Enrichment analysis of Alu elements with different spatial chromatin proximity in the human genome

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ABSTRACT

Transposable elements (TEs) have no longer been totally considered as “junk DNA” for quite a time since the continual discoveries of their multifunctional roles in eukaryote genomes. As one of the most important and abundant TEs that still active in human genome, Alu, a SINE family, has demonstrated its indispensable regulatory functions at sequence level, but its spatial roles are still unclear. Technologies based on 3C (chromosome conformation capture) have revealed the mysterious three-dimensional structure of chromatin, and make it possible to study the distal chromatin interaction in the genome. To find the role TE playing in distal regulation in human genome, we compiled the new released Hi-C data, TE annotation, histone marker annotations, and the genome-wide methylation data to operate correlation analysis, and found that the density of Alu elements showed a strong positive correlation with the level of chromatin interactions (hESC: $r = 0.9$, $P = 2.2 \times 10^{-16}$; IMR90: $r = 0.94$, $P < 2.2 \times 10^{16}$) and also have a significant positive correlation with some remote functional DNA elements like enhancers and promoters (Enhancer: hESC: $r = 0.997$, $P = 2.3 \times 10^{-4}$; IMR90: $r = 0.934$, $P = 2 \times 10^{-2}$; Promoter: hESC: $r = 0.995$, $P = 3.8 \times 10^{-4}$; IMR90: $r = 0.996$, $P = 3.2 \times 10^{-3}$). Further investigation involving GC content and methylation status showed the GC content of Alu covered sequences shared a similar pattern with that of the overall sequence, suggesting that Alu elements also function as the GC nucleotide and CpG site provider. In all, our results suggest that the Alu elements may act as an alternative parameter to evaluate the Hi-C data, which is confirmed by the correlation analysis of Alu elements and histone markers. Moreover, the GC-rich Alu sequence can bring high GC content and methylation flexibility to the regions with more distal chromatin contact, regulating the transcription of tissue-specific genes.

KEYWORDS chromatin interaction, alternative parameter of Hi-C data, open chromatin, methylation potential

INTRODUCTION

Transposable elements (TEs) are DNA sequences able to move and replicate within the genome of a single cell and
Alu elements enrichment associated with spatial chromatin proximity

RESEARCH ARTICLE

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have been found in virtually all eukaryotic genomes so far sequenced (Feschotte 2008; Kidwell and Lisch 2001). Large-scale genome sequencing has revealed that TEs comprise more than 45% of the human genome, and also represent an abundant part of the genomes in fungi and metazoans (3%–20% in fungi and 3%–45% in metazoans) (Wicker et al. 2007; Lander et al. 2001). According to the mechanism of transposition, TEs are usually divided into two classes (Class I or retrotransposons, and Class II or DNA transposons). Retrotransposons can be further grouped into three main orders (long terminal repeats (LTRs), long interspersed nuclear elements (LINE) and short interspersed nuclear elements (SINE)). In humans, Alu elements are the most abundant SINEs with over one million inserted copies, resulting from their continuous proliferation activity over the past ~65 million years (Myr) (Cordaux and Batzer 2009), and comprise about 10% of the DNA sequences of the human genome. According to the current model, modern Alus are ~300 bp in length and emerged from a head to tail fusion of two distinct fossil Alu monomers (FAMs) that originated from the 7SL RNA gene 55 Myr ago with the expansion of primates (Quentin 1999a, b). During the course of primate evolution, Alu elements have spread and formed several distinct subfamilies on the basis of different rates of amplification. The three major subfamilies are: the oldest AluJ, immediately aged AluS, and the youngest AluY. Previous studies have indicated that AluJ and AluS appeared in ancient genome 65–25 Myr, and AluY 25 Myr (Kapitonov and Jurka 1996). Due to the short evolutionary history of the AluY class, some AluY repeats have not went through the purify selection and are also unable to reach the fixation, resulting in the fact that, currently, most of Alu elements in the human genome belong to the AluS class.

Alu elements, together with other repetitive elements, were originally thought of as genomic parasites and had been long dismissed as selfish or ‘junk’ DNA (Kidwell and Lisch 2001; Brookfield 2005). However, studies have demonstrated that widespread Alu elements are distributed throughout the human genome in a non-random manner (Grover et al. 2003) and play an important role in genome (Kazazian 2004) and gene evolution (Nekrutenko and Li 2001), epigenetic and gene regulation (Feschotte 2008; Lynch et al. 2011; Teng et al. 2011; Polak and Domany 2006). For example, recent studies have indicated that Alu elements are predominant in isochores (Hackenberg et al. 2005) and segmental duplications (Jurka et al. 2004). Likewise, the distribution of Alu elements was found to have a highly positive correlation with local GC contents and the density of genes or introns (Grover et al. 2004). Moreover Polak et al. demonstrated that the upstream regions of the transcription start site (TSS) are enriched with Alu elements that contain many putative binding sites for transcription factors (Polak and Domany 2006). A subsequent study further highlighted that primate-specific Alu elements had derived many promoters and were thus considered to contribute a lot to lineage-specific patterns of gene expression in humans (Huda et al. 2011). In addition, except for the involvement of transcriptional regulation, several lines of evidence have shown that Alu elements also play a role in the post-transcriptional regulation by deriving target sites for miRNAs in the 3’ untranslated region (UTR). For example, Jordan and colleagues illustrated that nearly 20% of the human genes contain TEs in the 3’ UTR (Jordan et al. 2003), and it was also observed that some Alu elements within the 3’ UTR of human miRNAs are highly conserved and provide perfect complementary target sites for miRNAs (Smalheiser and Torvik 2006).

Transcription of eukaryotic genes is a highly complicated process requiring the ultra-precise cooperation of a batch of interactions among functionally diversified proteins and DNA sequences (Maston et al. 2006). Regulation of transcription is performed by a myriad of cis-regulatory elements, including enhancers, promoters, silencers and insulators, and fulfilled mainly by enhancers, which are portions of DNA that can activate transcription regardless of their location, distance or orientation relative to the promoters of genes by binding a variety of transcription factors (Ong and Corces 2011). This functional flexibility has indeed diversified the transcriptional regulation in different tissues or cell types. For example, a recent study examined the genome-wide histone H3 lysine 4 (H3K4) methylation patterns in two cancer cell lines—K562, a human erythroleukemia, and HeLa, a human cervical carcinoma, and predicted about 24,000 to 36,000 enhancers in each cell line, and found that only 5000 (14%–21%) are present in both, indicating the high cell-type-specificity of enhancer activity (Heintzman et al. 2009). Nevertheless, this flexibility impedes efforts to comprehensively investigate and record the full list of enhancers and promoters they regulated within the genome.

