with sequence similarity 151 member B
FAM47A	1.62E-12	0	0	0	0	chrX	34147868	family with sequence similarity 47 member A
FAM47B	0.00054	0	0	0	0	chrX	34960912	family with sequence similarity 47 member B
FAM47C	0.004982	0	0	0	0	chrX	37026431	family with sequence similarity 47 member C
FBXO34	0	0	0	0	0	chr14	55738871	F-box protein 34
ARHGAP36	0.012797	0	0	0	0	chrX	130192216	Rho GTPase activating protein 36
FOXP2	0.000221	1.42	0	0	0	chr7	114066556	forkhead box P2
GALNT11	0.004982	0	0	0	0	chr7	151722758	polypeptide N-acetylgalactosaminyltransferase 11
GPC3	0.001298	0	0	0	0	chrX	132669775	glypican 3
GPR101	0.025518	0	0	0	0	chrX	136112306	G protein-coupled receptor 101
GPR143	0.021164	0	0	1	0	chrX	9693452	G protein-coupled receptor 143
ADGRG2	0.02922	0	0	0	0	chrX	19007425	adhesion G protein-coupled receptor G2
GSPT2	0	0	0	0	0	chrX	51486480	G1 to S phase transition 2
HCFC1	0.0048	0	4.54	1	1	chrX	153213007	host cell factor C1
HDAC6	0.0252	0	2.05	1	0	chrX	48661069	histone deacetylase 6
HTATSF1	0.00174	0	0	0	0	chrX	135579670	HIV-1 Tat specific factor 1
IDS	0.004982	0	0	0	1	chrX	148568661	iduronate 2-sulfatase
IRS4	0.025518	1	0.78	0	0	chrX	107975726	insulin receptor substrate 4
ANOS1	0.00012	0.82	0.78	0	0	chrX	8528874	anosmin 1
KDM6A	0.000523	0	2.46	0	0	chrX	44732420	lysine (K)-specific demethylase 6A
KLF8	0	0	0	0	0	chrX	56258869	Kruppel-like factor 8
KLHL34	0.000963	0	0	0	0	chrX	21673466	kelch like family member 34
LAIR1	0.004884	0	0	0	0	chr19	54865232	leukocyte-associated IG-like receptor 1
LINC00894	0.004262	0	0	0	0	chrX	149938548	long intergenic non-protein coding RNA 894
MAGEE1	0	0	0	0	0	chrX	75648045	MAGE family member E1
MAGIX	0.001677	0	0	0	0	chrX	49019180	MAGI family member, X-linked
MECP2	1.62E-12	1.16	4.86	1	0	chrX	153295685	methyl-CpG binding protein 2
MED14	0	0	1.16	0	1	chrX	40508794	mediator complex subunit 14
MIR503HG	0.011216	0	0	0	0	chrX	134543337	MIR503 host gene
MIR503	0.025518	0	0	0	0	chrX	133680357	microRNA 503
NHSL2	0	0	0	1	0	chrX	71130937	NHS like 2
NLGN4X	0.014701	1	0	1	0	chrX	5808066	neuroligin 4, X-linked
PGR	0.032258	0	2.49	0	0	chr11	100900354	progesterone receptor
PKIA	0.031017	0	0	0	0	chr8	79428335	protein kinase (cAMP-dependent) inhibitor alpha
PLSCR1	9.89E-12	0	0	0	0	chr3	146232966	phospholipid scramblase 1
PRKX	0.004679	0	0	1	1	chrX	3522383	protein kinase, X-linked
RBMX	0.004996	0	1.95	0	0	chrX	135955605	RNA binding motif protein, X-linked
RBMX2	0.004262	0	0	0	0	chrX	129535942	RNA binding motif protein, X-linked 2
RBMXL3	1.62E-12	0	0	0	0	chrX	114423962	RNA binding motif protein, X-linked-like 3
RHOXF1	0.004903	0	0	0	0	chrX	119243010	Rhox homeobox family member 1
SCML1	0.027038	0	0	0	0	chrX	17755568	sex comb on midleg-like 1 (Drosophila)
SHROOM2	0.01758	0	0	1	0	chrX	9880411	shroom family member 2
SLC13A3	0.006976	0	0	0	0	chr20	45186461	SC fam 13 (Na+-dep dicarboxylate transporter)
SLC16A2	0	0	0	0	0	chrX	73641327	SC fam 16, member 2 (thyroid hormone transporter)
SLC9A7	0.005076	0	0	0	0	chrX	46458685	SC fam 9 (NHE7, cation proton antiporter 7)
SOX3	0.004343	0	0	0	0	chrX	139585151	SRY-box 3
SPAM1	1.63E-12	0	0	0	0	chr7	123565908	sperm adhesion molecule 1
SRPX	0	0	0	0	0	chrX	38008587	sushi-repeat containing protein, X-linked
SYN1	0.002401	0.82	0	1	1	chrX	47431299	synapsin I
TAF7L	1.87E-12	0	0	0	0	chrX	100523240	TATA-box binding protein associated factor 7 like
TNFSF13B	1.06E-09	0	0	0	0	chr13	108921976	tumor necrosis factor superfamily member 13b
TRDMT1	0.044798	0	0	0	0	chr10	17179334	tRNA aspartic acid methyltransferase 1

