

# Increased L1 Retrotransposition in the Neuronal Genome in Schizophrenia

Miki Bundo,<sup>1,2</sup> Manabu Toyoshima,<sup>3</sup> Yohei Okada,<sup>4</sup> Wado Akamatsu,<sup>4</sup> Junko Ueda,<sup>2</sup> Taeko Nemoto-Miyauchi,<sup>2</sup> Fumiko Sunaga,<sup>1</sup> Michihiro Toritsuka,<sup>5</sup> Daisuke Ikawa,<sup>5</sup> Akiyoshi Kakita,<sup>6</sup> Motoichiro Kato,<sup>7</sup> Kiyoto Kasai,<sup>8</sup> Toshifumi Kishimoto,<sup>5</sup> Hiroyuki Nawa,<sup>9</sup> Hideyuki Okano,<sup>4</sup> Takeo Yoshikawa,<sup>3</sup> Tadafumi Kato,<sup>2,\*</sup> and Kazuya Iwamoto<sup>1,10,\*</sup>

<sup>1</sup>Department of Molecular Psychiatry, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

<sup>2</sup>Laboratory for Molecular Dynamics of Mental Disorders

<sup>3</sup>Laboratory for Molecular Psychiatry

RIKEN Brain Science Institute, Saitama 351-0198, Japan

<sup>4</sup>Department of Physiology, Keio University School of Medicine, Tokyo 160-8582, Japan

<sup>5</sup>Department of Psychiatry, Nara Medical University, Nara 634-8521, Japan

<sup>6</sup>Department of Pathology, Brain Research Institute, Niigata University, Niigata 951-8585, Japan

<sup>7</sup>Department of Neuropsychiatry, Keio University School of Medicine, Tokyo 160-8582, Japan

<sup>8</sup>Department of Neuropsychiatry, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

<sup>9</sup>Department of Molecular Neurobiology, Brain Research Institute, Niigata University, Niigata 951-8585, Japan

<sup>10</sup>PRESTO, Japan Science and Technology Agency, Saitama 332-0012, Japan

\*Correspondence: [kato@brain.riken.jp](mailto:kato@brain.riken.jp) (T.K.), [kaziwamoto-ky@umin.ac.jp](mailto:kaziwamoto-ky@umin.ac.jp) (K.I.)

<http://dx.doi.org/10.1016/j.neuron.2013.10.053>

## SUMMARY

Recent studies indicate that long interspersed nuclear element-1 (L1) are mobilized in the genome of human neural progenitor cells and enhanced in Rett syndrome and ataxia telangiectasia. However, whether aberrant L1 retrotransposition occurs in mental disorders is unknown. Here, we report high L1 copy number in schizophrenia. Increased L1 was demonstrated in neurons from prefrontal cortex of patients and in induced pluripotent stem (iPS) cell-derived neurons containing 22q11 deletions. Whole-genome sequencing revealed brain-specific L1 insertion in patients localized preferentially to synapse- and schizophrenia-related genes. To study the mechanism of L1 transposition, we examined perinatal environmental risk factors for schizophrenia in animal models and observed an increased L1 copy number after immune activation by poly-I:C or epidermal growth factor. These findings suggest that hyperactive retrotransposition of L1 in neurons triggered by environmental and/or genetic risk factors may contribute to the susceptibility and pathophysiology of schizophrenia.