The systematic identification of a high-resolution cis-regulatory map between enhancers and their target promoters in mammalian systems has so far been limited. However, recently, 3C (chromosome conformation capture) based technologies have enabled large-scale spatiotemporal interactions, between distal sequence elements (mainly enhancers and promoters) within genomic loci located throughout the mammalian genome, to be routinely investigated by high-throughput microarray or deep sequencing approaches (Lieberman-Aiden et al. 2009; Wang et al. 2011; Eskeland et al. 2010; Kagey et al. 2010). 3C-based protocols were originally used to evaluate long-range chromatin interactions (Cis) between a pair of pre-specified genomic sites (Dekker et al. 2002). The extensions of the 3C approach include 4C (chromosome conformation capture-on-chip) (Simonis et al. 2006), 5C (chromosome conformation capture carbon copy) (Dostie et al. 2006), ChIA-PET (Chromatin Interaction Analysis with Paired-End Tag) (Fullwood et al. 2009) and Hi-C (Lieberman-Aiden et al. 2009) (reviewed in de Wit and de Laat 2012). Among these methods, Hi-C allows the detection of chromatin interactions between any pair of loci across an entire genome at a higher resolution (Lieberman-Aiden et al. 2009). In Hi-C experiments, CIs can be determined by the
number of Hi-C reads and the read count is negatively corre-
lated with the three-dimensional (3D) distance between two
loci, suggesting that Hi-C read count can serve as a powerful
proxy to measure the CI level (Lieberman-Aiden et al. 2009).
Recently, Dixon et al. reported an investigation of the 3D
organization of the human and mouse genome in four different
cell types at unprecedented resolution using Hi-C method
(Dixon et al. 2012) and identified large, mega-base-sized local
chromatin interaction domains, termed “topological domains”,
as a pervasive structural feature of the genome organization in
human and mouse. Such a pervasive structural characteristic
was subsequently confirmed by an independent study (Nora
et al. 2012). Further investigation reveals that the overall
domain structure is generally conserved during mammalian
evolution, and largely stable between different cell types,
whereas intra-domain contributes most (>96%) of dynamic
interacting regions, and thus potentially participate in cell-
type-specific regulatory events (Dixon et al. 2012). Furth-
more, they also observed that Alu/B1 and B2 elements in mice
and Alu elements in humans are enriched at boundary regions
of domains, indicating a role for TEs in the spatial organization
of the genome. In contrast to the intense research on the spatial
characteristics of TEs at boundary regions, TEs themselves, as the most abundant part in mammalian ge-
nomes, many unanswered questions associated with their
function and involvement in intra-domains have largely
remained uncharacterized. For example, whether TEs
actively take part in the cell-type-specific regulation in intra-
domains, and if so, how, is still mysterious.

To address these issues, in this study, we used newly
released, genome-wide chromatin interaction data provided
by Dixon et al. (Dixon et al. 2012) to investigate the possi-
ble roles for TEs, especially Alu elements, in intra-domain
regions, and further link the TEs with EP interactions in
human. First, we explored the association between TEs and
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Besides deriving enhancers and being involved in
enhancer-promoter interaction, the Alu elements also play
an important role in providing potential methylation sites.
Previous studies showed that G or C nucleotides will accu-
mulate in regions with more chromatin interactions (Dostie
et al. 2006). And the Alu elements, especially the old AluJ
and AluS, have been known to be enriched in GC-rich
regions ever since the sequencing of the human genome
(Lander et al. 2001). Our results not only prove the phe-
nomenon found in old studies again, but also for the first
time showed that the Alu elements may be derived from
increasing of GC content in high-CI regions, and CpG sites
also, which may raise the methylation potentiality as a result.
The high-CI regions are reported to be associated with open
chromatin (Smit 1999) and are highly related to tissue-
specific transcription by the regulation of distal DNA ele-
ments, nucleosome modification, and so on. The Alu ele-
ments can be therefore involved in tissue-specific regulation
by raising methylation flexibility and deriving binding sites for
transcriptional factors.

Taken together, our results show for the first time that Alu elements play a role in chromatin interactions, and its cov-
erage can be served as a surrogate of CI frequency and be
used to detect potential enhancer-promoter interaction. Ad-
ditionally, the more Alu elements in regions with more chromatin contacts will endow the regions with methylation
capacities and flexibilities, and may be important for tissue-
specific transcription. This means that the spatial charac-
teristics of TEs may play a more important role in confor-
mation of chromatin structure and gene activity.

RESULTS
SINE elements are over-presented in the genomic loci
of intra-domains with high frequencies of chromatin
interactions

The recently released whole-genome CI profiling data in
mammals include human embryonic stem cells (hESCs),
human IMR90 fibroblasts, mouse embryonic stem cells and
mouse cortex (Dixon et al. 2012), among which here we
focused our analysis on two human cell lines. Several lines
of evidence have previously demonstrated that the majority
of functional cis-regulatory interactions occur within the
same topological domain (Dixon et al. 2012; Nora et al.
2012). For example, although the distance between the Shh
enhancer and the target shh gene exceeds nearly 1 Mb, they
still locate within the same topological domain (Smallwood
and Ren 2013). The frequency of intra-domain CIs is higher
than inter-domain CIs and a higher frequency means a
higher probability to be a real interaction (Li et al. 2010),
suggesting that CIs between two genomic loci, located at
different domains, may be largely non-informative or back-
ground noise. In other words, frequency of inter-domain CIs
could serve as an effective threshold to remove false-posi-
tive CIs. Based on this assumption, we filtered the frequency
of intra-domain CIs using mean frequency of inter-domain ones as a threshold, and removed the bin-pairs whose frequencies are lower than the threshold in each of the two human cell lines (see MATERIALS AND METHODS). Furthermore, it was also observed that bin-pairs with extremely high frequencies are scattered, indicating them as possible outliers. We subsequently discarded the bin-pairs whose frequencies are larger than the thresholds of 153 and 133 in hESCs and IMR90 fibroblasts, respectively. The upper thresholds were chosen according to the following reasons: (1) they are the first window without interacting bin-pairs in each cell line, when we binned the frequency of intra-domain CIs by the step of 1; (2) after applying the upper threshold in each cell line, the removed bin-pairs only occupied 0.0085% and 0.0076% of valid ones in human ESCs and IMR90 fibroblasts, respectively (Fig. S1). Finally, we ambiguously retained 897,867 and 850,305 intra-domain CI bin-pairs (hereafter referred to as CI-bin-pairs, unless otherwise mentioned) in hESCs and IMR90 fibroblasts, respectively.