TSPAN6	0	0	0	0	0	chrX	99882104	tetraspanin 6
USP51	0	0	0	0	0	chrX	55511048	ubiquitin specific peptidase 51
USP9X	0.026016	0	0.78	1	1	chrX	40944887	ubiquitin specific peptidase 9, X-linked
UXT	0.000561	0	4.17	0	0	chrX	47511190	ubiquitously expressed prefoldin like chaperone
YY2	0.000536	0	0	0	0	chrX	21874104	YY2 transcription factor
ZBTB33	0	0	0	0	0	chrX	119384609	zinc finger and BTB domain containing 33
ZCCHC12	1.62E-12	0	0	0	0	chrX	117957705	zinc finger, CCHC domain containing 12
ZDHHC9	0	1.29	0	0	0	chrX	128937263	zinc finger, DHHC-type containing 9
ZFX	0	0	0	0	1	chrX	24167853	zinc finger protein, X-linked
ZNF716	0.031599	0	0	0	0	chr7	57509882	zinc finger protein 716

10 genes in the list (*RHOXF1*, *HTATSF1*, *ELF4*, *MED14*, *SOX3*, *TAF7L*, *DACH2*, *AR*, *ARX* and *YY2*) are transcription factors and altogether 27 genes (colored red in Fig 3) play a role in DNA-templated transcription. Three genes, *HDAC6*, *ZBTB33* and *MECP2* are related to histone deacetylation (4, 11, 12), *KDM6A* is a histone demethylase (13), while *UXT* is involved in chromosome remodeling (5). Two genes, *ZBTB33* and *MECP2* bind methylated CpG dinucleotides in chromosomal DNA (14, 15). In addition, *ZBTB33* promotes the formation of repressive chromatin structures (16). *MECP2* is also the key gene in Rett syndrome (17). Altogether these genes clearly indicate a dysfunctional transcriptional regulation, involving chromatin remodeling and abnormal methylation, the latter to be a well-known hallmark of schizophrenia.