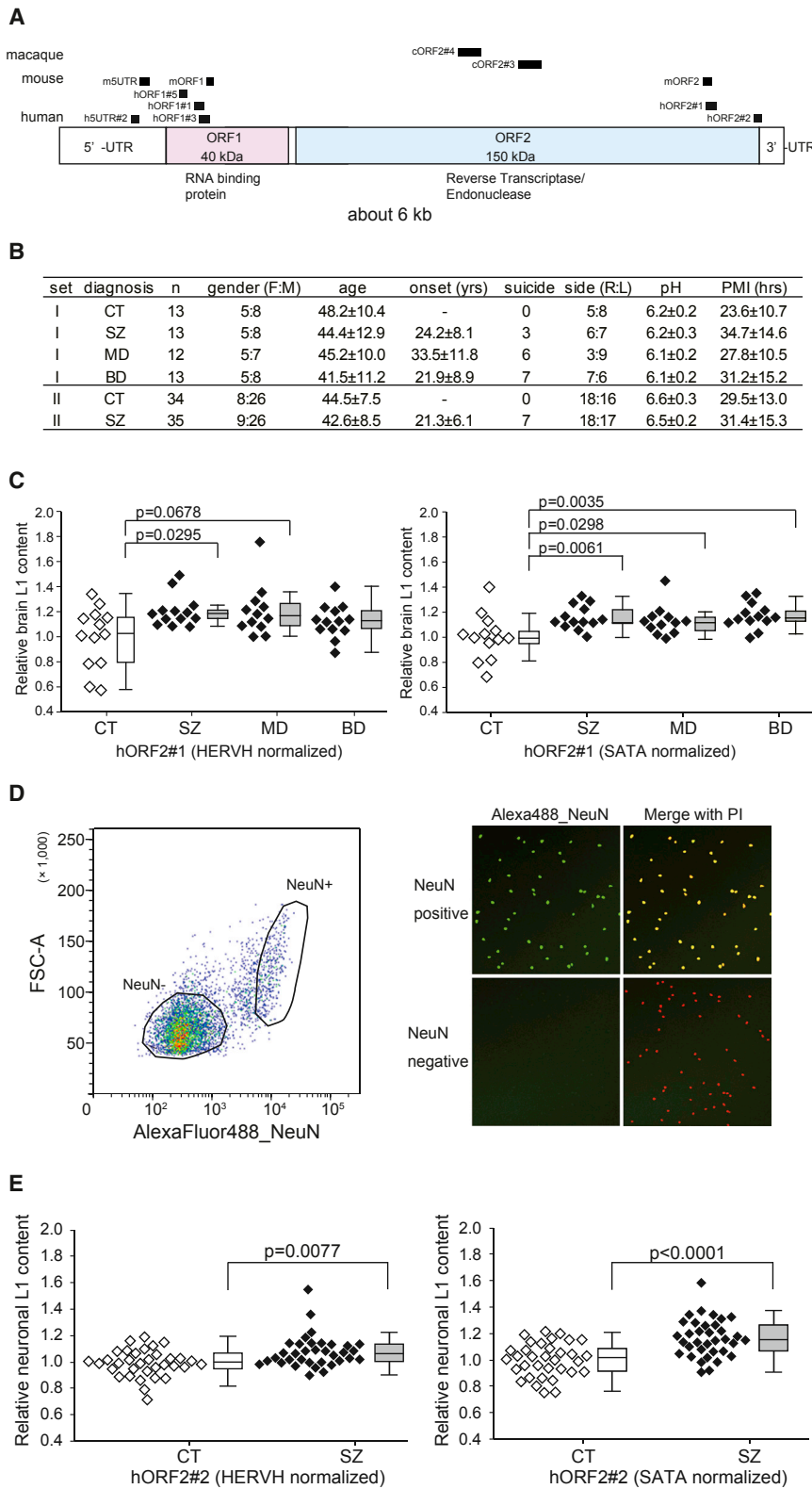
## INTRODUCTION

Mental disorders including schizophrenia, bipolar disorder, and major depression affect a large proportion of the global population and have a major negative economic impact. Twin, family, and adoption studies indicate the complex involvement of both genetic and environmental factors for these diseases (Keshavan et al., 2011). Despite their apparent heritability, however, causal

genetic factors are mostly unknown except for rare cases of schizophrenia associated with chromosomal abnormalities (Brandon and Sawa, 2011; Cook and Scherer, 2008; Karayiorgou et al., 2010). On the other hand, environmental risk factors including prenatal infection (Brown, 2006) and obstetric complications, such as neonatal hypoxia, embryonic ischemia, and gestational toxicosis (Lewis and Murray, 1987), are well-established risk factors for schizophrenia. However, it is not clarified how these environmental risk factors interact with genomic factors.

Accumulating evidence indicates that genomic DNA in the brain contains distinctive somatic genetic variations compared with nonbrain tissues (Poduri et al., 2013). These genetic signatures include brain-specific somatic mutations (Poduri et al., 2013), chromosomal aneuploidy (Rehen et al., 2005; Yurov et al., 2007), chromosomal microdeletion (Shibata et al., 2012), and the genome dynamics of nonlong terminal repeat (LTR) retrotransposons (Baillie et al., 2011; Evrony et al., 2012; Muotri and Gage, 2006). These observed somatic variations are hypothesized to contribute to the generation of functionally diversified brain cells (Muotri and Gage, 2006).

Among the known retrotransposons, only long interspersed nucleotide element-1 (L1) has autonomous retrotransposition activity. Full-length L1 elements include a 5' UTR, two open reading frames (ORFs), and a 3' UTR (Figure 1A). Encoded products from the ORFs contain activities required for retrotransposition and are employed in the insertion of new L1 copies as well as nonautonomous retrotransposons such as *Alu* and *SVA* (Cordaux and Batzer, 2009). Recent studies indicate that engineered L1 has retrotransposition activity in neural progenitor cells from rat hippocampus (Muotri et al., 2005), human fetal brain (Coufal et al., 2009), and human embryonic stem cells (Coufal et al., 2009). These in vitro findings were confirmed in human L1 transgenic mice in vivo (Muotri et al., 2005). Adult human brain cells also showed increased L1 copy number compared with non-brain tissues (Coufal et al., 2009). Moreover, retrotransposition



**Figure 1. Increase of Brain L1 Copy Number in Schizophrenia**

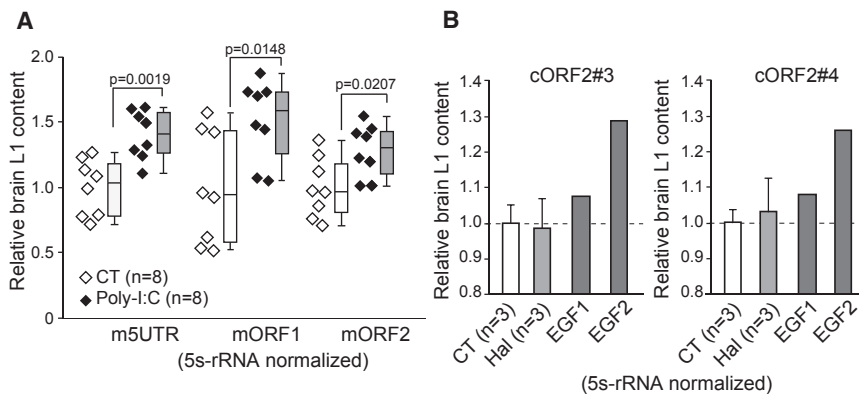
(A) Structure of L1 and map of the primers. Primers and probes are from previous studies (Coufal et al., 2009; Muotri et al., 2010) or designed for this study (Table S4). (B) Summary of the demographic variables of brain samples. (C) L1 copy number in set I. (D) Neuronal nuclei isolation. Left: example of NeuN-based nuclei sorting of brain cells from a patient with schizophrenia. Right: microscopic confirmation of isolated nuclei. The purity of each fraction was >95% and 99.9% for NeuN+ and NeuN-, respectively. (E) Neuronal L1 copy number in set II. In quantitative real-time PCR, L1 copy number was measured with HERVH or SATA as internal controls. The ratio of prefrontal cortex to liver (for set I) or neurons to nonneurons (for set II) was calculated and then normalized relative to the average value of control samples. Values were represented as open or closed diamonds as well as box plots. The  $\Delta$ Ct values of L1 and control probes were not significantly different between diagnostic groups in set I or set II. p values were determined by the Mann-Whitney U test. PMI, postmortem interval; CT, controls; SZ, schizophrenia; MD, major depression; BD, bipolar disorder; PI, propidium iodide. See also Tables S1 and S4 and Figures S1 and S2.

hypothesis that L1 retrotransposition may also be involved in the pathophysiology of mental disorders.

