



Waves of Retrotransposon Expansion Remodel Genome Organization and CTCF Binding in Multiple Mammalian Lineages

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SUMMARY

CTCF-binding locations represent regulatory sequences that are highly constrained over the course of evolution. To gain insight into how these DNA elements are conserved and spread through the genome, we defined the full spectrum of CTCF-binding sites, including a 33/34-mer motif, and identified over five thousand highly conserved, robust, and tissue-independent CTCF-binding locations by comparing ChIP-seq data from six mammals. Our data indicate that activation of retroelements has produced species-specific expansions of CTCF binding in rodents, dogs, and opossum, which often functionally serve as chromatin and transcriptional insulators. We discovered fossilized repeat elements flanking deeply conserved CTCF-binding regions, indicating that similar retrotransposon expansions occurred hundreds of millions of years ago. Repeat-driven dispersal of CTCF binding is a fundamental, ancient, and still highly active mechanism of genome evolution in mammalian lineages.

INTRODUCTION

In contrast to exons and structural RNA sequences, genomic regions bound by proteins such as transcription factors (TFs) can change rapidly in mammalian genomes. One apparent exception may be the sequences bound by CCCTC-binding factor (CTCF), a DNA-binding protein that can divide transcriptional and chromatin domains, help direct the location of cohesin, and orchestrate global enhancer-promoter looping (for reviews, see Dunn and Davie, 2003; Phillips and Corces, 2009). CTCF is an essential (Fedoriw et al., 2004; Heath et al., 2008; Splinter et al., 2006), widely expressed nuclear protein with an 11 zinc finger DNA-binding domain that is highly conserved

from fly to human (Burcin et al., 1997; Klenova et al., 1993; Moon et al., 2005). Originally identified as a transcriptional regulator for the *c-myc* oncogene (Baniahmad et al., 1990; Filippova et al., 1996; Lobanenkova et al., 1990), CTCF remains the only identified sequence-specific DNA-binding protein that helps establish vertebrate insulators (Bell et al., 1999). Additionally, CTCF has been implicated in transcriptional activation, repression, silencing, and imprinting of genes (Awad et al., 1999; Burcin et al., 1997; Filippova et al., 1996; Klenova et al., 1993; Lobanenkova et al., 1990).

Despite its importance to mammalian genome function and regulation, different preferred binding sequences for CTCF have been reported. A 15 to 20 bp core consensus sequence represented in nearly all CTCF-binding events was identified using genome-wide chromatin immunoprecipitation (ChIP) data (Kim et al., 2007). Subsequent studies have confirmed this result in different mouse, human, and chicken cells (Chen et al., 2008; Cuddapah et al., 2009; Heintzman et al., 2009; Jothi et al., 2008; Schmidt et al., 2010a). Earlier studies suggested that different combinations of zinc fingers might target sequences with lengths varying between 20 and 40 bp (Filippova et al., 1996; Ohlsson et al., 2001). Indeed, the DNase I footprint of CTCF at the *amyloid precursor protein* (*APP*) promoter is 40 bp in length (Quitschke et al., 2000). The apparent disconnect between in vivo binding specificity and the observed in vitro binding preferences has yet to be fully resolved (see Phillips and Corces, 2009).

How do CTCF-binding sequences change and emerge? The sequences bound by TFs evolve rapidly, most likely the result of genetic drift (ENCODE Project Consortium, 2007; Borneman et al., 2007; Dermitzakis and Clark, 2002; Kunarso et al., 2010; Odom et al., 2007; Schmidt et al., 2010b), whereas large, information-rich motifs, such as the one bound by CTCF, are likely to be selectively conserved. For example, CTCF's multiple roles, including division of chromatin and gene expression domains, have been reported to place strong purifying evolutionary pressure on bound regions (Kim et al., 2007; Xie et al., 2007; Bourque et al., 2008; Kunarso et al., 2010; Martin et al., 2011; Mikkelsen et al., 2010). Despite this likely selective pressure, evidence

