Human-Specific Hypomethylation of CENPJ, a Key Brain Size Regulator

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Abstract

Both the enlarged brain and concurrent highly developed cognitive skills are often seen as distinctive characteristics that set humans apart from other primates. Despite this obvious differentiation, the genetic mechanisms that underlie such human-specific traits are not clearly understood. In particular, whether epigenetic regulations may play a key role in human brain evolution remain elusive. In this study, we used bisulfite sequencing to compare the methylation patterns of four known genes that regulate brain size (ASPM, CDK5RAP2, CENPJ, and MCPH1) in the prefrontal cortex among several primate species spanning the major lineages of primates (i.e., humans, great apes, lesser apes, and Old World monkeys). The results showed a human-specific hypomethylation in the 5’ UTR of CENPJ in the brain, where methylation levels among humans are only about one-third of those found among nonhuman primates. Similar methylation patterns were also detected in liver, kidney, and heart tissues, although the between-species differences were much less pronounced than those in the brain. Further in vitro methylation assays indicated that the methylation status of the CENPJ promoter could influence its expression. We also detected a large difference in CENPJ expression in the human and nonhuman primate brains of both adult individuals and throughout the major stages of fetal brain development. The hypomethylation and comparatively high expression of CENPJ in the central nervous system of humans suggest that a human-specific—and likely heritable—epigenetic modification likely occurred during human evolution, potentially leading to a much larger neural progenitor pool during human brain development, which may have eventually contributed to the dramatically enlarged brain and highly developed cognitive abilities associated with humans.

Key words: DNA methylation, CENPJ, brain evolution, primate, CpG island, epigenetic regulation.

Introduction

A dramatic increase in brain size is one of the hallmarks of human evolution that serve to differentiate humans from other primates. Although this difference is a significant evolutionary trait, the causal molecular mechanisms underlying it remain unknown. Comparative genomic analyses between humans and nonhuman primates have implicated several potential explanatory mechanisms, such as the rapid evolution of protein-coding genes (Clark et al. 2003; Zhang et al. 2011), the emergence of human-specific segmental duplications and noncoding RNAs (Zhang et al. 2011; Xie et al. 2012), or transcriptome changes found in the human brain (Khaitovich et al. 2005; Fu et al. 2007; Hu et al. 2011; Liu et al. 2012; Shulha et al. 2012; Xie et al. 2012). Despite a myriad of genetic studies like these, little research has been done at the epigenetic level to determine whether DNA methylation may also play an important role in human brain evolution.

DNA methylation is a crucial epigenetic modification of genomic DNA and one that has been established to play a key role in gene regulation during stem cell differentiation (Spivakov and Fisher 2007), as well as synaptic plasticity and memory formation (Levenson and Sweatt 2005; Tsankova et al. 2007). Although a large number of studies have characterized gene expression differences between humans and nonhuman primates in the brain, few studies have attempted to test for a connection between potential epigenetic modification and human brain evolution (Khaitovich et al. 2005; Fu et al. 2007; Hu et al. 2011; Liu et al. 2012). Recently, a whole-genome DNA methylation mapping study examined the methylation divergence between humans and chimpanzees and found that the chimpanzee brains exhibited a higher level of DNA methylation as compared with that found in human brains (Zeng et al. 2012). Unfortunately, as no outgroup primate species (e.g., rhesus macaque) was included in that study, identifying human-specific methylation from their results proved difficult.