In this study, we quantified L1 copy number in genomic DNA derived from postmortem brains of patients with major mental disorders. We report significant increases of L1 content in the prefrontal cortex of patients with schizophrenia. To confirm this finding, we quantified L1 copy number in neurons and nonneurons from a second, independent patient cohort using NeuN-based cell sorting (Iwamoto et al., 2011; Rehen et al., 2005; Spalding et al., 2005) and found that L1 copy number in neurons was increased in patients with schizophrenia. We next quantified L1 copy number in the animal models that are known to disturb early neural development. These included maternal polyriboinosinic-polyribocytidilic acid (poly-I:C) injection in mice (Meyer and Feldon, 2012; Giovanoli et al., 2013) and chronic epidermal growth factor (EGF) injection to infant macaques (Nawa et al., 2000). We found that genomic DNA of brains from both animal

models showed increased L1 copy number, addressing the importance of environmental factors during perinatal and postnatal stages. We also found that the increased L1 copy number

is active in MeCP2 mouse models and patients with Rett syndrome, indicating a role for this mechanism in this Mendelian disorder (Muotri et al., 2010). Together, these findings suggest the



**Figure 2. Increase of Brain L1 Copy Number in Animal Models**

(A) Brain L1 content in the maternal poly-I:C model. p values were determined by the Mann-Whitney U test. Values were represented as open or closed diamonds as well as box plots. (B) Brain L1 content in chronic EGF or haloperidol-treated macaque models. Error bars indicate SDs. The comparative Ct method, with 5S-rRNA as an internal control, was used. The ratio of prefrontal cortex to liver (for poly-I:C model) or prefrontal gray matter to NeuN-sorted nonneurons in white matter (for macaque models) was calculated and then normalized relative to the average value of control samples. See also Table S4.

in the neurons derived from induced pluripotent stem (iPS) cells of schizophrenia patients with 22q11 deletion. The 22q11 deletion is a well-defined genetic factor and is one of the highest risk factors for schizophrenia, affecting about 1%–2% of schizophrenia patients (Karayiorgou et al., 2010). Finally, we performed whole-genome sequencing (WGS) analysis of brain and liver in controls and patients. Comparison of brain-specific L1 insertion sites revealed that brain-specific L1 insertion in patients is enriched in or near genes related to synaptic function and neuropsychiatric diseases. These results suggest that increased retrotransposition of L1 in neurons, which was triggered by genetic component and/or environmental factors at the early neural development, could contribute to the susceptibility and pathophysiology of schizophrenia.

## RESULTS

### Increased Brain L1 Content in Schizophrenia

We used postmortem prefrontal cortex samples of patients with schizophrenia, bipolar disorder, and major depression as well as control subjects for analysis in set I. The demographic variables are summarized in Figure 1B. We quantified L1 copy number of postmortem prefrontal cortex and liver in each subject by quantitative RT-PCR with two different internal controls, which were designed for human endogenous retrovirus (HERVH) and alpha-satellite (SATA). We found a significant increase in the brain L1ORF2 content in patients with schizophrenia (Figure 1C). A tendency toward copy number increase was also observed in mood disorders and in other L1 probes in schizophrenia (Figure S1 available online).

Somatic L1 retrotransposition was primarily found in neuronal cells (Kuwabara et al., 2009). To confirm the increased brain L1 copy number in schizophrenia and address whether this copy number increase is due to alteration of the neuronal genome, we examined an independent prefrontal cortex sample set (set II). We separated neuronal and nonneuronal nuclei from frozen brains using NeuN-based cell sorting (Figure 1D) (Iwamoto et al., 2011). NeuN is expressed in vertebrate neurons, and its antibody can be used for labeling neuronal nuclei (Mullen et al., 1992). We quantified L1ORF2 copy number of genomic DNA derived from neurons (NeuN-positive nuclei) and nonneurons (NeuN-negative nuclei) and then calculated the neuron-to-non-

neuron ratio. We found a significant increase of neuronal L1ORF2 content in schizophrenia in two different internal controls (Figure 1E). The copy number of the other L1 probes tested also showed significant increase in schizophrenia compared to controls in SATA-normalized data, and similar tendency toward copy number increase was observed in HERVH-normalized data (Figure S1 and data not shown).

### Assessment of Confounding Factors

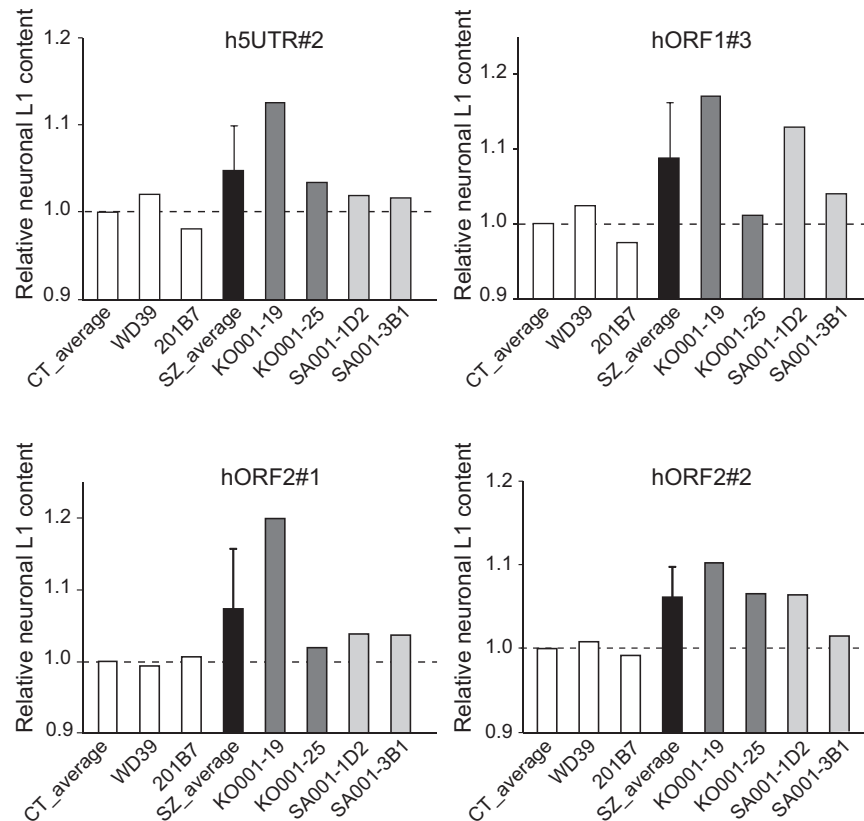
We assessed the effect of confounding factors on L1 content (Table S1). Among the demographic variables tested, sample pH showed a weak correlation with L1ORF2 content in set II but not in set I. Several variables also showed weak correlations, but none showed consistency across the different internal control probes or across the two different sample sets.

To consider the possible effect of antipsychotics, we examined L1 copy number in a human neuroblastoma cell line cultured with haloperidol or risperidone for 8 days. Both antipsychotics did not modify the L1 copy number at their low or high concentrations (Figure S2). Together with the fact that the lifetime intake of antipsychotics, which was estimated as fluphenazine milligram equivalents, did not correlate with L1 copy number in both brain sets (Table S1), medication status did not affect our results.