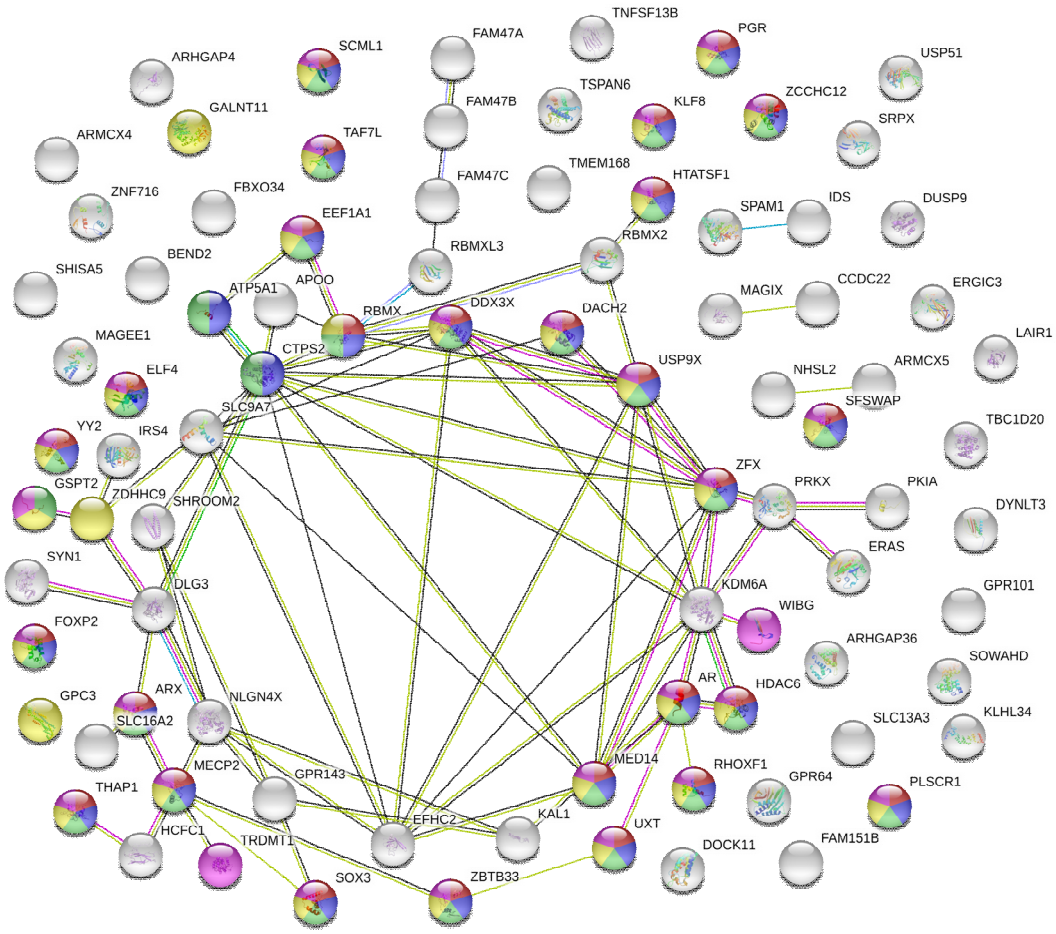

Figure 3. Protein-protein interactions (PPIs) among the protein-coding genes according to STRING (18). Proteins shown in red are involved in "DNA-templated transcription", blue is for "macromolecule biosynthesis process", green for "nucleobase-containing compound biosynthetic process and yellow is for "RNA metabolic process". Most genes are located on chromosome X, 4 genes (*FOXP2*, *GALNT11*, *SPAM1* and *ZNF716*) on chromosome 7 and one pesudogene, *CYorf15A*, on the Y chromosome.

Synaptic genes

The list also contains SYN1, synapsin I, a synaptic vesicle associated phosphoprotein, involved in the fine regulation of neurotransmitter release (19). According to Genecards (20), there are four more synaptic genes in our list: *DLG3*, *MECP2*, *NLGN4X* and *TSPYL2* (21-24). As schizophrenia is called the disease of the synapse (25), this finding warranted a more in-depth analysis. In a recent work Piton et al collected and resequenced all X-chromosome synaptic genes (26). After filtering our results with their list, we found that 15 genes, 1/5th of those listed in Table 1 are synaptic genes.