To overcome the difficulty in determining a potential role of human-specific brain methylation, primary microcephaly (OMIM#251200), a rare human genetic disorder characterized by a marked reduction in brain size, provides an ideal model to delineating key genes involved in human brain development and evolution. To date, a total of seven genes have been implicated in causing primary
microcephaly: MCPH1/BRT1 (BRCT-repeat inhibitor of human ert gene expression, which encodes microcephalin; MCPH1) (Jackson et al. 2002; Lin and Ell ledge 2003), WDR62 (WD repeat domain 62; MCPH2) (Bilguvar et al. 2010; Nicholas et al. 2010; Yu et al. 2010), CDK5RAP2 (cyclin-dependent kinase 5 regulatory associated protein 2; MCPH3) (Bond et al. 2005), CEP152 (centrosomal protein 152 kDa; MCPH4) (Guernsey et al. 2010), ASPM (abnormal spindle like microcephaly associated protein; MCPH5) (Bond et al. 2002), CENPJ (centromeric protein J; MCPH6) (Bond et al. 2005), and STIL (SCL/TAL1 interrupting locus; MCPH7) (Kumar et al. 2009). Previous reports showed that among these microcephaly genes, ASPM, CDK5RAP2, CENPJ, and MCPH1 underwent rapid evolution at the protein sequence level due to Darwinian positive selection during primate evolution and human origin, which in turn suggests that these genes are likely key players in the evolution of the human brain (Evans, Anderson, Vallender, Choi, et al. 2004; Evans, Anderson, Vallender, Gilbert, et al. 2004; Wang and Su 2004; Evans et al. 2006). To test whether the microcephaly genes have accumulated genetic divergences in DNA methylation over the course of primate evolution—and particularly during human origin—we studied the methylation pattern of four microcephaly genes (ASPM, CDK5RAP2, CENPJ, and MCPH1) in several representative primate species: humans (Homo sapiens), chimpanzees (Pan troglodytes), eastern hoolock gibbons (Hoolock leuconedys), and rhesus macaques (Macaca mulatta). Alongside having evolved under Darwinian positive selection with rapid protein sequence changes during primate evolution and human origin (Zhang 2003; Evans, Anderson, Vallender, Gilbert, et al. 2004; Koupriina et al. 2004; Wang and Su 2004; Evans et al. 2006; Montgomery et al. 2011), these four genes are all associated with major changes in relative cerebral cortex size across primates (Evans, Anderson, Vallender, Choi, et al. 2004; Evans, Anderson, Vallender, Gilbert, et al. 2004; Wang and Su 2004; Evans et al. 2006). Likewise, knock-out mouse models of all of these genes displayed a marked reduction in brain size (Al-Dosari et al. 2010; Barrera et al. 2010; Buchanan et al. 2010; Pulvers et al. 2010; Gruber et al. 2011), which suggests that they are all, at some level, functionally important to brain development.

In this study, we demonstrated a human-specific hypomethylation of CENPJ that correlates with a much higher expression of CENPJ in humans compared with nonhuman primates during brain development.

Results

DNA Methylation Patterns of Four Microcephaly Genes in Primates

DNA samples were collected from the prefrontal cortex (PFC) of four primate species, including seven male humans, two male chimpanzees, one male gibbon, and six rhesus macaques (four males and two females), a summary of which is in table 1. CpGPlot/CpGReport was used to identify CpG islands (CGIs) (http://www.ebi.ac.uk/Tools/emboss/cpgplot/, last accessed December 7, 2013) (Larsen et al. 1992) and analyze a 2-kb putative cis-regulatory region upstream of the translational start sites of the four studied genes. Analysis yielded a total of five CGIs: two in MCPH1, one in ASPM, one in CDK5RAP2, and one in CENPJ (table 2 and supplementary fig. S1, Supplementary Material online). Next, bisulfate sequencing allowed us to generate methylation maps of these genes for humans, chimpanzees, and rhesus macaques, which showed that two genes—ASPM and MCPH1—were completely or near completely demethylated with no observable between-species differences (supplementary data S1 and S2, Supplementary Material online). In contrast, results for both CDK5RAP2 and CENPJ showed between-species differences in DNA methylation. Targeting these genes for further study, we conducted bisulfate sequencing of a gibbon brain sample to provide an extra outgroup species, thus covering all the major lineages of primates (humans, great apes, lesser apes, and Old World monkeys). For CDK5RAP2, among the 37 CpG sites tested, 6 sites showed between-species divergence in DNA methylation levels, whereas for CENPJ, 6 of the 10 CpG sites were differentially methylated among different species, although most particularly between humans and nonhuman primates.