### L1 Quantification in Animal Models

To assess the potential roles of environmental factors on increased L1 copy number, we employed two different animal models that mimic environmental risk factors that affect early neural development. They included maternal poly-I:C injection in mice and chronic EGF injection to neonatal macaques. The poly-I:C, which mimics viral double-stranded RNA, injection to pregnant mice induces elevated maternal immune activation, and the offspring is known to show schizophrenia-like behavioral alterations such as impairments of prepulse inhibition and social behavior at the later stage (Meyer and Feldon, 2012). Pregnant mice received a single intraperitoneal injection of poly-I:C. L1 copy number in the prefrontal cortex of offspring was tested at postnatal day 21. We found that significant elevation of L1 copy number at all the tested probes compared to controls (Figure 2A).

We then examined the L1 copy number in macaques treated with EGF during neonatal period. Perinatal and postnatal



**Figure 3. L1 Content in Neurons Derived from iPS Cells of Schizophrenia Patients with 22q11 Deletions**

The comparative Ct method, with SATA as an internal control, was used. The ratio of NeuN-sorted neurons to nonneurons was calculated and then normalized relative to average value of control samples. Error bars indicate SDs. See also [Table S4](#) and [Figure S3](#).

perturbation of EGF is known to evoke schizophrenia-like phenotypes, including deficits in prepulse inhibition, latent inhibition, social interaction, and working memory, in adulthood ([Nawa et al., 2009, 2000](#)). The neonatal macaques ( $n = 2$ ) subcutaneously received EGF for seven times over 11 days. After 4 and 7 years from treatment, L1 copy number in the prefrontal cortex was tested. In addition, chronic haloperidol-treated macaques ( $n = 3$ ) were also tested. Due to unavailability of other tissues, we isolated nonneuronal nuclei from frozen white matter and calculated the grey matter-to-nonneuron ratio in each subject. Although statistical approach could not be applied, we observed increase of L1 copy number in EGF-treated macaques, but not in the haloperidol-treated macaques, compared to controls ([Figure 2B](#)). Taken together, these results suggest that early environmental factors play important roles in the L1 content in the brain. We further confirmed that chronic haloperidol treatment did not influence L1 copy number in this model.

#### L1 Quantification in the iPS Cells of Schizophrenia Patients with 22q11 Deletion

We next assessed the importance of genetic risk factor on the L1 copy number in brain. We quantified L1 copy number in the neurons derived from iPS cells of schizophrenia patients with 22q11 deletion ( $n = 2$ ) as well as controls ( $n = 2$ ) ([Figure S3](#)). The iPS cells were established from the fibroblasts according to the previously

developed method ([Imaizumi et al., 2012](#); [Takahashi et al., 2007](#); M.T., unpublished data). To estimate the L1 copy number, we used two independently established iPS cell lines per patient. After induction of neuronal cells ([Imaizumi et al., 2012](#)), we isolated neuronal nuclei by NeuN-based sorting ([Figure S3](#)). We then examined L1 copy number and calculated the neuron-to-nonneuron ratio. Compared to controls, we observed consistent increase of L1 copy number in iPS cell-derived neurons of patients with schizophrenia with 22q11 deletion ([Figure 3](#)). These results suggest that the well-defined strong genetic risk factor also plays an important role in the L1 content in the brain.

#### Identification and Comparison of Brain-Specific L1 Transposition

We next performed WGS of brain and liver DNA from same subjects by self-assembling DNA nanoarray technology ([Drmanac et al., 2010](#)). For this experiment, schizophrenia patients ( $n = 3$ ) and control subjects ( $n = 3$ ) were selected to match age, PMI, gender, brain pH, and race from set I. Selected patients exhibited increased L1 content by quantitative RT-PCR assay, compared to average L1 content of the controls and selected control subjects. The WGS metrics and identified variations were summarized in [Table S2](#). Distribution of the detected mobile elements was almost equal between the tissues and across subjects, and over the half of the identified elements was related

A				
Control				
insertion site	CT1	CT2	CT3	average ratio
intergenic (low)	0.64	0.76	0.69	0.69
intragenic (low)	0.36	0.24	0.31	0.31
intron (low)	0.90	1.00	0.99	0.96
exon (low)	0.10	0.00	0.01	0.04
intergenic (high)	0.65	0.81	0.69	0.72
intragenic (high)	0.35	0.19	0.31	0.28
intron (high)	0.88	1.00	0.99	0.96
exon (high)	0.12	0.00	0.01	0.04
Schizophrenia				
insertion site	SZ1	SZ2	SZ3	average ratio
intergenic (low)	0.72	0.63	0.58	0.64
intragenic (low)	0.28	0.37	0.42	0.36
intron (low)	0.99	0.98	0.98	0.98
exon (low)	0.01	0.02	0.02	0.02
intergenic (high)	0.74	0.63	0.57	0.65
intragenic (high)	0.26	0.37	0.43	0.35
intron (high)	0.99	0.98	0.98	0.98
exon (high)	0.01	0.02	0.02	0.02
B				
Control				
Term	Count	p value		
GO:0005856~cytoskeleton	74	5.92E-04		
GO:0005509~calcium ion binding	56	0.0031		
GO:0005930~axoneme	9	0.0095		
GO:0035085~cilium axoneme	7	0.0289		
GO:0003779~actin binding	26	0.0322		
GO:0044425~membrane part	240	0.0387		
GO:0016010~dystrophin-associated glycoprotein complex	6	0.0405		
Schizophrenia				
Term	Count	p value		
GO:0045202~synapse	57	3.09E-09		
GO:0030054~cell junction	64	8.57E-06		
GO:0044459~plasma membrane part	187	1.49E-05		
GO:0004674~protein serine/threonine kinase activity	58	2.27E-05		
GO:0044456~synapse part	38	4.44E-05		
GO:0004672~protein kinase activity	72	7.45E-05		
GO:0030554~adenyl nucleotide binding	147	1.09E-04		
GO:0005856~cytoskeleton	126	1.19E-04		
GO:0005488~binding	820	1.25E-04		
GO:0006468~protein amino acid phosphorylation	76	1.75E-04		
GO:0006796~phosphate metabolic process	100	1.98E-04		
GO:0006793~phosphorus metabolic process	100	1.98E-04		
GO:0016773~phosphotransferase activity, alcohol group as acceptor	80	2.31E-04		
GO:0001882~nucleoside binding	148	2.52E-04		
GO:0005524~ATP binding	138	2.63E-04		
GO:0001883~purine nucleoside binding	147	2.77E-04		
GO:0032559~adenyl ribonucleotide binding	139	3.43E-04		
GO:0017076~purine nucleotide binding	169	4.19E-04		
GO:0000166~nucleotide binding	190	0.0011		
GO:0014069~postsynaptic density	17	0.0011		
GO:0032553~ribonucleotide binding	161	0.0012		
GO:0032555~purine ribonucleotide binding	161	0.0012		
GO:0043167~ion binding	323	0.0015		
GO:0016043~cellular component organization	204	0.0018		
GO:0043169~cation binding	318	0.0021		
GO:0016310~phosphorylation	83	0.0021		
GO:0046872~metal ion binding	315	0.0025		
GO:0016301~kinase activity	85	0.0032		
GO:0005737~cytoplasm	489	0.0037		
GO:0008092~cytoskeletal protein binding	58	0.0045		
GO:0007155~cell adhesion	74	0.0049		
GO:0022610~biological adhesion	74	0.0052		
GO:0019898~extrinsic to membrane	54	0.0053		
GO:0043687~post-translational protein modification	108	0.0169		
GO:0030030~cell projection organization	45	0.0219		
GO:0005509~calcium ion binding	88	0.0234		
GO:0015629~actin cytoskeleton	33	0.0439		
GO:0016772~transferase activity, transferring phosphorus-containing groups	90	0.0473		
GO:0045211~postsynaptic membrane	21	0.0487		
C				
Control				
Term	Count	p value	FE	
height	4	0.0132	7.7	
scoliosis	3	0.0316	10.3	
Schizophrenia				
Term	Count	p value	FE	
schizophrenia;				
schizoaffective disorder;	5	0.0125	5.2	
bipolar disorder				
schizophrenia	29	0.0135	1.6	
hypertension	20	0.0194	1.7	
bipolar disorder	13	0.0373	1.9	