Seven of the synaptic genes (*DLG3*, *MECP2*, *KAL1*, *SYN1*, *ARHGAP4*, *NLGN4X* and *AR*) are listed by Genecards as schizophrenia-related (27-33). *DLG3* is critical for synaptogenesis and required for learning most likely through its role in synaptic plasticity (34). Piton et al. found that *MECP2*, the main Rett syndrome gene, showed an accumulation of non-synonymous rare variants in schizophrenics (26). *KAL1* is the Kallman-syndrome gene (35), its relation to schizophrenia is so far largely hypothetical, based on a single patient with dual pathologies (29). *ARHGAP4*, the Rho GTPase Activating Protein 4 has been found to be associated with schizophrenia in the Han Chinese population (31). Interestingly the gene is in close proximity of *MECP2*, both located on chromosome Xq28 (31).

One of the synaptic genes with the most hypomethylated probes is *NLGN4X*, a neuroligin and synaptic cell-adhesion molecule that connects presynaptic and postsynaptic neurons at synapses (23). While it has more known association with autism (36) than with schizophrenia, a recent large-scale study by the Psychiatric Genomics Consortium identified the region as being one of 108 loci with highest numbers of mutations in schizophrenics (37). It is also a "hub" gene, having a very high number of, 3960, interacting partners in the brain (38), as is true of many of the synaptic genes (37, 38). *NLGN4X* has more than 20 times more interacting partners than the 2nd highest ranking gene, *MECP2* in Table 1, with 178 interacting partners.

Chromatin remodeling in the cluster of six

The most striking feature in the six patient samples is the remarkably similar profile of hypomethylated genes on chromosome X. Another characteristic is the apparently high occurrence of hypomethylated genes with functions leading to chromatin remodeling as 13 out of the 86 genes in our list of differentially methylated genes in the six patients are annotated with chromatin remodeling by Genecards (39-49). Many of the affected genes in Table1 show the hallmarks of chromatin regulation/remodeling, such as *MECP2*, *HDAC6*, a histone deacetylase and *KDM6A*, a histone demethylase. Sex hormone receptors (*AR* & *PGR*) are also known to influence chromatin remodeling by binding to chromatin (50, 51).

Several more genes in our list in Table 1 have chromatin remodeling functions, either directly recognized, such as for MED14, mediator complex subunit 14 (52), *UXT*, ubiquitously expressed prefoldin-like chaperone (40) or by association in proteomics studies, such as *IRS4*, insulin receptor substrate 4, *RBMX*, RNA binding motif protein, X-linked and *USP9X*, Ubiquitin Specific Peptidase 9, X-Linked (49). *EEF1A1*, elongation factor 1 alpha subunit and *ATP5A1*, a mitochondrial ATP synthase were linked to chromatin remodeling by an RNA interference screen (48).

Further evidence for chromatin remodeling on chromosome X of the 6 patients is provided by the high level of hypomethylation for probes overlapping experimentally verified G-quadruplexes on their X chromosomes whereas the opposite trend is observed for the rest of the patients and the rest of the chromosomes. As noted by others, G-quadruplexes may serve as genome regulators by playing a role in chromatin remodeling (53, 54).

Genes overlapping with X-inactivation escape regions

We also compared our list of hypomethylated genes with a recent compendium of 114 genes for which the authors found experimental evidence that these genes tend to escape X-chromosome inactivation in women (9). Unexpectedly, 14 of the 68 chromosome X-located genes in our list in Table 1 are also listed in their compendium as X-escapee genes (Figure 4). What is more, most of the 68 genes are in the same broadly defined genomic regions outlined in (9). As noted by the authors, there is heterogeneity in escape between the studied European and Yoruban populations regarding the affected genes but there is a consensus for the genomic locations for the two groups. In Fig 4 where we showed the distributions of their 114 and our 66 genes, **respectively**, there is an overall consensus between the two groups of genes, with a Pearson correlation of 0.532 (p-value= 0.00041) for the gene occurrences in the 41 bins that we used to represent the layout of the two gene sets.