Human-Specific Hypomethylation of CENPJ

The CENPJ CGI is about 1.7 kb upstream of the translational start site (supplementary fig. S1, Supplementary Material online) but after the transcription start site. Its DNA sequence is well conserved across different living primate species as well as in two archaic human species (Neanderthal and Denisovan) (fig. 1A). Among the nine CpG sites, six (CpG1–6) are conserved in DNA sequences among the human, Neanderthal, Denisovan, chimpanzee, and gibbon samples, while four of the six sites have sequence substitutions in macaques (fig. 1A). In addition, there are two macaque-specific CpG sites (CpGm2 and CpGm3m4) and one macaque-gibbon shared CpG site (CpGm1) (fig. 1A). Interestingly, at the two DNA sequence conserved CpG sites (CpG3 and CpG5), we observed a clear pattern of human-specific hypomethylation. In all nonhuman primate species, more than 80% of these two CpG sites are methylated, whereas less than 40% are methylated in humans (P < 0.01, pairwise t test; fig. 1B). A similar pattern was also observed for the other four partially conserved sites (CpG1, CpG2, CpG4, and CpG6), with the exception of CpG1, wherein gibbons show a lower level of methylation than humans (fig. 1C). The other three nonconserved CpG sites are all highly methylated in rhesus macaques, consistent with the high methylation levels of the two conserved CpG sites (CpG3 and CpG5) in macaques (supplementary fig. S2, Supplementary Material online). The observed human-specific hypomethylation of CENPJ in the brain was also confirmed in previously published data, which showed a significant methylation difference between humans and chimpanzees (13.8% vs. 19.2% in the CENPJ genomic region) (table 3) (Zeng et al. 2012). To check whether the DNA sequences of the CpG sites are conserved in extant human populations, we analyzed data
Brain Expression Divergence of CENPJ between Humans and Nonhuman Primates

Following our analysis of CENPJ’s methylation, we next checked the interspecific gene expression divergence of CENPJ in the brain using the published RNA-seq data from adult individuals of humans, chimpanzees, and macaques (Liu et al. 2012). As expected, the expression level of CENPJ in the human brain is about 3–10 times higher than that found in either brain of chimpanzee or rhesus macaque (P = 4.1e–11 for human vs. chimpanzee; P = 2.5e–13 for human versus macaque, one-way ANOVA followed by a multiple t-test with Bonferroni correction; fig. 3A). Similar results were also reported by an earlier study conducted by Zeng et al. (2012), in which humans, as compared with chimpanzees, showed significantly higher CENPJ expression in PFC (P = 0.0325, Bayesian t test) but not in the other three microcephaly genes (table 3).

Using publically available human brain expression data (www.brainspan.org, last accessed December 7, 2013), we found that during the course of human brain development, there is a peak of CENPJ expression in the fetal brain at roughly 9 gestational weeks, which corresponds to the onset of neurogenesis (fig. 3B). After birth, the expression level decreases sharply and remains at a relatively stable level through adulthood (fig. 3C), suggesting that CENPJ may potentially be a key regulator for neurogenesis. A similar pattern also exists in both chimpanzees and macaques, but the overall expression levels of the gene during gestational development are much lower than in humans (fig. 3C). Additionally, previous studies indicated that during fetal brain development in humans, at 13–16 gestational weeks the neocortex showed a higher expression level of CENPJ in the ventricular zone (VZ) and subventricular zone (SVZ) as compared with that in the cortical plate (CP) (Fietz et al. 2012; Genin et al. 2012). As VZ and SVZ are the brain layers with active neural progenitor proliferations, the high expression of CENPJ in these brain layers suggests its close involvement in neurogenesis. To confirm some of these findings, we also analyzed the microarray database (www.brainspan.org, last accessed December 7, 2013) of brain gene expression during human prenatal time and observed a similar pattern (supplementary fig. S3, Supplementary Material online).