We first sought to examine whether a similar distribution pattern of intra-domain CI frequency (hereafter referred to as CI frequency, unless otherwise mentioned) exists in four different TE families. To do this, we divided CI frequencies into 50 bins (referred to as frequency bin), and computed density for each of the four TE families in each frequency bin. The density is the fraction of TEs belonging to the same family in one CI-bin-pair, divided by the maximum possible length of each CI-bin-pair (80 kilobases) to the mean length of that TE family (Fig. 1). The density was calculated for each of the four TE families and plotted across different bins in hESCs and IMR90 fibroblasts, respectively (see Table S1 for TE families with calculated density in each cell line). Higher density implies that more same-family TEs are in a frequency bin, whereas a lower one means less same-family TEs. We noticed that two TE families, LTR and DNA transposons, have comparable low densities, yet SINE and LINE have higher densities across different bins in both cell lines (Fig. 1). This phenomenon may be due to the reason that DNA transposons and LTRs either are currently not mobile or have a very limited activity in the human genome compared to SINEs and LINEs, where their subfamilies, such as Alu and L1s, are still active in humans and have proliferated during primate evolution (Cordaux and Batzer 2009). Then, the Pearson correlation coefficient (PCC) score was computed for each of four different TE families in each cell type, and we found that SINE density is positively correlated with CI frequency in hESC ($r = 0.89, P < 2.2 \times 10^{-16}$, Fig. 1A), and this trend for SINEs is also observed in IMR90 fibroblasts ($r = 0.91, P < 2.2 \times 10^{-16}$, Fig. 1B). But the densities of other three TE families (LINE, LTR, and DNA) are negatively correlated in both cell lines (Fig. 1C–H). To evaluate whether the above results will be affected by bin lengths, we further separated CI frequency into 20 and 100 bins, respectively. The results illustrated that the trends of different TE families, previously observed in the bin size of 50, were still hold true for the bin sizes of 20 and 100 (Figs. S2 and S3).

Since the above results are not affected by the different bin sizes, we then tested whether these results are significantly different from those of non-intra-domains. The correlation between density and CI frequency in non-intra-domains were subsequently calculated for each of the four TE families, respectively. We found that no TE families are correlated with interaction frequency in non-intra-domains, except for DNA transposons in IMR90 fibroblasts with a weakly negative correlation ($r = -0.377, P = 0.013$, Table S1). We further compared the correlations of DNA transposon between intra- and non-intra-domains using a z-test, and found that they are significantly different ($P = 0.0016$). Thus, these results clearly demonstrate that the density of four TE families in intra-domains are significantly different from those in non-intra-domains, and SINE is the only TE family whose distribution shows strong correlations with the frequency of intra-domain CIs in both investigated cell lines. Such correlation is also supported by a recent study, in which they indicated that multigene chromatin interacted regions are enriched with SINE density.

**Alu is the major player of TEs in intra-domain chromatin interactions**

As Alus are the most successful active TEs in the human genome in terms of copy number, and occupy two-thirds of the SINE elements, this prompted us to contemplate whether Alus contributed the most to the correlation between SINE density and CI frequency in intra-domains. To prove this hypothesis, we divided SINE elements into two subgroups, Alus and SINE/non-Alus, and investigated them separately. Compared with SINE/non-Alus, we found that Alu density showed very strong correlations with CI frequencies in both hESC ($r = 0.90, P < 2.2 \times 10^{-16}$) and IMR90 fibroblasts ($r = 0.94, P < 2.2 \times 10^{-16}$), in sharp contrast with PCC scores of $-0.082 (P = 0.57)$ and $-0.4 (P = 0.0044)$ for SINE/non-Alus in hESC and IMR90 fibroblasts, respectively (Fig. 2A and 2B). Likewise, we noticed that SINE/non-Alus are even not associated with CI frequencies due to the insignificant P-value ($P > 0.05$). The difference between Alus and SINE/non-Alus may be due to the reason that, except for Alus, most of SINEs have lost their mobilization activity in mammals, and such a strong correlation between Alu density and CI frequency in both investigated cell lines is consistent with our assumption that Alus are the major contributors of SINE elements in intra-domain interactions.

As mentioned previously, Alus can be grouped into three major groups (old $Alu_J$, middle $Alu_S$, and the young $Alu_Y$) that emerged at different time points during the course of primate evolution. We further ask whether a similar tendency can be observed in different Alu subfamilies. To do this, we investigated the characterizations of each Alu subfamilies by calculating their density across different frequency bins. The distribution of density of three subfamilies in two cell types
was plotted in Fig. S4. The densities of three major subfamilies are all positively correlated with CI frequencies in both hESC and IMR90 fibroblasts, suggesting that they have similar distribution tendency in intra-domain interactions.

Considering that Alus are active TEs in the human genome, we doubted whether other active TE families in the human genome shared a similar pattern as Alus. Besides Alus, there are two other TE subfamilies, L1 and SVA, which can actively transpose in humans (Chen et al. 2005). Since SVA is very sparse in the human genome with only 1750–3500 copies, we only focused on the L1 family by exploring the relationship between L1 density and CI frequency. Fig. 2C and 2D clearly demonstrated that L1 densities are not correlated with CI frequencies in hESC ($r = 0.11, P = 0.47$), and negatively correlated in IMR90 fibroblasts ($r = -0.42, P = 0.0021$). Although L1s and Alus are both active in the human genome, they show no similar distribution patterns in each of the two cell lines. Such a huge difference between them may be due to the reason that Alus have a greater tendency to be retained in the genomic loci with higher frequency of intra-domain interactions, whereas L1 elements do not or even tend to deplete in the higher frequency loci, although both of them maybe integrate into genome randomly. Our finding strongly indicated that more than 81% ($r^2$) of the variation in Alu density could be explained by CI frequency, suggesting that Alus may play an

Figure 1. Mean coverage (MC) of four TE types in each 50 frequency bin. (A) The MC of SINE in 50 frequency bins in hESC. (B) The MC of SINE in 50 frequency bins in IMR90. (C) The MC of LINE in 50 frequency bins in hESC. (D) The MC of SINE in 50 frequency bins in IMR90. (E) The MC of LTR in 50 frequency bins in hESC. (F) The MC of SINE in 50 frequency bins in IMR90. (G) The MC of DNA in 50 frequency bins in hESC. (H) The MC of SINE in 50 frequency bins in IMR90.
important functional role in intra-domain interactions. Taken together, our results strongly emphasized that Alu are over-presented in genomic loci of intra-domains with high CI frequencies. Abundant Alus in highly interacted CI regions contribute more GC content and CpG sites, and probably facilitate to maintain an open chromatin status.