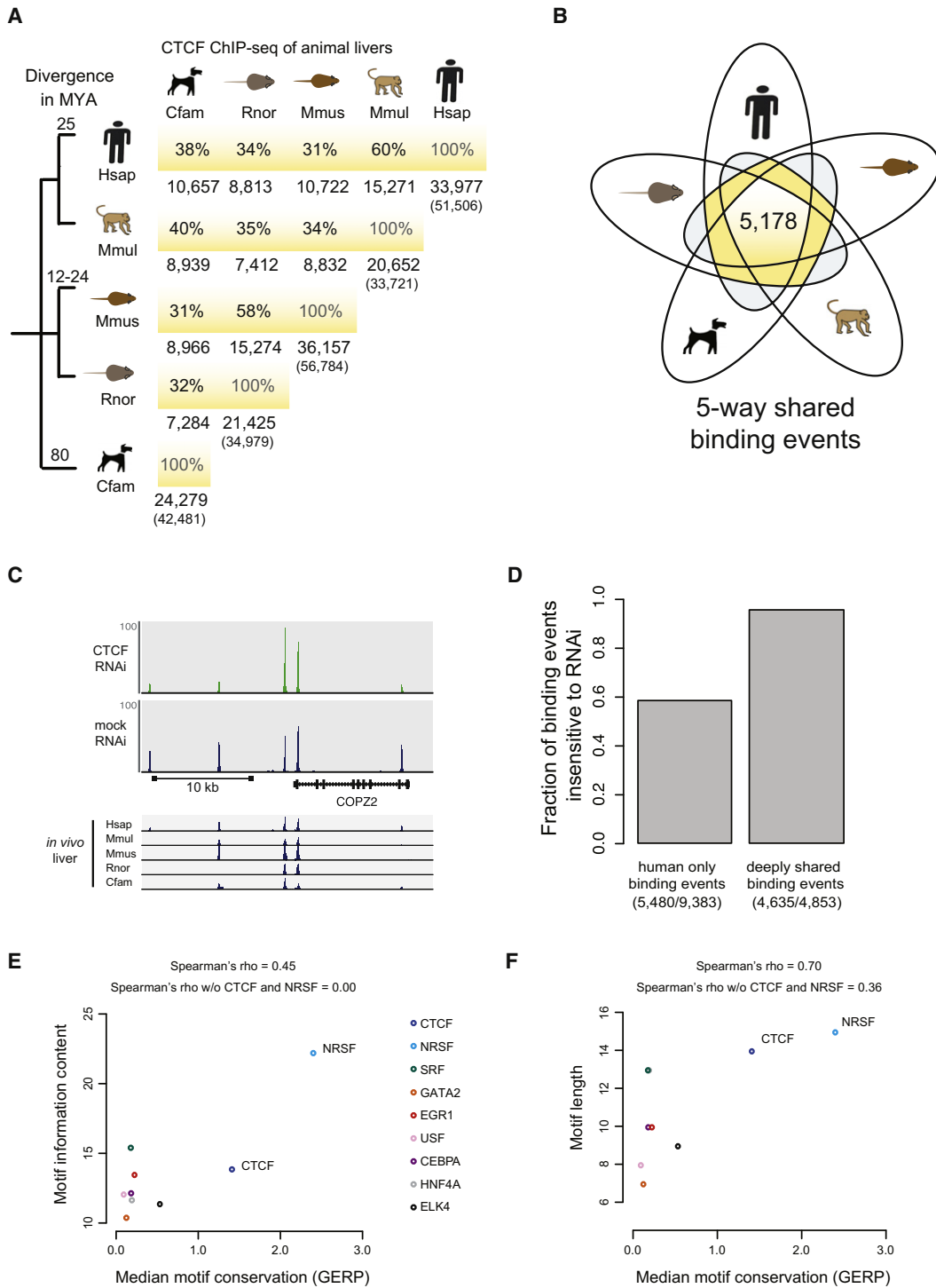


Figure 1. CTCF Occupancy in Five Placental Mammalian Genomes Reveals a Large Core Set of Conserved Binding

(A) The total numbers of CTCF-binding events found in orthologous locations between each pair of placental species are shown as row-column intersections. The right-most numbers for each species represent all alignable CTCF-binding peaks (total peaks are in parentheses). Percentages are percentage-averages between pairwise species (Experimental Procedures).