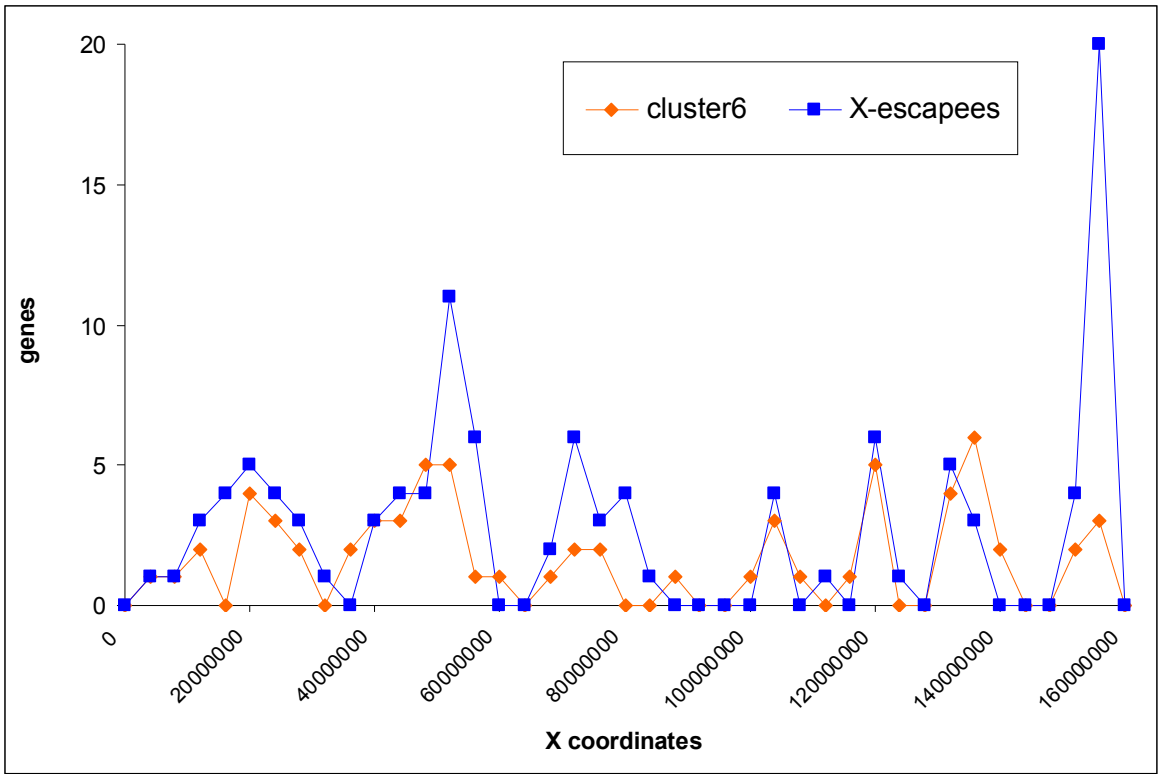

Figure 4. The distribution of the hypomethylated genes in the cluster of six male schizophrenics in (7) and X-escapee genes in (9) on the X chromosome in 41 equal-length (4 Mbase) bins.

Comparing cluster 6 with two outlier controls

As two controls behave similarly to our cluster 6 samples, the question arises if we can distinguish the two groups based on the methylation values. We used another t-test when we compared the individual counts for each hypomethylated gene for the cluster 6 patients against the counts for the two controls. However, the result was significant only for 18 genes out of the 82 in Table1 (indicated in bold).

In the next step we compared the *hypermethylated* probes against both the two outlier controls and the rest of the controls, using again Student's t-test. This gave a more consistent result (Table 2) as we found 9 genes (out of a total of 11 genes **in the two comparisons**) that were significantly more hypermethylated in cluster 6 in both comparisons. These genes have been incorporated already in Fig 3. Interestingly, two hypermethylated genes have protein-protein interactions with hypomethylated genes, such as *ATP5A1*, a mitochondrial ATP synthase also implicated in schizophrenia (55), which interacts with *CTPS2*, CTP synthase 2 and *WIBG*, a key regulator of the exon junction complex (56), which interacts with *KDM6A*. The antagonistic methylation differences for these two pairs raises the possibility that they have genetic, rather than protein-protein interactions.