The SINE elements are long known to be appeared in GC-rich regions that are associated to open chromatin (Smit 1999). Previous studies have indicated that chromatin interactions are enriched in higher GC-rich regions (Li et al. 2012), so as the SINE elements (Korenberg and Rykowski 1988). However, it is still a mystery what relationships there are among CI frequency, GC content and SINE density. To answer this question, we first divided total GC content (background) in each CI-pair into three major groups (Alu, SINE/non-Alu and non-SINE) according to their origins, and then calculated the arithmetic mean of GC content values for each CI frequency bin. We found that high CI frequencies are significantly enriched in GC-rich regions in both hESC (r = 0.92, P < 2.2 × 10⁻¹⁶, green triangle) and IMR90 (r = 0.9, P < 2.2 × 10⁻¹⁶, green triangle), which is consistent with the previous study (Li et al. 2012) (Fig. 3A and 3B). Interestingly, further investigation indicates that the GC content contributed by Alus are significantly correlated with CI frequencies.

**Figure 2.** MC of SINE/Alu, SINE/non-Alu, and LINE/L1 in each frequency bin. (A) In hESC, the MCs of Alu were positively correlated with CI frequencies (r = 0.90, P < 2.2 × 10⁻¹⁶), but the MCs of SINE/non-Alu were not correlated with CI frequencies (r = −0.082, P = 0.57). (B) Although the MCs of Alu were also positively correlated with CI frequencies in IMR90 fibroblasts (r = 0.94, P < 2.2 × 10⁻¹⁶), the MCs of SINE/non-Alu in IMR90 fibroblasts were negatively correlated with CI frequencies (r = −0.40, P = 0.0044). (C) Different patterns of the correlation of the MCs of Alu and L1 with CI frequencies were observed in hESC, with a positive correlation of Alu and no correlation of L1 (r = 0.11, P = 0.47). (D) In IMR90 fibroblasts, on the contrary of the positive correlation of the MCs of Alu, the MCs of L1 were negatively correlated with CI frequencies (r = −0.42, P = 0.002).
frequencies in hESC (\(r = 0.92, \ P < 2.2 \times 10^{-16}\), red square) and IMR90 (\(r = 0.93, \ P < 2.2 \times 10^{-16}\), red square), whereas those contributed by SINE/non-Alus are not or negatively correlated with CI frequencies in hESC (\(r = -0.19, \ P = 0.19\), blue diamond) and IMR90 (\(r = -0.32, \ P = 0.022\), blue diamond), respectively (Fig. 3A and 3B). On the other hand, GC contents derived from Alus, on average, are generally higher than those from SINE/non-Alus, suggesting that large

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**Figure 3.** The GC content, CpG content, and methylation rate of bin-pairs and SINEs. (A and B) GC content of genome background, Alu elements regions, SINE/non-Alu regions (other transposable elements in SINE type) and non-SINE regions (regions that aren’t covered by SINES) in each frequency bin (A: hESC; B: IMR90 fibroblasts). (C and D) The ratio of CpG density of Alu regions and genome non-Alu regions (Alu regions + SINE/non-Alu regions + non-SINE regions) increased as the increasing of CI frequencies. (C: hESC; D: IMR90 fibroblasts). (E and F) The fraction of normalized CpG sites in Alu regions and non-Alu regions (Alu regions + SINE/non-Alu regions + non-SINE regions) (E: hESC; F: IMR90 fibroblasts). (G and H) Methylation density (the ratio of the methylated cytosine in CpG site) of three Alu subfamilies, AluJ, AluS, and AluY (G: hESC; H: IMR90 fibroblasts).
proportion of GC contents in SINE elements are contributed by Alus. We also observed that there are strong negative correlations between GC contents contributed by non-SINEs and CI frequencies in both cell types ($r = -0.71, P = 6.7 	imes 10^{-19}$ for hESC; $r = -0.81, P = 1.5 	imes 10^{-12}$ for IMR90, Fig. 3A and 3B). These results demonstrated that the enrichment of CI frequencies in GC-rich regions is solely due to the increased copies of Alu elements, suggesting that enrichment of Alu elements in high CI frequency loci may not be just a result of higher GC contents but a cause of it. We concluded that the tendency toward overrepresentation of Alus in high CI frequency regions maybe due to important functional roles in the formation of chromatin structure and transcriptional regulation.