(B) Five-way comparison of CTCF binding in five placental mammals identified a shared set of 5,178 CTCF-binding events.

(C) The upper track shows CTCF binding after CTCF knockdown (CTCF) in human MCF-7 cells (Figure S1F). The track immediately below shows CTCF binding with control RNAi (mock). The bottom five tracks show CTCF-binding data in liver of five mammalian species in syntenic regions, demonstrating that highly conserved CTCF-binding events are less sensitive to perturbation by RNAi knockdown.

suggests that CTCF binding is also evolving rapidly, most notably the discovery that mouse embryonic stem cells (ESCs) have thousands of CTCF-binding events that cannot be conserved in the human genome, as they are found in rodent-specific B2 repeat elements (Bourque et al., 2008). This is consistent with early models (McClintock, 1950) and recent examples of repetitive elements driving regulatory divergence in eukaryotic genomes (Bejerano et al., 2006; Bourque et al., 2008; Britten, 1997; Han et al., 2004; Kunarso et al., 2010; Markljung et al., 2009; Mikkelsen et al., 2010; Wang et al., 2007; Lynch et al., 2011).

By analyzing the evolution of CTCF binding in six representative mammals, we found that CTCF binds a 33/34 bp motif with a two-part profile that is conserved across mammals, providing an explanation for the observed CTCF target sequence discrepancies. Remarkably, the bound sequences exhibit a hierarchy conserved across mammals, wherein frequently used motif instances underlie CTCF-binding events that are both most conserved and most resilient to changes in nuclear concentration of CTCF after RNAi knockdown. Moreover, in most species examined, we found that CTCF-binding events are associated with repeat element expansions, revealing the mechanism by which they are born. Functional studies illustrate that both newborn and conserved CTCF-binding events act as chromatin and gene expression barriers with similar frequency. Together, our results support a repeat-driven mechanism for functional CTCF-binding expansion, which is currently active in multiple mammals and was active in our common ancestor, thus creating the CTCF-binding events shared across mammals.

RESULTS

CTCF-Binding Events Are Markedly More Conserved among Mammals than Tissue-Specific TF Binding in Mammalian Genomes

We used ChIP followed by sequencing (Table S1 available online) to determine CTCF binding in livers isolated from five eutherian mammals (human, macaque, mouse, rat, and dog) and the metatherian gray short-tailed opossum and confirmed that CTCF binding is mainly directed by genetic sequence rather than nuclear environment (Wilson et al., 2008) (Figures S1A and S1B).

We first compared CTCF-binding conservation with matched genome-wide data available for the TFs HNF4A and CEBPA in mouse, dog, and human (Schmidt et al., 2010b). Consistent with prior reports (Kunarso et al., 2010), we observed substantially higher conservation among CTCF-binding events than among liver-specific TFs, even near direct liver-specific target genes. For example, HNF4A and CEBPA binding has extensively diverged around the CEBPA target gene *APOA2* (Schmidt et al., 2010b), yet the CTCF-binding events in the same region are uniformly conserved in all three mammals (Figure S1C). Globally, CTCF binding is shared five times as often among human, dog,

and mouse, as are CEBPA and HNF4A; conversely, CTCF has proportionally less lineage-specific binding (Figure S1D).

The inclusion of rat and macaque allowed us to compare closely related species, which overlapped by up to 60% in shared CTCF binding. In fact, as might be expected, CTCF-binding divergence generally corresponded with evolutionary distance (Figure 1A).