Methylation Status of CENPJ in Other Tissues

To test whether the human-specific hypomethylation also exists in other tissues, we analyze the methylation patterns of the CENPJ CpG sites in liver, kidney, and heart. Interestingly, similar to the pattern seen in the brain, humans also show a
Fig. 1. DNA sequence and methylation level comparison of the CENPJ promoter between humans and nonhuman primates. (A) Nucleotide sequence alignment of the CENPJ promoter of human, Denisovan, Neanderthal, chimpanzee, gibbon, and macaque. The shared CpG sites among species are indicated with no. 1–6. The macaque-specific CpG sites are m2, m3, and m4, and gm1 indicates the CpG site shared between gibbon and macaque. (B) Average methylation percentages of CpG_3 and CpG_5 in PFC of humans (n = 4), chimpanzees (n = 2), gibbon (n = 1), and macaques (n = 3). (C) Average methylation percentages of CpG1–6 in PFC of humans (n = 4), chimpanzees (n = 2), and gibbon (n = 1). Bars represent average methylation levels with SD. “*” indicates a significant difference (P < 0.01, pairwise t-test) of average methylation levels between humans and nonhuman primates (chimpanzees, gibbons, and macaques). “#” indicates a significant difference (P < 0.01, pairwise t-test) of average methylation levels between humans and chimpanzees.

Table 3. Methylation-C Seq Data from PFC of Humans and Chimpanzees.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Human Methylation</th>
<th>Chimpanzee Methylation</th>
<th>Human Expression</th>
<th>Chimpanzee Expression</th>
<th>Expression Difference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPM</td>
<td>0.24</td>
<td>0.32</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>CDK5RAP2</td>
<td>0.20</td>
<td>0.28</td>
<td>4.47</td>
<td>4.36</td>
<td>0.03</td>
<td>0.86</td>
</tr>
<tr>
<td>CENPJ</td>
<td>0.13</td>
<td>0.19</td>
<td>5.03</td>
<td>3.19</td>
<td>0.94</td>
<td>0.03</td>
</tr>
<tr>
<td>MCPH1</td>
<td>0.06</td>
<td>0.05</td>
<td>5.54</td>
<td>4.36</td>
<td>1.18</td>
<td>0.24</td>
</tr>
</tbody>
</table>
lower methylation levels in all six CpG sites as compared with nonhuman primates (fig. 4). The only exceptions are the comparatively low methylation levels of CpG-1 in both gibbons and chimpanzees (fig. 4B and C). Notably, the observed methylation differences between humans and nonhuman primates are far more pronounced in the brain than in the liver, kidney, or heart. In the brain, the average methylation difference between humans and nonhuman primates is 60%, although it is only 33% for the liver, 45% for kidneys, and 38% for the heart. CpG-1 was excluded in these calculations, due to the exceptionally low methylation levels observed in gibbons and chimpanzees.

We further checked the level of histone methylation at H3K4me3 (H3-trimethyl-lysine4, an epigenetic marker that reflects the transcriptional activity of the regulated gene) (Shilatifard 2006; Zhou et al. 2011) for ASPM, CDKSRAP2, CENPJ, and MCPH1 using published genome-wide H3K4me3 data for the PFC of humans, chimpanzees, and macaques (Shulha et al. 2012). We found that only CENPJ showed interspecific difference where both humans and chimpanzees had positive H3K4me3 signals while macaques do not (fig. 5 and supplementary fig. S4, Supplementary Material online), a result consistent with both the data showing relatively higher expression levels of CENPJ in humans and chimpanzees as compared with macaques as well as the observed hypomethylation in humans.

**Discussion**

CENPJ encodes the centromere protein CPAP, which is enriched at centrosomes and is also present in the cytoplasm of proliferating cells (Tang et al. 2009). Truncated mutations of CENPJ would accordingly not only cause primary microcephaly but also be associated with Seckel syndrome (OMIM#210600), a rare autosomal recessive disorder characterized by intrauterine and postnatal growth delay, microcephaly with mental retardation, and facial dysmorphisms (Shanske et al. 1997; Bond et al. 2005; Gul et al. 2006; Al-Dosari et al. 2010). During the development of the brain, neural progenitors undergo symmetrical divisions in which the spindle is usually positioned parallel to the ventricular surface (Rakic 2009). CENPJ has been shown to contribute to centriolar location and centriole formation by generating overly long centrioles (Kitagawa et al. 2011). Likewise, CENPJ could also physically interact with STIL (also called MCPH7) and CEP152 (also called MCPH4) during the early phase of procentriole assembly (Tang et al. 2011). The expression level of CENPJ may accordingly be important for maintaining conserved in the DNA sequences among all the tested primate species, including the 6 CpG sites that show between-species methylation differences (fig. 6A). In total, there are five CpG sites specific for rhesus macaques (CpGm1, CpGm3, CpGm4, CpGm5, and CpGm6) and two other CpG sites shared among part of the studied species (CpGhdncg2 and CpGcg7). The seven nonconserved CpG sites are all hypomethylated, with no observed interspecific differences. Among the six CpG sites (CpG_20, CpG_26, CpG_27, CpG_28, CpG_29, and CpG_30) showing between-species methylation differences, five displayed a significant methylation divergence between humans and nonhuman primates; in humans, they are relatively hypermethylated (>10%), whereas in the three nonhuman primate species they are totally demethylated, with the exception of CpG30, which shows a high methylation level in gibbons (>40%) (fig. 6B).