Higher GC content can not only stable the DNA structure, but also provide more CpG sites for methylation. In mammals, cytosine methylation is, currently, the only covalent DNA modification and restricted to CpG sites. CpG islands are genomic regions that contain a high frequency of CpG dinucleotides, and commonly represent promoters, which are usually located in GC dense regions. Recent research has demonstrated that strong CpG island promoters are mostly unmethylated, even when inactive, whereas CpG poor or weak CpG island promoters are largely hypermethylated, or are preferential targets for de novo methylation in human, respectively (Weber et al. 2007). This result indicates that CpG islands with CpG sites tend to be hypomethylated to allow an open chromatin organization and facilitate neighboring gene expression. As Alus encompass ~25% of all CpG sites in the human genome, Xie et al. investigated the epigenetic status of CpG sites of Alu elements in human ependymomas and found that methylation status of the majority of CpG sites within or adjacent to Alu elements remained unchanged, and differentially methylated CpG sites, in normal control and ependymomas, are enriched in the loci with low CpG density in the flanking regions of Alu elements, rather than within the Alu sequences themselves (Xie et al. 2010), suggesting CpG sites within Alu elements may be more resistant to alteration in methylation than those in the flanking regions. Based on this evidence, we then asked whether the increased GC contents contributed by Alus in high CI frequency loci result in elevated CpG density and further function in maintaining an open chromatin structure for highly active CIs. To tackle this question, we analyzed CpG density contributed by three above-mentioned categories (Alu, SINE/non-Alu and non-SINE) and compare them with background (see MATERIALS AND METHODS). We found that background CpG density is correlated positively with CI frequency ($r = 0.90, P < 2.2 	imes 10^{-16}$ for hESC, Fig. S5A; $r = 0.95, P < 2.2 	imes 10^{-16}$ for IMR90, Fig. S5B). Similar to GC content, strong correlations between CpG density contributed by Alus and CI frequency were observed ($r = 0.88, P < 2.2 	imes 10^{-16}$ for hESC, Fig. S5A; $r = 0.95, P < 2.2 	imes 10^{-16}$ for IMR90, Fig. S5B). In contrast, CpG density contributed by SINE/non-Alus is not correlated with CI frequency and, on average, is much lower than that from Alus, suggesting that the majority of CpG sites contributed by SINEs come from Alu elements. We then performed the correlation analysis comparing the CpG sites provided by Alu sequence and the rest sequence termed “non-Alu sequences”. The CpG sites provided by Alu sequences increase significantly faster than the non-Alu sequences as the increase of CI frequencies (Fig. 3C and 3D). We then looked into the proportion of how much the GC content becoming CpG sites; the results showed that the proportion of CpG sites in the GC nucleotide in Alu elements is stable as the increase of CI scores, but is increased in the region not covering by Alu elements (Fig. 3E and 3F). This suggests to the increase of GC content and CpG sites may be connected with the growth in Alu number, but not the changing of the Alu sequences. The following analysis of the methylation level of the CpG sites in Alu elements showed that older Alu elements have higher methylation level than younger ones (Fig. 3G and 3H).

Our result demonstrated that the increased parts of CpG sites in higher CI frequency loci are mainly contributed by Alus, which is consistent with our assumption. In summary, our analysis strongly suggested that, in human, GC content that is enriched in higher CI frequency loci are mainly due to the tendency towards overrepresentation of Alu elements and further significantly increased CpG density in these regions. Such elevated CpG density may protect the regions from methylation and allows an open chromatin structure to increase binding probability of ubiquitous transcription factors (Deaton and Bird 2011).

Alu-derived Enhancers and Promoters are significantly enriched in higher CI frequency regions

According to our results, Alu coverage showed strong positive correlation with CI frequency (Fig. 2), highlighting that the enrichment of Alus in high CI frequency regions will leads to elevated GC contents and CpG sites that might function in maintaining an open chromatin structure to increase the binding probability of transcriptional factors (Fig. 3A–D). However, such enrichment will also evolve in distal DNA elements regulation directly, like deriving enhancers and promoters that forming distal interactions. Previous studies indeed supported this hypothesis that enhancers are enriched in TE-derived genomic sequences. For example, an Alu-containing enhancer regulates the CD8a gene in human T cells (Hambor et al. 1993). Likewise, promoter regions are also hotspots of TE that provided alternative promoters or binding sites for transcription factor (TF). For instance, the LTR-derived promoter significantly increases the expression of human EBR gene in placenta (Medstrand et al. 2001), and Alu elements provide binding motifs for three zinc-finger TFs (Sp1, estrogen receptor alpha, and YY1) (Oei et al. 2004). Because chromatin interaction is usually used to exam enhancer-promoter interactions, we determined to investigate the relationship between enhancer-promoter interactions and CI frequency using histone modification marks. Since active
enhancers are marked by mono-methylation of H3 lysine 4 (H3K4me1) together with acetylation of H3 lysine 27 (H3K27ac), and active promoters are associated with trimethylation of H3 lysine 4 (H3K4me3), we analyzed the data downloaded from the UCSD Human Reference Epigenome Mapping Project (see MATERIALS AND METHODS). We first computed the expression levels of H3K4me3-marked genes, and found that these genes indeed expressed higher than non-active ones (Fig. S6), which is consistent with the previous evidence (Santos-Rosa et al. 2002; Santos-Rosa et al. 2003; Schneider et al. 2004).

After mapping active enhancers and TSSs to the chromatin interaction bin-pairs, we found that the number of enhancers and TSSs are positively correlated with CI frequencies in both hESC and IMR90 cell lines, indicating that the enhancers and promoters are enriched in the interacted genomic loci with high CI frequencies (Fig. 4A and 4B). Since Alu coverage increased with growing CI frequency, we asked whether Alu elements contribute a lot to the increasing trends of enhancers and promoters during the increase of CI frequencies. To answer this question, we divided the genome into 40 kb bins and assigned each bin to 5 groups based on the Alu coverage of the sequence. We then compared the distribution of regulatory elements in the bins of the 5 groups. By correlation analysis of the coverage of Alu elements and the number of enhancers and TSSs, we found that both the enhancers and TSSs showed striking positive correlation with Alu coverage (Enhancer: hESC: $r = 0.997, P = 2.3 \times 10^{-5}$; IMR90: $r = 0.934, P = 2 \times 10^{-2}$; Promoter: hESC: $r = 0.995, P = 3.8 \times 10^{-5}$; IMR90: $r = 0.996, P = 3.2 \times 10^{-4}$, Fig. 4C and 4D), suggesting the Alu coverage can partially indicate the enrichment of active enhancers and TSSs. This result was confirmed by further examination by using two other independent data sources, the experimental reviewed enhancers in VISTA enhancer browser, and predicted enhancers of K562 and HeLa cell lines predicted by Heintzman ND et al. (Heintzman et al. 2009). We grouped the enhancers based on whether they are covered by Alu elements, and analyzed the 40 kb sequence centered on the enhancers. The results demonstrated that the regions around Alu-derived enhancers have significantly high Alu coverage compared with the randomly selected regions in both two cell lines and VISTA dataset ($P < 0.05$). The result was verified in flanking regions of the enhancers of different length (Fig. S8). The enrichment of active enhancers and TSSs in regions with more distal chromatin interaction and higher Alu coverage showed that Alu elements may be able to predicted distal enhancer-promoter interaction as a complement of Hi-C data, and may have some biological function in distal regulation of gene transcription.