**Figure 4. Insertion Site, Gene Ontology, and Disease Association Analyses**

(A) L1-insertion site analysis. Proportion of intergenic and intragenic L1 insertion and that of intronic and exonic L1 insertion are given. The low and high mean estimated proportions based on both less and stringent criteria are given. Note that ratios are not significantly different between patients and controls. (B) Gene ontology analysis. p values indicate Bonferroni-corrected modified Fisher's exact test p value. The terms showing  $p < 0.05$  are shown for both groups. (C) Disease-association analysis. p values indicate noncorrected modified Fisher's exact test p value. FE, fold enrichment. In both analyses, gene lists generated by the stringent criteria were used. See also [Tables S2](#) and [S3](#) and [Figure S4](#).

to the L1-Hs ([Figure S4](#)). Among the detected mobile element insertion sites in each sequenced sample, we first identified brain-specific L1 insertions in each subject ([Tables S2](#) and [S3](#)). Although the total number of brain-specific L1 insertion tended to be higher in schizophrenia patients, this was not statistically significant, most likely due to the limited sample size and high interindividual variation. We then compared genomic locations of the insertion sites of brain-specific L1 between patients and controls ([Figure 4A](#)). The inter-to-intragenic L1 insertion ratio as well as exonic-to-intronic L1 insertion ratio did not differ between patients and controls. We then compared the affected genes by brain-specific L1 insertion by gene ontology approach. This

analysis revealed that the number of enriched terms is higher in schizophrenia than controls, in spite that the number of brain-specific L1 insertions did not significantly differ. We found that neuronal function-related terms such as synapse and protein phosphorylation are clearly overrepresented in schizophrenia compared to controls ([Figure 4B](#)). In addition, disease-association analysis revealed that affected genes in patients are specifically enriched in terms related to schizophrenia and bipolar disorder, while those in controls are enriched in nonneuropsychiatric terms such as height and scoliosis ([Figure 4C](#)). These results were consistently confirmed when we used less stringent definition of brain-specific L1 insertion ([Figure S4](#)). In



addition, enrichment of the L1-inserted genes to the terms related to neuropsychiatric disorders in schizophrenia was also detected by the ingenuity pathway analysis (IPA) (Figure S4).

## DISCUSSION

We report that the neuronal genome of schizophrenia contains higher copy number of a retrotransposon, L1. To validate this finding, we utilized iPS cells from patients with schizophrenia carrying the 22q11 deletion and observed an increase in L1 copy number in iPS cell-derived neurons. Moreover, using WGS, we found that L1 preferentially inserted into genes related to synaptic functions and schizophrenia. Animal model studies showed that environmental factors related to infection or inflammation that disturbs early neurodevelopmental processes increase L1 copy number in the brain. Collectively, these results suggest that hyperactive L1 retrotransposition into critical genes during neural development, triggered by genetic and/or environmental factors, contribute to the pathophysiology of schizophrenia. Our results significantly expand the range of neuropsychiatric illnesses linked to aberrant L1 retrotransposition, from Mendelian disease patients with *MECP2* mutations in Rett syndrome (Muotri et al., 2010) and *ATM* mutations in ataxia telangiectasia (Coufal et al., 2011) to schizophrenia, a complex mental disorder.

The observed increase of L1 content in schizophrenia was not due to, or modulated by, biological or experimental artifacts, because changes were measured in two independent patient cohorts and each result was confirmed with two different internal controls. Although the L1 region showing significant increases differed between the two brain sets, this is attributable to cohort differences amplified by the strict threshold we employed. Actually, a significant increase of L1 content was widely observed in all probes in the SATA-normalized data in set II, where neuronal L1 copy number was directly examined (Figure S1). In addition, from the data analysis utilizing lifetime intake of antipsychotics of patients, and from the cell culture and macaque experiments, we conclude that antipsychotics do not affect L1 copy number in the brain. A significant increase was also observed in patients with mood disorders in one internal control in set I (Figure 1C). Future work will clarify whether there are L1 content increases in other mental disorders using larger and/or stratified patient cohorts.

L1 retrotransposition has been detected during adult neurogenesis in the rat hippocampus, indicating that neural progenitor cells retain retrotransposition activity even in adult stages (Muotri et al., 2009). However, we analyzed potential confounding factors, including age, age of onset, and duration of illness, and did not observe any significant correlation with L1 copy number in the brain. The transcript level of L1 in adult brain sample was also increased in patients compared to controls (data not shown). However, elevated expression is unlikely to contribute to increase of L1 copy number in patients, as significant increase of L1 transcripts was detected only in the 5' region of L1 such as 5' UTR and ORF1. These results suggest that L1 copy number does not globally increase with aging and that the variation of L1 copy number in patients is probably confined to early neurodevelopmental stages, at least in the prefrontal cortex. This

prediction would be consistent with the neurodevelopmental hypothesis of schizophrenia, where abnormalities during critical early periods of brain development may trigger the later appearance of clinical symptoms (Bloom, 1993; Murray et al., 1992; Weinberger, 1987).