More importantly, we observed a core set of over 5,000 CTCF-binding events shared by all five eutherian mammals (Figure 1B) and found across numerous human tissues (Figure S1E). Conserved CTCF-binding events are less sensitive than species-specific binding events to reduced levels of the CTCF protein. We analyzed CTCF binding before and after RNAi knockdown in human MCF-7 cells (Schmidt et al., 2010a) (Figures 1C, S1F, and Extended Experimental Procedures) and found that virtually all binding events conserved across five species were resistant to knockdown, compared to only 60% of human-specific binding events (Figure 1D). Thus, conserved binding events are highly stable protein-DNA interactions, suggesting that they play functional roles in many cell types.

Although higher conservation among CTCF-binding events, relative to tissue-specific TFs, could be due solely to the information content or length of the bound motif, we found that conservation of CTCF-binding events across mammalian genomes is not purely the result of a longer target motif. We observed an increase in shared binding events between human, mouse, and dog with motif lengths from CEBPA (4%), to HNF4A (5%), to CTCF (24%), but we did not see a significant dependence when looking at a collection of sequence-specific factors. The median sequence conservation of a cross-section of motifs (ENCODE Project Consortium, 2011) revealed some degree of correlation with the motifs' length and information content; however this was not statistically significant and was largely due to the inclusion of CTCF and NRSF/REST (see Extended Experimental Procedures and Figures 1E and 1F). After excluding CTCF and NRSF, the other TFs showed very little to no correlation. Together, these data show that over 5,000 CTCF-binding events are highly biochemically and evolutionarily stable across mammalian species.

CTCF Binds a DNA Motif Containing a Two-Part Profile

Our genome-wide data for CTCF binding in livers of five eutherian species allowed us to identify *de novo* DNA sequences associated with CTCF binding at hundreds of thousands of locations. In addition to the known 20 bp motif, we further discovered a second 9 bp motif present at high frequency and with consistent spacing in each species. Both halves of the motif are unchanged across 180 million years of evolution, consistent with the high conservation of CTCF's DNA-binding domain (Figure S2), and create together a two-part 33/34 bp binding motif, which occurs in a quarter to a third of CTCF-binding events (Figures 2A and 2B). The second motif is downstream by either 21 or 22 bp from the center of the previously identified motif in

(D) The fraction of binding events found only in human (human only) or shared among all placental (five-way) were characterized by their sensitivity to RNAi knockdown of CTCF protein. Very few deeply shared CTCF-binding events were affected by CTCF knockdown.

(E) Relation between motif information content and motif sequence conservation for nine TFs in human. (F) Relation between motif length and motif sequence conservation for the same TFs as in (E).

See also Figure S1 and Table S2.