**DISCUSSION**

**Alu elements are highly evolved in genome-wide chromatin interaction**

Alu elements, the active SINE in human genome, are highly enriched in regions with high level of chromatin contact and can be an additional criterion to conduct Hi-C data quality control. Transposable elements have been regarded as noncoding sequences with potential regulatory functions in genomes instead of the orthodoxy “junk DNA” for quite a time. Previous studies of mammalian genomes revealed important functions of TEs, including promoting the evolution of mammalian pregnancy pathway (Lynch et al. 2011; Xie et al. 2010) and tissue-specific transcriptions (Bourque et al. 2008; Faulkner et al. 2009; Lowe et al. 2007; Jin et al. 2012), rewiring the regulatory network in embryo development (Kunarso et al. 2010; Gifford et al. 2013), the microRNA mediated post-transcriptional regulation (Ahn et al. 2013; Berezikov 2011), and the arrangement of CTCF (CCCTC-binding factor) binding sites (Schmidt et al. 2012). The newly studied distal regulatory roles of SINE, the wide-spread retrotransposons in the human genome, are enlighted by chromatin construction capture methods, but only find SINE enriched in regions that separate interaction-rich domains (Lieberman-Aiden et al. 2009; Wang et al. 2011; Eskeland et al. 2010; Kagey et al. 2010). By testing the correlation between different TE types and chromatin interaction levels, we found only SINE but not other TEs enriched in region with high CI scores. Besides, further analysis ruled out non-Alu elements in SINE, elucidating that Alu element tend to enriched in regions with high CI score for the first time, which suggestion that the Alu elements may be significant for distal regulations. High level of chromatin interactions indicates more adjustable transcriptional regulatory events like enhancer-promoter interaction, and has strong nexus with tissue-specific expression (Dixon et al. 2012). There are more than 1 million Alu copies in human genome. Besides causing diseases in most time they transpose (Batzer and Deininger 2002; Ule 2013; Winkler et al. 2013; Gallus et al. 2010), the fixed ones often went through selection, and some of them have obtained various important functional roles including inducing enhancers and transcriptional factor binding sites in promoters (Huda et al. 2011; Pastor and Pagani 2011; Antonaki et al. 2011; Cui et al. 2011), deriving alternative spliced exons (Sorek et al. 2002; Shen et al. 2011), and forming alternative promoters, which lead to tissue-specific expression (Faulkner et al. 2009; Lin et al. 2008). Although L1 of LINE, the other active TE in human genome, also can provide source for expression change though mechanisms like generating pseudogenes (Esnault et al. 2000), exonization (Kaer et al. 2011), our results showed that it is Alu elements that play the role in distal controlling of genome but not the other TEs, even the other activate ones.

We further suggest Alu elements be a new complementary parameter to CI scores on the basis of the significantly positive correlation between the density of Alu elements and the score of chromatin interaction (Fig. 2). The Hi-C approach for knowing the genome-wide chromatin contact is undoubtedly remarkable, which makes the search for good ways to eliminate the bias and screen out high-quality pairs important. More than one model was raised to ensure the
Figure 4. Mean numbers of enhancers and promoters demonstrated correlation with CI frequencies. (A and B) The mean numbers of active enhancer regions and active TSS regions were both positively correlated with CI frequencies in two cell lines (A: hESC; B: IMR90 fibroblasts). (C and D) The mean numbers of active enhancer regions and active TSS regions were both positively correlated with Alu coverage of the bin-pairs they belonged to in two cell lines (C: hESC; D: IMR90 fibroblasts). (E) The Alu coverage in the 40 kb regions centered on the predicted enhancer peaks of K562 cell line, HeLa cell line, and in VISTA enhancer browser. The Alu-overlapped enhancers in the two cell lines and VISTA enhancer browser were all significantly different from the background, which is the mean Alu coverage of a random 40 kb region in human genome (Binomial test, \( P < 0.01 \)), and the non-Alu-overlapped enhancers in HeLa cell line were also significantly different from the random distribution (Binomial test, \( P < 0.05 \)).
The high GC content in chromatin interaction hotspot is provided by Alu elements

Alu elements are GC-rich sequences, of which the presence will raise the GC content of certain sequences, provide more CpG sites for methylation, resulting in the remaining of open chromatin status and higher regulatory flexibility. The sequencing step in the Hi-C method for the data we used suffered from the interference of nucleotide composition, also regarded as GC content, which needed to be converted and normalized to limit the effects (Dixon et al. 2012; Yaffe and Tanay 2011). However, although the Hi-C data we investigated have been normalized to rule out GC effect, it still showed a significant correlation with CI scores (Fig. 3). This phenomenon should be caused by functional DNA elements enrichment, which is consistent with other studies. Investigation of the contribution Alu elements made to GC content demonstrated that the correlation of Alu-provided GC is also positively correlated with CI score, and the pattern is share consistency with the genome background, while the regions not covered by Alu elements contribute less GC content to the regions with higher CI scores. The increasing total GC content consisting of increased share of Alu regions and decreased share of non-Alu regions illustrate clearly that the presence of Alu elements raise the GC content in the regions with more chromatin contacts.

The arguments for the intriguing and complicated question why the GC content of Alu-surrounded DNA in human genomes is high have lasted for more than a decade. Despite the Alu elements making use of the insertion pathway of LINEs, which have a preference for AT-rich integration sites and lead to the accumulation of LINEs in AT-rich region, the human genome project strongly supports that the Alu elements tend to be distributed in GC-rich regions (Lander et al. 2001; Jurka 1997). Additionally, further analysis showed that the distribution of “young” Alu elements were still in accordance with the pattern of LINE, while the “old” Alu elements tended to accumulated in GC-rich regions, indicating the functional role Alu may play (Lander et al. 2001). Other studies soon followed up trying to determine the reason why the Alu distribution is relative to the GC content (Kunarso et al. 2010; Brookfield 2001), but none of them denied the phenomenon that most Alu elements, especially the old ones, have a surrounded area with high GC content. As expected, our results also proved the old finding that there are more Alu elements displayed in regions with higher GC content. However, it seemed that it is the GC-rich Alu elements themselves raise the GC content of the whole region, but not the sequences not belonging to Alus. That is to say, the regions with high CI frequencies own more Alus that have more guanine and cytosine, and the whole GC content of these regions are higher than the regions with less chromatin contact as a result (Fig. 5). To summarise, the finding that the Alu contributes more GCs in regions with higher CI scores combined and answered the two long controversial questions together, pointed out that the Alu elements are the reason why active interacted region have a higher GC content, and it is the Alu elements gathered together that raise the GC content of the region, not only the surrounding DNA but also itself.