In Rett syndrome, increased L1 copy number in human brain was linked to mutations in *MECP2* (Muotri et al., 2010) and *MeCP2* knockout mice also showed increased L1 content (Muotri et al., 2010). It has also been suggested that *SOX2* and *MECP2* regulate L1 transcription in neurons (Muotri et al., 2005; Yu et al., 2001). However, we did not observe a significant correlation between *MECP2* or *SOX2* expression and brain L1 content, by using the previously performed gene expression analyses on the same sample sets (Iwamoto et al., 2004, 2005) (data not shown). In addition, patients with high levels of L1 copy number (two schizophrenia and one major depression in set I, and two schizophrenia patients in set II) did not show altered *MECP2* or *SOX2* expression levels (data not shown). These findings suggest that the molecular mechanism of increased L1 in schizophrenia is different from Rett syndrome.

In this study, we found that both early environmental and well-defined strong genetic factors for schizophrenia are involved in the increase of L1 copy number in the brain. A recent study using the poly-I:C model indicated that the offspring of this model had exacerbated schizophrenia-like phenotypes, if they were exposed to environmental stress during puberty, suggesting that early environmental factors can lower the threshold for onset of schizophrenia (Giovannoli et al., 2013). Therefore, increased L1 insertions induced by environmental factors may increase the susceptibility to schizophrenia by disrupting synaptic and schizophrenia-related genes in neurons, rather than being a direct cause of the disease. On the other hand, the pathological consequences of increased L1 content in neurons derived from iPS cells of schizophrenia patients with 22q11 deletions remain unclear. We chose patients with 22q11 deletions to examine L1 dynamics where there is a well-defined strong genetic risk for schizophrenia. In *MeCP2*-knockout mice, Rett-like behavioral abnormalities could be rescued by the re-expression of wild-type *MeCP2* at both young and adult stages (Cobb et al., 2010; Ehninger et al., 2008), suggesting that L1 content itself may not be directly causal to disease phenotypes but instead modulate phenotypic variability among patients (Muotri et al., 2010). Similarly, we speculate that the L1 increase in schizophrenia patients with 22q11 deletions is likely to modulate phenotypes of schizophrenia rather than a direct cause, because many genes related to schizophrenia, such as *TBX-1*, *SEPT5*, *COMT*, and *PRODH*, are located within the deletion (Hiroi et al., 2013; Karayiorgou et al., 2010). Nevertheless, our findings will facilitate further studies of the mechanism of increased L1 retrotransposition associated with schizophrenia.

Our WGS analysis could not detect increased brain-specific L1 insertions in schizophrenia; however, we found that L1 insertions were more frequent in genes for synaptic function and schizophrenia relative to controls. Evrony et al. cloned one L1 insertion event from 300 single neurons and showed that 2 of 83 cortical neurons from an individual had this insertion, but detection of such a low level mosaic insertion in bulk brain tissue of the same individual was difficult and needed optimization

(Evrony et al., 2012). Thus, rare L1 insertion events could be missed in our WGS analysis. Apart from L1, nonautonomous retrotransposons such as *Alu* and *SVA* also show an increased copy number in the brain, possibly via the aid of L1 ORF products (Baillie et al., 2011) and their copy number might also be increased in patients. Further studies on the neuronal genome of patients with mental disorders, and supporting mechanistic evidence from animal and cellular models, may establish a broader role for instability of neural genome in the pathophysiology of schizophrenia. We expect that our findings will promote the further study of genomic instability in disease etiology due to L1 retrotransposition in brain development.

## EXPERIMENTAL PROCEDURES

### Postmortem Samples

Postmortem brain and liver samples were obtained from the Stanley Medical Research Institute. The demographics are summarized in Figure 1B and are described at the web site (<http://www.stanleyresearch.org/>). Ethics committees of RIKEN and the University of Tokyo Faculty of Medicine approved the study.

### Animal Models

Animal experiments were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and guidelines of relevant facilities. For poly I:C model, pregnant mice (C57BL/6) received either a single intraperitoneal injection of poly-I:C (2 mg/ml, Sigma-Aldrich) dissolved in PBS (20 mg/kg) or an equivalent volume of PBS at embryonic day 9.5. At postnatal day 21, tissues were dissected from pups. For macaque models, cynomolgus monkeys (*Macaca fascicularis*) (4 years old; all males) were given oral haloperidol (0.25–0.5 mg/kg; Wako Pure Chemical Industries) or vehicle for 2 months (Shibuya et al., 2010). After transiently separating two male monkey neonates (2 weeks old) from dams, neonates received subcutaneous administration of human recombinant EGF (0.3 mg/kg, Funakoshi) for seven times over 11 days and then quickly returned to their dams. Preliminary behavioral assessment of the EGF-treated monkeys was performed at ages of 4 and 6 years and reported (Nawa et al., 2009). These monkeys were sacrificed at the age of 4 and 7 years with the overdose of pentobarbital (26 mg/kg; 65 mg/ml). Experiments were subjected to review by the Ethical Committee of Shinn Nippon Biomedical Lab.

### iPS Cells

All procedures for skin biopsy and iPS cell production were approved by the Keio University School of Medicine ethics committee and RIKEN ethics committee. The 201B7 iPS cells were kindly provided by Dr. Yamanaka (Takahashi et al., 2007). For the control WD39, a skin-punch biopsy from a healthy 16-year-old Japanese female obtained after written informed consent was used to generate iPS cells (Imaizumi et al., 2012). 22q11.2 deletion syndrome iPS cells (SA001 and KO001) were generated from a 37-year-old Japanese female patient (Toyosima et al., 2011) and a 30-year-old Japanese female patient, respectively, using the same method used to generate the WD39 (M.T., unpublished data). 22q11 deletion was characterized by the CGH array analysis (Figure S3). Production and maintenance of iPS cells were performed according to the previous studies (Imaizumi et al., 2012; Takahashi et al., 2007). All the iPS cells and differentiated neuronal cell lines were characterized with immunofluorescence staining and their morphologies (Figure S3).