Table 2. 9 genes significantly hypermethylated (p-value<0.05) in the cluster 6 patients with relative person-wise counts of probes min. 5 times higher (in cluster 6) than in the control samples (except in the two outlier controls).

Gene	p-val(ttest)	SZsc	CRsc	Chr	5' end	Description
ATP5A1	0.040979	1	0.78	chr18	43664109	ATP synthase, H+ transporting
EEF1A1	0	0	0.94	chr6	74225472	eukaryotic translation elongation factor 1 alpha 1
ERGIC3	0.025155	0	0	chr20	34129777	ERGIC and golgi 3
SFSWAP	0.00092	0	0	chr12	132195631	Splic. factor, suppressor of white-apricot family
SHISA5	0.004122	0.58	0	chr3	48509196	shisa family member 5
TBC1D20	0.025155	0	0	chr20	416120	TBC1 domain family member 20
THAP1	0.004122	0	0	chr8	42691816	THAP domain, apoptosis associated protein 1
TMEM168	0.025155	0	0	chr7	112402436	transmembrane protein 168
PYM1	0.025155	0	0	chr12	56295196	PYM homolog 1, EJC associated factor

We also observed an extremely hypermethylated single probe (cg00470565) in 4 of the 6 samples in our cluster, on chromosome 22 (Fig 5) deviating from the control mean by more than 9 SDs in all four of them. While the region is quite gene-rich, there is no gene annotation for this CpG probe. However, locating the probe in the UCSC genome browser (hg19, Fig 5) shows that it lies between an evolutionary highly conserved region and an open chromatin region with a high number of transcription factor binding sites.