The amount of GC can affect the chromatin structure directly, while a large part that forms its biological function is that DNA can be methylated at CpG sites, which will accumulate when there is more GC as our results show (Fig. S5). By comparing the genome-wide CpG density in different part of the human genome, we found that the although the CpG density of each region showed positive correlation with CI score regardless of whether the sequences are covered by Alu elements, the higher the CI score is, the more CpG comes from Alu elements than non-Alu regions (Fig. 3B), which indicates that Alu elements not merely generate GC
content rise in regions with high CI score, they also provide more CpG sites that can be methylated. When we looked into the proportion of how much the GC content becomes CpG sites, the results showed that the increasing GC content and CpG sites are connected with the growth in Alu number, but not the changing of the Alu sequences (Fig. 3C). The results shown above highlight again that the more Alu elements in regions with more chromatin contact can provide additional GC content as well as CpG sites.

The CpG sites in vertebrates are known for typical DNA methylation. The methylation process at CpG sites is by adding a methyl group to cytosine and turning it to 5-methylcytosine, which may change the structure of chromatin and result in various biological consequences. For example, the methylation surrounding TSS will block the initiation of transcription, while the methylation in the gene body will not block the transcription but even sometimes stimulate it (Suzuki and Bird 2008). The TEs are often highly methylated since most transposable events are deleterious, and the host genome must try to suppress the activation of TEs (Yoder et al. 1997; Bestor 1998). In our results, the Alus showed a very high level of methylation in the whole genome with more than 90% of the CpG sites in Alu regions methylated regardless the cell types (Fig. S5C and S5D). This is consistent with the previous studies, and elucidated clearly that the transposition of Alu is not welcomed by genome and should be under control. The methylation rate showed a slightly negative correlation in hESC cell line, which maybe result from the several abnormal values at the high-CI regions, and it is not correlated with CI correlation in IMR90 fibroblasts. The high methylation in Alu elements also raises the methylation level of promoters and gene bodies when Alu is inserted into genes (Fig. S7C–F),
which is quite reasonable because the transposition of Alu elements in actively transcribing genes should be strictly restricted. Nevertheless, the promoter region without Alu elements possessed more percentage of methylated CpG sites in higher-CI regions. Obviously, the promoters in regions with more chromatin contacts are actively regulated by DNA elements, so they have loose chromatin structures with less methylation. But the significant decreasing pattern of methylation did not happen in the gene body, which only had a weak decreasing trend as the CI score increased. As mentioned above, the methylation in CpG sites will not affect the transcription in the gene body, sometimes even promote it, which make our results rational no matter where the gene is actively regulated of trancribing, the methylation density in the gene body will not change much. Moreover, the methylation density in the gene body is always higher than the promoter as our results demonstrate (Fig. S7A and S7B), which confirmed again that the CpG islands are barely methylated when located around transcription start sites (TSSs), but will be methylated when in gene bodies in a tissue-specific manner.

Different Alu subfamilies follow the chronological methylation

Alu elements were noticed to have the GC-rich region preference after the human genome was sequenced, with the “old” ones tending to accumulate in GC-rich regions and “young” ones AT-rich regions. (Lander et al. 2001). Here we tested the methylation rate of the CpG sites in Alu elements with different ages, and found that besides the difference in preference for GC content, the “old” Alu elements were more highly methylated than the “young” ones (Fig. 3D). Those young Alu elements are generally more transcriptionally active than old ones results from various reasons. One of the reasons is the accumulation of mutations in Alu elements disables the recognition of transposase; others include methylation and RNA interference by the host genome, which are more important. The mutations will accumulate as time passes, so the older ones must contain more mutated sites; however the methylation status will not always become increasingly intensive. The host genome cannot recognize new Alu repeats immediately after insertion, and forbid its future transposition with methylation, so the young AluY may have less methylated CpG sites and have less mutations in key regions related to transposition as our results indicate. As the Alu elements stay in the genome for more time, the host genome will discover this sequence and methylate it more often, and the sequence will change at a certain rate itself. Under the pressure of evolution, the methylation of CpG will eventually reach saturation or just enough to suppress the move and remain stable, and the mutation will not stop. When the sequence is converted so much that it could not be identified by transposase and will be silent forever, the deleterious nature of the Alu is fully removed, and the host genome may release it from its high methylation status as time goes by. According to our investigation of old AluJ, median AluS and young AluY subfamilies, the AluJ have a slightly higher methylation density than AluS, and both of them are almost fully methylated with more than 90% CpG sites methylated, while only less than 80% CpG sites in AluY were methylated. The average age of AluJ and AluS is 81 million years and 36 million years (Kapitonov and Jurka 1996), while AluY sequences are very young and still fully active in human genome. If the methylation rate of the host genome is stable, we can estimate that the methylation rate of CpG sites in Alu elements is about 0.004% per million-year using the methylation density in our result, which means in another 10 million years, all the AluY will be fully methylated and silent as the presents AluJ. However, the new insertions will still be active and may form another subfamily that can still affect the host genome in various ways.

CONCLUSIONS

The Alu elements are highly enriched in the genome region with high levels of chromatin interaction. Correlation analysis reveals that the sequence character of Alu elements and its enrichment have endowed the actively regulating regions with a higher GC content, together with more CpG sites, and also a higher methylation density. The increase in methylation potentialtality will provide flexibility to the sequences and transcriptional regulation as shown in Fig. 5. That is why the high CI frequencies always relate to tissue-specific expression. In contrast, other TEs including the active L1 in human genome are not enriched in high-CI regions and show a random distribution when evaluated by CI frequency.

MATERIALS AND METHODS

Processing chromatin interaction data

The recently released genome-wide chromatin interaction profiling of two cell lines in human, includes human embryonic stem (ES) cells and human IMR90 fibroblasts corresponding to pluripotent and differentiated cells. In these two cell lines, the datasets with the bin size of 40 kilobases (kb) were adopted, since chromatin interaction (CI) frequencies were normalized for biases in the data. The coordinates of domain-boundary annotations were converted from Hg18 to Hg19 using the UCSC Genome Browser liftover utility (http://genome.ucsc.edu/cgi-bin/hgLiftOver). We discarded the domains and bins, of which coordinates failed to remap, which is about 6.88% of the bins of Hg18. We then calculated the mean inter-domain CI frequencies in each cell line and used these values as the lower threshold for each cell line (3.34 for hESC and 3.06 for IMR90) and discarded all the bin-pairs whose CI frequencies are lower than the threshold in the corresponding cell lines (assuming they are non-informative or background noises in hESC and IMR90 cell lines). We then calculated the number of bin-pairs to be filtered out by setting upstream thresholds increased from the downstream threshold by 1 as the step each time, and finally removed the bin-pairs whose CI frequencies are larger than 153 in hESC and 133 in IMR90, respectively (assuming they are outlier in each cell line). Finally, we were
able to retain 897,867 bin-pairs for hESC and 850,305 bin-pairs for IMR90.