With the use of previously published data (Liu et al. 2012), we were able to compare the mRNA expression levels of CDKSRAP2 in the brain among humans, chimpanzees, and rhesus macaques. Compared with chimpanzees and macaques, humans have slightly higher mRNA expression level, which seems to contradict the observed overall higher methylation levels observed in humans (fig. 6D and supplementary fig. S5, Supplementary Material online) because the higher methylation in humans would, in theory, predict a relatively lower mRNA expression among humans compared with nonhuman primates. Accordingly, the observed DNA methylation divergence of CDKSRAP2 does not seem to affect the gene expression in the brain and may therefore not be functionally important for human brain evolution, although further evaluation is likely needed to reach any definitive conclusions.
Fig. 3. Brain expression divergence of CENPJ between humans and nonhuman primates. (A) Comparison of CENPJ expression levels (indicated by RPKM values) among human \((n = 14)\), chimpanzee \((n = 13)\), and macaque \((n = 15)\). (B) The curve of CENPJ expression changes in PFC during human brain development. The black star indicates the peak expression at gestational week 9. (C) Comparison of CENPJ expression changes during brain development among human, chimpanzee, and macaque. Statistical analysis was performed by one-way ANOVA followed by a multiple t-test with Bonferroni correction.
symmetric divisions of neural progenitor cells, and a higher expression of \textit{CENPJ} may result in a larger pool of neural progenitors, which would eventually lead to a larger brain.

A transgenic mouse study confirmed the functional outcome of \textit{CENPJ} expression level during brain development in mice, where \textit{CENPJ} is also strongly expressed in the neuroepithelium during neurogenesis (Bond et al. 2005). Recently, McIntyre et al. (2012) generated a \textit{CENPJ} hypomorphic mouse model (mice that express a low level of the \textit{CENPJ} protein) and showed that the transgenic mouse recapitulates many of the clinical features of Seckel syndrome, including intrauterine dwarfism, microcephaly with memory impairment, ossification defects, and ocular and skeletal abnormalities. These findings suggest that the level of \textit{CENPJ} expression during neurogenesis is crucial for normal brain development.

Studies of molecular evolution have similarly shown that \textit{CENPJ} is one of the microcephaly genes that have undergone rapid protein sequence evolution in primates, as compared with that in rodents and carnivores (Montgomery et al. 2011). However, the evolutionary rates of \textit{CENPJ} in various primate lineages show a generalized rate increase, as opposed to a specific rate increase along the lineage that led to humans (Montgomery et al. 2011). Unlike the other microcephaly genes that show accelerated protein sequence evolution along the lineage leading to humans (e.g., \textit{ASPM}) (Evans, Anderson, Vallender, Gilbert, et al. 2004; Kouprina et al. 2004), the protein sequence change of \textit{CENPJ} in the human lineage does not seem to explain the dramatically enlarged brain present during human origin. The human-specific DNA methylation change of \textit{CENPJ} in the brain may then be what led to the significantly increased gene expression of \textit{CENPJ} accompanying neurogenesis, which in turn explains the comparatively larger neuro-progenitor pool present during human brain development and the subsequently larger brain size possessed by humans.