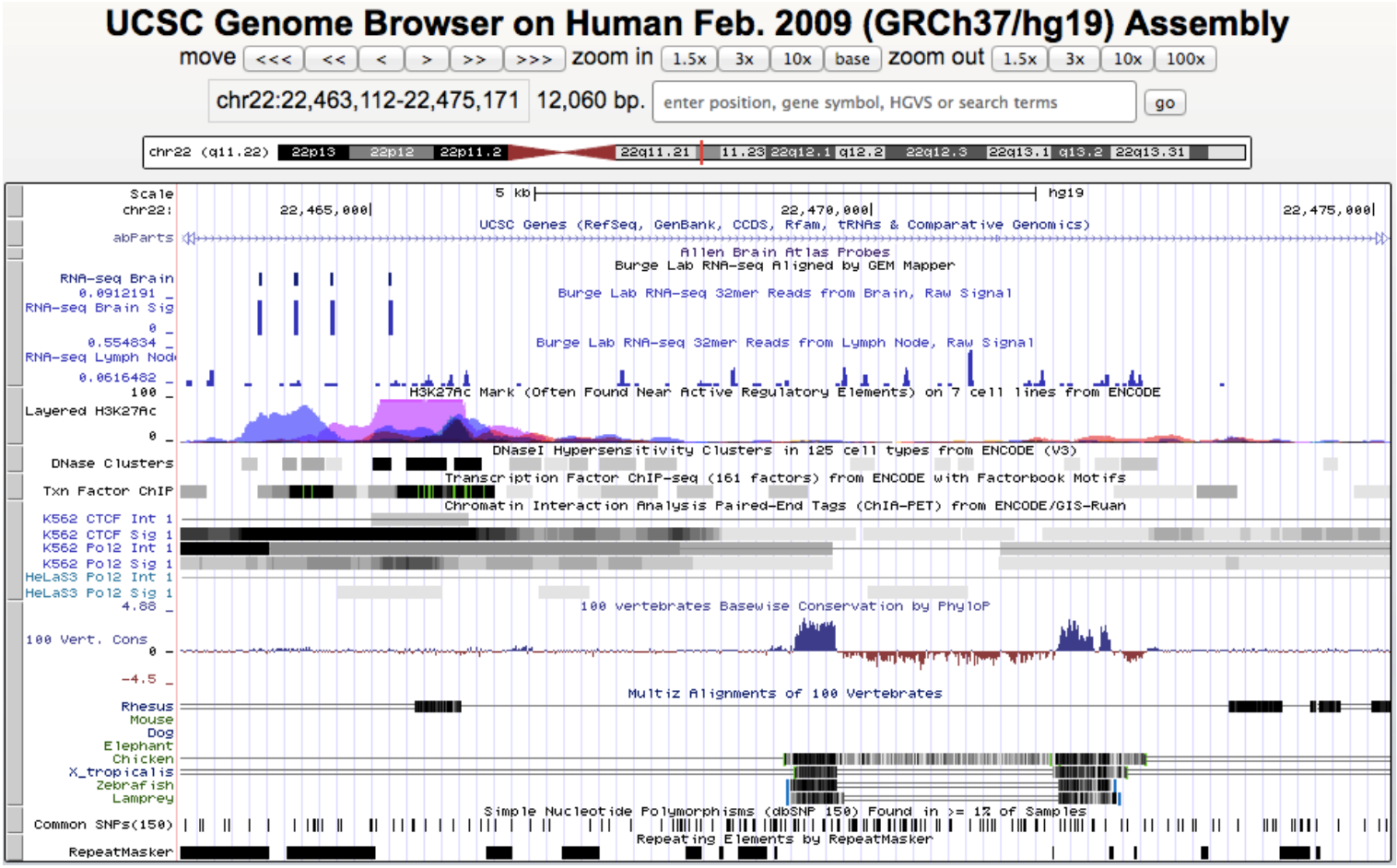

Figure 5. The genomic region around Illumina 450K probe cg00470565 on chromosome 22 at position 22,469,147 (using the coordinates of the human genome version hg19). There is no gene annotation for the probe but it lies in a highly conserved region, surrounded by open chromatin regions with a high number of transcription factor binding sites.

Discussion

In this work we analyzed a recent large-scale methylome study of schizophrenics and controls where the authors queried the full methylome of 225 patient and 287 adult control samples taken from the prefrontal cortex, the brain region most implicated in schizophrenia.

We identified six male patient samples with a distinct methylation profile where the most differentially methylated probes were identified on chromosome X, most of which have been hypomethylated. We also identified two controls (one of them a female) with profiles very similar to the cluster of 6 but with less extreme hypomethylation values. While this chromosome X behavior may not necessarily lead to schizophrenia, it apparently predisposes the person to it, as the frequency ratio between schizophrenic and control population is more than 10:1 in males (restricting the calculations to adult males in the cohort in (7): 6 out of 119 patients vs. 1 out of 208 controls = 10.49).

Chromatin remodeling has been observed previously in neurodevelopmental and intellectual disorders (57), including schizophrenia (28), however it usually implicates one of four protein complex families as described in (58), none of which appears in our list of genes. However, the consistent hypomethylation of 68 X-coded genes in Table 1 in the six patient samples clearly indicates a pervasive chromatin remodeling of the X chromosome, apparently involving a new mechanism and new genes, different from the previously described ATPase-dependent chromatin remodelers (57).

The most unexpected result in our study is the apparent overlap (14 genes) between the hypomethylated genes in the six schizophrenic males and those that escape X-inactivation in women. As noted by Peeters et al. (59) X-inactivation has long served as a paradigm for heterochromatin formation, therefore the abnormal hypomethylation in the six schizophrenics is a further confirmation of chromatin remodeling in their X chromosomes. It shows that the same mechanism that lets the genes escape X-inactivation by unwinding the packaging of chromosome X in women makes it possible for these genes or genomic regions to escape epigenetic control in men, apparently leading or predisposing to schizophrenia.