Compiling TEs

The coordinates of TEs in the human genome were downloaded from RepeatMasker (http://www.repeatmasker.org, release version 3.3.0, Repbase library version 20110920) (Hubley and Green 1996). Based on the classification of RepeatMasker program, we divided all the TEs to four major types (LINE, SINE, LTR, and DNA) and discarded the TEs with uncertain categories. The coverage of certain TE type or TE family was defined as the number of nucleotide in the chromatin interaction bin-pairs by using the length and annotation of TEs. For the Alu subfamilies, AluJ, AluS, and AluY, we counted the number of the TE copies of certain Alu subfamilies in chromatin interaction bin-pairs regarded as the enrichment score. The reason why we used coverage for four TE types, but used enrichment score for Alu subfamilies is that the average lengths of the four TE types are different, while those of the Alu subfamilies are nearly the same. The coverage of TE copies and the enrichment score of Alu subfamilies in bin-pairs with different CI frequencies were then used in the Pearson correlation coefficient analysis with other parameters.

Determination of the methylation density

The coordinates of methylated CpG sites of both hESC cell line and IMR90 fibroblasts were based on the results of recent work (http://neomorph.salk.edu/human_methylome) (Lister et al. 2009) and were converted from Hg18 to Hg19 by using UCSC Genome Browser lift-over tool (http://genome.ucsc.edu/cgi-bin/hgliftOver). To evaluate the methylation level, we calculated the percentage of methylated sites of all the CpG sites in each bin-pair, termed methylation density. Similarly, the CpG density was defined as the percentage of CpG sites in all the C/G nucleotide in each bin-pair, while the GC content is the percentage of C/G nucleotide in all the nucleotide in each bin-pair.

Identification of active genes and cis-elements

The active genes were identified in both hESC cell lines and IMR90 fibroblasts by combining the gene annotation and histone markers. We used annotated mRNAs in RefSeq genes and UCSC genes, and de-redundant by keeping the longest gene loci in gene loci with the same gene symbols. The ChIP-Seq data of H3K4me1, H3K27ac, and H3K4me3 histone markers were downloaded from GEO database (GSE16256), and processed by Model-Based Analysis for ChIP-Seq (MACS) to rule out the false positive peaks with the false discovery rate (FDR) value over 5% (Heintzman et al. 2009; Zhang et al. 2008; Hawkins et al. 2010). We then combined the processed transcription start sites (TSS) annotation and H3K4me3 peaks to obtain the active markers. The active enhancer regions were defined by the overlapping regions of H3K4me1 and H3K27ac markers. Active TSSs were defined by H3K4me3 peaks on TSS. If a gene locus has multiple TSS, we choose the TSS with most H3K4me3 peak as the promoter of the whole gene locus. To evaluate the difference between enhancer regions and TSS regions in different cell lines, we regarded the enhancers or promoters from two datasets a same one if the distance between two peaks is less than 250 bp. The hESC and IMR90 groups share 2126 enhancers, which are 8.2% in hESC and 7.2% in IMR90. The hESC and IMR90 groups share 5292 TSSs, which are 80.7% in hESC and 83.8% in IMR90 (Fig. S9A).

Correlation analysis between TEs and other chromatin characters

The PCC between the coverage of different TE families and the CI frequencies in each bin-pairs were calculated. The number of TE copies of Alu subfamilies and the CI frequencies were also evaluated by PCC. The number of GC content, CpG content and methylation density were investigated by PCC with CI frequencies in different regions in each bin-pairs including regions covered by Alu elements, regions covered by SINE elements but not Alu, and regions not covered by SINE. The PCC between the number of promoters and enhancers annotation by histone markers and the CI frequencies were calculated.

Processing enhancer data of K562 and HeLa cell lines, and VISTA database

The predicted enhancers of K562 and HeLa cell lines were adopted from Bing Ren’s study (Heintzman et al. 2009). We also downloaded the reviewed data in the VISTA enhancer browser (http://enhancer.lbl.gov/frmt_page_n.shtml). We then grouped all the enhancers to Alu-derived class and non-Alu-derived class depending on whether their peaks are in Alus, and then calculated the mean Alu coverage in 20 kb upstream to 20 kb downstream in the two classes. We evaluated the enhancers in three datasets by comparing the distance between their peaks. If the distance between two peaks is less than 250 bp, we regarded them as a same enhancer. The three groups had very different patterns that K562 and HeLa groups share 1200 enhancers, which are 3.3% in HeLa and 4.9% in K562. The K562 and VISTA groups share 22 enhancers, which are 0.09% in K562 and 2.7% in VISTA data. The HeLa and VISTA groups share 28 enhancers, which are 0.08% in HeLa and 3.5% in VISTA data. And only 4 enhancers are shared by all three groups, which are 0.01% in HeLa, 0.02% in K562, and 0.5% in VISTA data (Fig. S9B).

AUTHOR CONTRIBUTIONS

Y. Zhong and P. Nan conceived and initiated the project. Z. Gu and K. Jin planed and performed the experiments and statistic analysis. Z. Zhang, Y. Zhang, and M. J. C. Crabbe contributed in data analysis. Z. Gu, K. Jin, Yanyan Huang, Mengyi Hua, M. J. C. Crabbe, and Y. Zhong wrote the manuscript. All authors reviewed and contributed to the final manuscript.

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ABBREVIATIONS

3C, chromosome conformation capture; 3D, three-dimensional; Cis, chromatin interactions; CTCF, CCCTC-binding factor; FAMs, fossil Alu monomers; FDR, false discovery rate; hESCs, human embryonic stem cells; LINE, long interspersed nuclear elements; LTRs,
long terminal repeats; Myr, million years; SINE, short interspersed nuclear elements; TEs, transposable elements; TSS, transcription start site; UTR, untranslated region.

COMPLIANCE WITH ETHICS GUIDELINES

Zhuoya Gu, Ke Jin, M. James C. Crabbe, Yang Zhang, Xiaolin Liu, Yanyan Huang, Mengyi Hua, Peng Nan, Zhaolei Zhang, and Yang Zhong declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by the any of the authors.

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