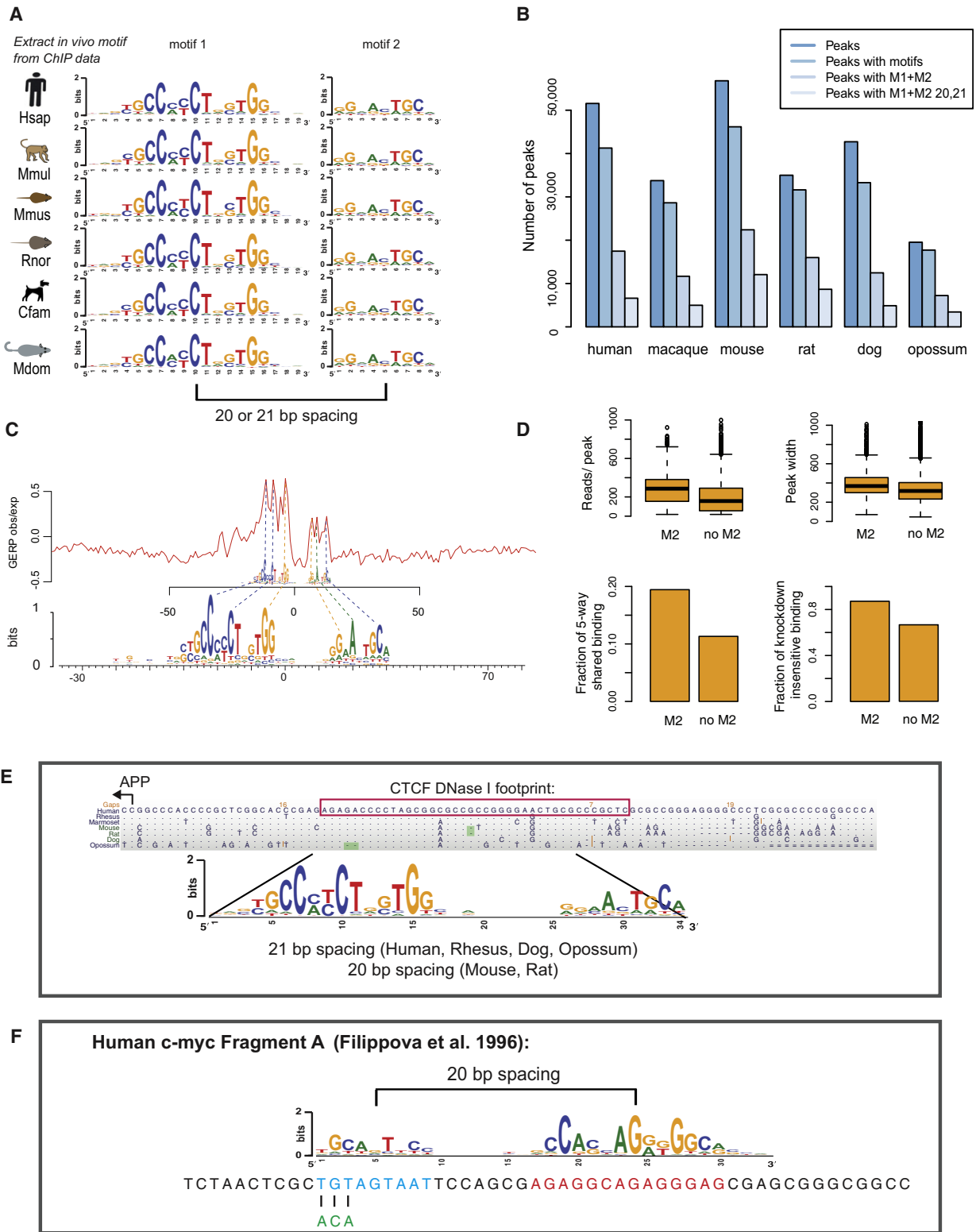


Figure 2. CTCF Binding Often Occurs at a Highly Conserved Motif, Consisting of a Two-Part Profile

(A) Motifs (M1 and M2) identified de novo from CTCF-binding events.

(B) Binding event counts and number of binding events with at least one motif (M1 and M1+M2) in all six species. M1+M2 20,21 represents the preferred spacing patterns of these two submotifs.

approximately equal proportions in all studied species, except mouse and rat (Figure 4). Henceforth, we will refer to the canonical 20 base motif as M1 and to the 9 base motif as M2. The M2 motif has previously been found in CTCF DNase footprints, but the role of this motif is unknown (Boyle et al., 2011). The variable presence of the shorter and less information-rich M2 agrees with earlier suggestions that CTCF may have multiple binding modalities (Burcin et al., 1997; Filippova et al., 1996).

To characterize the importance of M2 for CTCF binding, we first identified binding events conserved in five placental mammals that contain both M1 and M2. Then we searched for evidence of positional sequence conservation of the M2 submotif. Plotting the frequency of all unchanged bases in the multiple species alignment revealed that the bases associated with both M1 and M2 were much less likely to see sequence changes compared to both the spacer and surrounding sequences where background levels are observed (Figures 2 and S2). We used genomic evolutionary rate profiling (GERP), a specific model of evolutionary constraint at the sequence level, to confirm this observation of purifying selection on both the previously known and the newfound motif bases (Cooper et al., 2005) (Figure 2C).