### L1 Copy Number Estimation

We performed either Taqman-based quantitative real-time PCR according to Coufal et al. (2009) with minor modifications (100 or 500 pg DNA as starting material and single amplicon analysis) or SYBR-Green-based quantitative real-time PCR according to Muotri et al. (2010). SYBR-Green assay was performed using 500 pg DNA and Power SYBR Green PCR Master Mix (Life Technologies). Primers, probe location, and reaction chemistry are listed in Figure 1A and Table S4. Quantification was performed in triplicate. A nonpara-

metric Mann-Whitney U test was employed for two group comparison and  $p < 0.05$  was considered significant.

### Whole-Genome Sequencing

WGS of brain and liver samples from controls and schizophrenia patients was performed by Complete Genomics, with the paired-end library preparation and sequencing-by-ligation using self-assembling DNA nanoball (DNB) (Drmanac et al., 2010). Data process, mapping, and detection of variations were performed using the software developed by the Complete Genomics (version 2.2.0.26 and format version 2.2). Among the detected mobile insertion elements, we compared the genomic location of L1 insertion between brain and liver within an individual and identified brain-specific L1 insertions.

Further experimental details are available in the [Supplemental Experimental Procedures](#).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2013.10.053>.

## ACKNOWLEDGMENTS

This work was supported in part by the Grant-in-Aid for Scientific Research on Innovative Areas (Unraveling the microendophenotypes of psychiatric disorders at the molecular, cellular, and circuit levels) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to T.Y., H.N., T.K., and K.I., and a Grant-in-Aid from Ministry of Health, Labour and Welfare to T.K. This work was also supported by JST, CREST to T.K. and by JST, PRESTO to K.I. This work was also supported in part by Leading Project for Realization of Regenerative Medicine from MEXT and “Funding Program for World-Leading Innovative R&D on Science and Technology” to H.O., and by the “Development of biomarker candidates for social behavior” carried out under the Strategic Research Program for Brain Sciences from MEXT to T.Y. and K.K. This work was also supported in part by the Collaborative Research Project of the Brain Research Institute, Niigata University. Postmortem samples were donated by the Stanley Medical Research Institute, courtesy of Drs. Michael B. Knable, E. Fuller Torrey, Maree J. Webster, and Robert H. Yolken. We thank Tomoko Toyota and Atsuko Komori-Kokubo at RIKEN BSI for their technical assistance. We also thank Kenji Ohtawa at Research Resources Center at the RIKEN BSI for the cell-sorting analysis. M.B., F.S., and K.I. belong to the Department of Molecular Psychiatry, which is endowed by Daiinippon Sumitomo Pharma and Yoshitomiya-kuhin. H.O. is a scientific consultant for San Bio, Eisai, and Daiichi Sankyo. T.K. received a grant from Takeda Pharmaceutical. These companies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Accepted: October 18, 2013

Published: January 2, 2014

## REFERENCES

- Baillie, J.K., Barnett, M.W., Upton, K.R., Gerhardt, D.J., Richmond, T.A., De Sapio, F., Brennan, P.M., Rizzu, P., Smith, S., Fell, M., et al. (2011). Somatic retrotransposition alters the genetic landscape of the human brain. *Nature* 479, 534–537.
- Bloom, F.E. (1993). Advancing a neurodevelopmental origin for schizophrenia. *Arch. Gen. Psychiatry* 50, 224–227.
- Brandon, N.J., and Sawa, A. (2011). Linking neurodevelopmental and synaptic theories of mental illness through DISC1. *Nat. Rev. Neurosci.* 12, 707–722.
- Brown, A.S. (2006). Prenatal infection as a risk factor for schizophrenia. *Schizophr. Bull.* 32, 200–202.
- Cobb, S., Guy, J., and Bird, A. (2010). Reversibility of functional deficits in experimental models of Rett syndrome. *Biochem. Soc. Trans.* 38, 498–506.