The results of our study highlight the reality that human-specific hypomethylation of \textit{CENPJ} cannot simply be explained by either a gain or loss of CpG sites during primate evolution, because the CpG sites with between-species methylation divergence are highly conserved in the DNA sequences we studied (fig. 1A). Similarly, the flanking sequences of the CpG sites do not seem to explain the diverged methylation either, as there is only one human-specific sequence substitution (C to T mutation at site-157, fig. 1A) in the analyzed region of \textit{CENPJ}. This substitution does not seem to affect methylation because it is not located within either a CpG site or a known sequence motif affecting DNA methylation, although further functional tests are needed. If neither of these explanations is workable, a trans-regulatory human-specific change may explain the observed human-specific hypomethylation of \textit{CENPJ}, although the responsible gene(s) have not yet been identified and would, as such, necessitate further study. Another possibility is that the species-specific environmental and behavioral influence may be

\begin{figure}
\centering
\includegraphics{figures.png}
\caption{Comparison of methylation levels of \textit{CENPJ} among different human tissues. (A) Human (n = 7); (B) chimpanzee (n = 2); (C) gibbon (n = 1); (D) macaque (n = 6). The dashed line indicates 80% methylation.}
\end{figure}
at work, because both the living environment and life style
of humans are totally different from those of nonhuman
primates. A previous study showed that environmental and
behavioral changes are capable of altering the methylation
pattern of genes in the brain (Levenson and Sweatt 2005;
Tsankova et al. 2007; Graff and Mansuy 2008). Although an
attractive possibility, this scenario does not seem to explain
the sharp difference of \textit{CENPJ} expression during fetal
development—wherein humans show a much higher ex-
pression level as compared with nonhuman primates (fig. 3C)—because the developmental environment of fetuses
should be similar between both humans and nonhuman
primates. The high expression of \textit{CENPJ} would predict a
hypomethylation status of \textit{CENPJ} during fetal brain devel-
opment in humans compared with nonhuman primates,
but clearly further testing and more precise investigations
are needed to confirm this notion. Additionally, consider-
ing the human-specific hypomethylation and the relatively
low within-species variations of methylation levels of \textit{CENPJ}
in the brain (fig. 1B and C), the methylation pattern of
\textit{CENPJ} in the brain is possibly heritable, which may con-
tribute to the dramatically enlarged brain during the origin
of humans. Again, however, such a proposal—provocative
as it may be—requires experimental verification that is
beyond the scope of this study.

In summary, comparative bisulfate sequencing identified
a human-specific low methylation of \textit{CENPJ}, a key gene for brain
development. Our analysis illustrates how the methylation
status of \textit{CENPJ} can influence its expression in vitro, which
correlates well with the noted expression divergence of \textit{CENPJ}
in the brain between humans and nonhuman primates.
Taken on the whole, these results suggest that human-specific
epigenetic changes in the brain may be among the key con-
tributors that were at work in the origin of human cognition,
a feature that has become one of the defining differences
between humans and nonhuman primates.

\textbf{Materials and Methods}

\textbf{Tissue Samples}

Frozen tissues were obtained from the cerebral cortexes of
seven humans, two chimpanzees, one gibbon, and six
rhesus macaques, all of which had no known neuronal
diseases or history of drug abuse. A summary of sample
information is shown in table 1. For the human subjects,
informed written consents were obtained from the relatives
of the human subjects prior to sample collection or anal-
ysis. All protocols of this study were approved by the in-
ternal review board of Kunming Institute of Zoology,
Chinese Academy of Sciences.
Promoter Sequence Analysis