We found that binding events containing the full 33/34 bp motif show stronger ChIP enrichment, are more conserved, and remain less sensitive to CTCF knockdown compared to binding events containing only the M1 motif (Figure 2D). Moreover, the CTCF-binding peak is offset from the center of the M1 motif, consistent with CTCF binding to a larger, directional 33/34 bp motif. In cases where the M2 motif is present, this effect is slightly stronger (Figure S2C). These results indicate that CTCF directly binds M1+M2 in a highly conserved manner (Figure S2D).

Hierarchical Motif-Word Usage of CTCF Is Conserved among Mammals

The position weight matrix of CTCF's binding motif is composed of thousands of specific sequences, or motif-words. We tested whether CTCF has a preferred set of motif-words by analyzing their frequency of occurrence. We clustered highly similar motif-words using the 14 most informative bases of the M1 motif, which together capture over 95% of the motif's information content. A set of 33,994 different 14-mer motif-words (out of a possible 69,865) are used by CTCF at least once in the five placental mammals. We found that a small subset of these tens of thousands of motif-words are disproportionately often bound by CTCF within and between different species (Figure 3). For example, the top 200 bound motif-words are responsible for 4,006 binding events in the human genome; in fact, just 2,492

words (3.6% of the possible words) account for over half of the binding events in the human genome. CTCF motif-word usage is strikingly conserved between the species (Spearman rank correlation > 0.76) and recapitulates both the evolutionary distances of the species as well as key characteristics of the CTCF-binding events (Figure 3). In particular, we observed that the frequency of a word's usage positively correlates with both the likelihood of a binding event being shared among all five species and the strength of the ChIP enrichment (Figure 3). A similar analysis for a typical tissue-specific TF (HNF4A) showed considerably lower correlation of motif-word usage (Figure S3A) and no correlation between word frequency and either conservation or ChIP enrichment (Figure S3B). Collectively, these results reveal a functional hierarchy of CTCF-bound motif-words maintained during evolution.

Lineage-Specific Repeats Drive Divergence of CTCF Binding in Many Mammalian Lineages

The existence of a motif-word usage hierarchy as well as thousands of highly conserved CTCF-binding events is inconsistent with prior models proposing rapid TF birth by neutral mutation and natural selection (MacArthur and Brookfield, 2004).

We therefore searched for an alternative mechanism for the de novo creation in a common mammalian ancestor of the thousands of CTCF-binding events now found throughout mammals. Despite the generally high conservation of CTCF motif-word usage, we noted that specific sets of motif-words were overrepresented in rodents (mouse and rat), dog, and opossum (Figure 4A). We found that the vast majority of these overrepresented motif-words are embedded within SINE transposons (Figures 4B and S4).

In mouse, this observation is consistent with previous reports showing that the CTCF motif was carried to over 10,000 locations in the mouse genome by the B2 SINE family (Bourque et al., 2008), which has expanded significantly in rodents (Kass et al., 1997). We further discovered that CTCF binding has been spread to thousands of locations in the rat genome, also via muridae-specific B2 SINEs (Figure 4B). About 2,000 binding events found within B2 elements are shared by mouse and rat, whereas approximately 5,300 B2-associated binding events are found uniquely within mouse and over 1,200 solely in rat (Figures 4C and S4). Thus, the B2 expansion was active before the speciation of rats and mice and remained active in both lineages after speciation. The thousands of rodent-expanded B2-associated CTCF-binding events, most of which contain a full 33 bp CTCF motif with a 20 bp spacing between M1 and M2, have profoundly influenced the occurrence of specific bound CTCF motifs (Figure 4D). However, the B2-associated

(C) The DNA sequence constraint around the CTCF motif in human was plotted by observed/expected genomic evolutionary rate profiling (red, GERP scores (Cooper et al., 2005)). The frequencies of unchanged bases in five-way shared CTCF-binding events are shown as position weight matrix (PWM) below the GERP profile.