X chromosome inactivation (XCI), a dosage compensation process in female mammals has been extensively studied in the last 50 years but still is not completely understood (60). The main factor is *XIST*, a long non-coding RNA that gradually coats and thus inactivates one of the two X chromosomes in females (59). It is known that about 15% of X-coded genes escape inactivation, which, however, vary both between and within species (9) but also share some common genes between human and mouse (59), some of which also appear in our list of hypomethylated genes (e.g. *DDX3X* and *KDM6A*). The exact cause of the escape from inactivation for these genes is not known but *XIST* has an antagonistic long noncoding RNA, *TSIX*, which raises the possibility that an escape from inactivation is also facilitated in part by long noncoding RNAs. We have the long noncoding RNA MGC16121/MIR503HG/H19X in our list, one of the most extensively hypomethylated genes in the six patients, which may have some role in the escape process. The gene was named H19X by Necsulea et al. (61) because it was co-expressed with H19, a conserved, maternally imprinted lncRNA (62) highly expressed in the brain. Several other lncRNAs have been suggested to play a role in X-inactivation and contribute to disease.

While we did not detect any aberrant methylation patterns in the six patients for the remodeling protein complexes mentioned above, we did find that X chromosome probes overlapping with G-quadruplexes (experimentally verified in (8)) are extremely hypomethylated in them whereas in other chromosomes in the same (as well as other) patients such probes tend to be more methylated than non-overlapping ones. This observation raises the possibility that chromatin remodeling in these patients is facilitated by these G-quadruplexes, acting perhaps as a molecular switch between euchromatin and heterochromatin. It has been recently observed that G-quadruplexes do play a role in the regulatory functions of chromatin (63).

As we also observed this phenomenon in two controls it raises the possibility that X chromosome remodeling may play a role in other diseases/syndrome especially when there is an observed difference between males and females such as autism, where the male: female occurrence ratio is about 3 (64). As we also observed the same X-related hypomethylation pattern in a female patient sample (but excluded it from further analysis as it did not pass quality threshold in the original publication), the possibility that the described X chromosome remodeling may cause schizophrenia also in women cannot be excluded.

Methods

We downloaded methylation data accompanying ref. (7) from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74193. It contains methylation values of the Illumina 450K commercial chip querying 480 thousands locations across the human genome in the frontal cortex of 225 schizophrenic and 450 control samples. After removing samples with less than 17 years of age at the time of death we calculated statistics (mean, standard deviation (SD)) for the remaining 287 control samples for each of the 450k probes using an in-house Perl script (wherever not mentioned explicitly, we used such Perl scripts that are available from the authors).

Clustering patients based on their hypo- and hypermethylated probe counts

We counted the differentially methylated probes for each chromosome for each patient and each adult control and compared the chromosome-wise profiles with one another calculating the Pearson correlation of the chromosome-wise counts, separately for the hypomethylated and hypermethylated probes (using the 2 SD thresholds to delineate the differentially methylated probes). We calculated Pearson correlations for every pair of samples of the 287 adult controls and the 225 patients using the chromosome-wise profiles of hypomethylated and hypermethylated probes using the 2SD thresholds.

We also counted the gene-wise differentially methylated probes for each sample. We took the 50 most hypomethylated patient samples (with the highest number of hypomethylated probes using the 2SD thresholds) and the 30 most hypomethylated control samples and generated clusters among them using Exploration Graph Analysis (EGA). EGA takes in an array of values for several samples and establishes the similarities among the samples by grouping them into a number of clusters. It is incorporated in R and Rstudio (10).

Determining G-quadruplexes overlapping with hypomethylated probes

We used the G-quadruplex definitions established by Balasubramanian et al. (8) downloaded from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63874 and identified the probes on the Illumina 450K commercial chip that overlap with any of the G-quadruplexes in (8) using the intersect function of bedtools (39).

For Figure 2 we used the appropriate **Boxplot** R function.

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