CpGPlot/CpGReport (http://www.ebi.ac.uk/Tools/emboss/cpgplot/, last accessed December 7, 2013) (Larsen et al. 1992) was used to identify CGIs in putative cis-regulatory regions 2 kb upstream of the translational start sites of the genes of interest. The default parameters were used for ASPM, CDK5RAP2, and MCPH1, including 1) the observed CpG/expected CpG ratios >0.6, 2) %C + %G >50%, and 3) sequence length >200 bp. For CENPJ, because the GC content of its promoter region is 48.26%, we used a %C + %G >40% cutoff in the analysis, and the other parameters remained the same. The promoter sequences of four microcephaly genes (ASPM, CDK5RAP2, CENPJ, and MCPH1) from humans, Neanderthals, chimpanzees, gibbons, and macaques are retrieved from Ensembl (http://www.ensembl.org, last accessed December 7, 2013). The Denisovan promoter sequences were provided by Dr Martin Kircher of the Max Planck Institute for Evolutionary Anthropology (Meyer et al. 2012). Sequence alignment was conducted with Clustal W (BioEdit 7.0.5.2). As shown in table 2, most of the amplicons are longer than the predicted CGIs. The reasons are as follows: 1) It was hard to design primers only covering the predicted CGI regions and make them suitable for bisulfate sequencing. Accordingly, we looked for effective primers upstream or downstream of the predicted CGI regions. 2) We intended to cover the entire predicted CGI regions, and consequently, most of the amplicons (ASPM, MCPH1 CGI-1 and CGI-2, and CDK5RAP2) regions were longer than the predicted CGIs. For CENPJ, the amplicon is slightly shorter than the predicted CGI, also due to the primer design strategy.
Bisulfite Sequencing
We used EpiTect Bisulfite Kits (Qiagen, Valencia, CA) to conduct bisulfite conversions of DNA following the manufacturer's instructions. Sodium bisulfite converts unmethylated cytosine to uracil, which is then PCR amplified as thymidine while methylated cytosine remains cytosine. PCR products from bisulfite-treated DNA were cloned into pMD19T vector (TaKaRa, Tokyo, Japan). PCR primers were designed using Methyl Primer Express 1.0 (ABI). A total of 10–30 clones were sequenced using an ABI 3130 Sequencer after PCR, ligation, and cloning. Cloned sequences were then analyzed using the BiQ Analyzer software (Bock et al. 2005) to determine the methylation levels, following manual checking. Methylation primers are listed in supplementary table S1, Supplementary Material online.

Human Brain Development Expression Data Analysis
We downloaded human brain development expression RNA-seq data and microarray data of CENPJ gene from BRAIN SPAN (atlas of the developing human brain) (www.brainspan.org, last accessed December 7, 2013), covering the developing stages ranging from 5 to 7 postconceptional weeks to over 40 years of age. The RPKM (reads per kilobase per million) value is used to indicate the expression level of CENPJ.

Transient Transfection and Luciferase Reporter Assay
All transfections were carried out in triplicate in 24-well plates (Corning, NY, USA). About 2 × 10⁵ cells were seeded for 24 h prior to transfection. Equal numbers of cells were plated in 24-well and 6-well plates and grown to 80% confluency. The indicated amounts of vectors were mixed in OPTI-MEM medium (Gibco) with Lipofectamine 2000 (Invitrogen). The solution was then incubated for 30 min at room temperature and then placed on the cultured cells. After 6 h, the medium was changed into Dulbecco’s Modified Eagle Medium (Gibco) with 10% fetal bovine serum (HyClone). For the luciferase assay, cells were grown in 24-well plates and transfected with the indicated amounts of vectors, including pTK-Renilla as an internal control, by Lipofectamine 2000 (Invitrogen). Luciferase activity was assayed 28 h after transfection. The luciferase activity of the cell extract was determined by Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the protocols supplied by the manufacturer. The relative light units were measured using a luminometer. Each experiment was repeated at least three times to ensure accuracy.

The human CENPJ promoter constructs were generated by PCR amplification and subcloned into pGL3 basic vector (Promega, Madison, WI) to construct pGL3-CENPJ. The sequences of the cloned DNAs were verified by sequencing of the entire region. The constructs were either unmethylated or fully methylated at all CpGs by incubation with SssI Methylase (NEB) in the presence of 160 μM S-adenosylmethionine in NEB buffer 2 (50 mM NaCl, 10 mM Tris–HCl pH 7.9, 10 mM MgCl₂, and 1 mM DTT) at 37 °C for 16 h. All methylated CENPJ promoter regions were verified by bisulfite sequencing.

Data Analysis
Statistical analysis was performed using the R program (http://www.r-project.org/, last accessed December 7, 2013), and the graph was generated using the R ggplot2 package.

Supplementary Material
Supplementary figures S1–S5, tables S1 and S2, and data S1 and S2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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