- Cook, E.H., Jr., and Scherer, S.W. (2008). Copy-number variations associated with neuropsychiatric conditions. *Nature* **455**, 919–923.
- Cordaux, R., and Batzer, M.A. (2009). The impact of retrotransposons on human genome evolution. *Nat. Rev. Genet.* **10**, 691–703.
- Coufal, N.G., Garcia-Perez, J.L., Peng, G.E., Yeo, G.W., Mu, Y., Lovci, M.T., Morell, M., O’Shea, K.S., Moran, J.V., and Gage, F.H. (2009). L1 retrotransposition in human neural progenitor cells. *Nature* **460**, 1127–1131.
- Coufal, N.G., Garcia-Perez, J.L., Peng, G.E., Marchetto, M.C., Muotri, A.R., Mu, Y., Carson, C.T., Macia, A., Moran, J.V., and Gage, F.H. (2011). Ataxia telangiectasia mutated (ATM) modulates long interspersed element-1 (L1) retrotransposition in human neural stem cells. *Proc. Natl. Acad. Sci. USA* **108**, 20382–20387.
- Drmanac, R., Sparks, A.B., Callow, M.J., Halpern, A.L., Burns, N.L., Kermani, B.G., Carnevali, P., Nazarenko, I., Nilsen, G.B., Yeung, G., et al. (2010). Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. *Science* **327**, 78–81.
- Ehninger, D., Li, W., Fox, K., Stryker, M.P., and Silva, A.J. (2008). Reversing neurodevelopmental disorders in adults. *Neuron* **60**, 950–960.
- Evrony, G.D., Cai, X., Lee, E., Hills, L.B., Elhosary, P.C., Lehmann, H.S., Parker, J.J., Atabay, K.D., Gilmore, E.C., Poduri, A., et al. (2012). Single-neuron sequencing analysis of L1 retrotransposition and somatic mutation in the human brain. *Cell* **151**, 483–496.
- Giovanoli, S., Engler, H., Engler, A., Richetto, J., Voget, M., Willi, R., Winter, C., Riva, M.A., Mortensen, P.B., Schedlowski, M., and Meyer, U. (2013). Stress in puberty unmasks latent neuropathological consequences of prenatal immune activation in mice. *Science* **339**, 1095–1099.
- Hiroi, N., Takahashi, T., Hishimoto, A., Izumi, T., Boku, S., and Hiramoto, T. (2013). Copy number variation at 22q11.2: from rare variants to common mechanisms of developmental neuropsychiatric disorders. *Mol. Psychiatry* **18**, 1153–1165.
- Imaizumi, Y., Okada, Y., Akamatsu, W., Koike, M., Kuzumaki, N., Hayakawa, H., Nihira, T., Kobayashi, T., Ohyama, M., Sato, S., et al. (2012). Mitochondrial dysfunction associated with increased oxidative stress and  $\alpha$ -synuclein accumulation in PARK2 iPSC-derived neurons and postmortem brain tissue. *Mol. Brain* **5**, 35.
- Iwamoto, K., Kakiuchi, C., Bundo, M., Ikeda, K., and Kato, T. (2004). Molecular characterization of bipolar disorder by comparing gene expression profiles of postmortem brains of major mental disorders. *Mol. Psychiatry* **9**, 406–416.
- Iwamoto, K., Bundo, M., and Kato, T. (2005). Altered expression of mitochondrial-related genes in postmortem brains of patients with bipolar disorder or schizophrenia, as revealed by large-scale DNA microarray analysis. *Hum. Mol. Genet.* **14**, 241–253.
- Iwamoto, K., Bundo, M., Ueda, J., Oldham, M.C., Ukai, W., Hashimoto, E., Saito, T., Geschwind, D.H., and Kato, T. (2011). Neurons show distinctive DNA methylation profile and higher interindividual variations compared with non-neurons. *Genome Res.* **21**, 688–696.
- Karayorgou, M., Simon, T.J., and Gogos, J.A. (2010). 22q11.2 microdeletions: linking DNA structural variation to brain dysfunction and schizophrenia. *Nat. Rev. Neurosci.* **11**, 402–416.
- Keshavan, M.S., Nasrallah, H.A., and Tandon, R. (2011). Schizophrenia, “Just the Facts” 6. Moving ahead with the schizophrenia concept: from the elephant to the mouse. *Schizophr. Res.* **127**, 3–13.
- Kuwabara, T., Hsieh, J., Muotri, A., Yeo, G., Warashina, M., Lie, D.C., Moore, L., Nakashima, K., Asashima, M., and Gage, F.H. (2009). Wnt-mediated activation of NeuroD1 and retro-elements during adult neurogenesis. *Nat. Neurosci.* **12**, 1097–1105.
- Lewis, S.W., and Murray, R.M. (1987). Obstetric complications, neurodevelopmental deviance, and risk of schizophrenia. *J. Psychiatr. Res.* **27**, 413–421.
- Meyer, U., and Feldon, J. (2012). To poly(I:C) or not to poly(I:C): advancing pre-clinical schizophrenia research through the use of prenatal immune activation models. *Neuropharmacology* **62**, 1308–1321.
- Mullen, R.J., Buck, C.R., and Smith, A.M. (1992). NeuN, a neuronal specific nuclear protein in vertebrates. *Development* **116**, 201–211.
- Muotri, A.R., and Gage, F.H. (2006). Generation of neuronal variability and complexity. *Nature* **441**, 1087–1093.
- Muotri, A.R., Chu, V.T., Marchetto, M.C., Deng, W., Moran, J.V., and Gage, F.H. (2005). Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. *Nature* **435**, 903–910.
- Muotri, A.R., Zhao, C., Marchetto, M.C., and Gage, F.H. (2009). Environmental influence on L1 retrotransposons in the adult hippocampus. *Hippocampus* **19**, 1002–1007.
- Muotri, A.R., Marchetto, M.C., Coufal, N.G., Oefner, R., Yeo, G., Nakashima, K., and Gage, F.H. (2010). L1 retrotransposition in neurons is modulated by MeCP2. *Nature* **468**, 443–446.
- Murray, R.M., O’Callaghan, E., Castle, D.J., and Lewis, S.W. (1992). A neurodevelopmental approach to the classification of schizophrenia. *Schizophr. Bull.* **18**, 319–332.
- Nawa, H., Takahashi, M., and Patterson, P.H. (2000). Cytokine and growth factor involvement in schizophrenia—support for the developmental model. *Mol. Psychiatry* **5**, 594–603.
- Nawa, H., Someya, T., and Sakai, M. (2009). A novel schizophrenia model established by subcutaneously injecting a cytokine to a cynomolgus monkey neonate. *Schizophr. Bull.* **35** (Suppl 1), 252.
- Poduri, A., Evrony, G.D., Cai, X., and Walsh, C.A. (2013). Somatic mutation, genomic variation, and neurological disease. *Science* **341**, 1237758.
- Rehen, S.K., Yung, Y.C., McCreight, M.P., Kaushal, D., Yang, A.H., Almeida, B.S., Kingsbury, M.A., Cabral, K.M., McConnell, M.J., Anliker, B., et al. (2005). Constitutional aneuploidy in the normal human brain. *J. Neurosci.* **25**, 2176–2180.
- Shibata, Y., Kumar, P., Layer, R., Willcox, S., Gagan, J.R., Griffith, J.D., and Dutta, A. (2012). Extrachromosomal microDNAs and chromosomal microdeletions in normal tissues. *Science* **336**, 82–86.
- Shibuya, M., Komi, E., Wang, R., Kato, T., Watanabe, Y., Sakai, M., Ozaki, M., Someya, T., and Nawa, H. (2010). Measurement and comparison of serum neuregulin 1 immunoreactivity in control subjects and patients with schizophrenia: an influence of its genetic polymorphism. *J. Neural Transm.* **117**, 887–895.
- Spalding, K.L., Bhardwaj, R.D., Buchholz, B.A., Druid, H., and Frisén, J. (2005). Retrospective birth dating of cells in humans. *Cell* **122**, 133–143.
- Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872.
- Toyosima, M., Maekawa, M., Toyota, T., Iwayama, Y., Arai, M., Ichikawa, T., Miyashita, M., Arinami, T., Itokawa, M., and Yoshikawa, T. (2011). Schizophrenia with the 22q11.2 deletion and additional genetic defects: case history. *Br. J. Psychiatry* **199**, 245–246.
- Weinberger, D.R. (1987). Implications of normal brain development for the pathogenesis of schizophrenia. *Arch. Gen. Psychiatry* **44**, 660–669.
- Yu, F., Zingler, N., Schumann, G., and Strätling, W.H. (2001). Methyl-CpG-binding protein 2 represses LINE-1 expression and retrotransposition but not Alu transcription. *Nucleic Acids Res.* **29**, 4493–4501.
- Yurov, Y.B., Iourov, I.Y., Vorsanova, S.G., Liehr, T., Kolotii, A.D., Kutsev, S.I., Pellestor, F., Beresheva, A.K., Demidova, I.A., Kravets, V.S., et al. (2007). Aneuploidy and confined chromosomal mosaicism in the developing human brain. *PLoS ONE* **2**, e558.