(D) Peaks containing the M2 motif in preferred spacing are stronger in ChIP enrichment both by read count and peak width, are more highly shared among mammals, and are resistant to RNAi-mediated knockdown.

(E) A multiple mammalian sequence alignment of a CTCF peak at the APP gene is shown. The DNase I footprint (red box, Quitschke et al., 2000) encompasses a complete 34 bp M1 and M2 CTCF motif.

(F) DNA sequence of the human *c-myc* promoter (Human *c-myc* Fragment A) bound by CTCF in vivo and in vitro (Filippova et al., 1996). The sequence contains the canonical M1 CTCF motif (red) and the M2 motif (blue). A 3 bp mutation in the M2 motif that eliminates CTCF binding in vitro is indicated in green.

See also Figure S2.

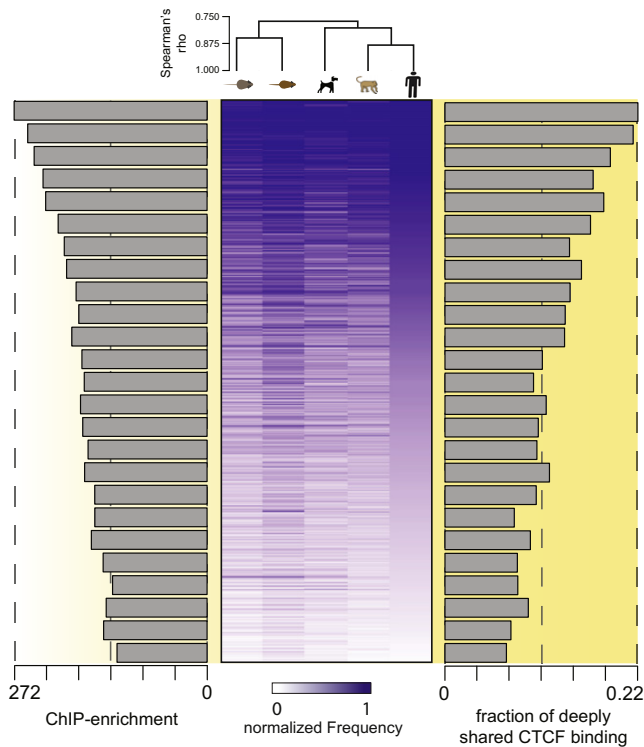


Figure 3. CTCF Motif Usage Shows a Conserved Hierarchy among Placental Mammals

Heat map of the 2,492 CTCF motif-words found at least five times in any species anchored to human; words are normalized by their background occurrences within each genome. This set of words is found in 27,543 human-binding events. The data are sorted in the human column by decreasing frequency, and spearman rank correlations after one-dimensional hierarchical clustering of the rows are shown. The average ChIP-enrichment of the motif-words separated into bins containing 100 words is shown as a bar chart (left). Similarly, the fraction of five-way conserved CTCF-binding events within the same bins are shown as a bar chart (right). See also Figure S3.

CTCF-binding events seem not to be enriched near mouse- or rodent-specific genes compared to other binding events (Figure S4).

Similarly, we found that the SINEC-Cf member of the canoidea-specific SINE repeat family LYS has carried CTCF-binding events through the dog genome (Figure 4). In contrast to rodents, the dog-specific expansion appears more limited, resulting in well under a thousand binding events and a sequence that is centered solely on the M1 motif (Figure 4E).

Similar to rodents and dogs, word-level analysis of the CTCF-binding events revealed a set of motif-words overrepresented in opossum, frequently associated with opossum-specific SINE repeats MAR_Mdo (MIR family) (Figures 4A and 4B). Opossum is the closest out-group to the eutherian mammals, and its genome is rich in transposable elements (Mikkelsen et al., 2007). The expansion of CTCF-binding events numbers in the hundreds, and the CTCF-bound MIR elements in opossum contain only M1 motifs, with no evidence of associated M2 motifs (Figure 4D).

Perhaps surprisingly, we found no evidence of enrichment of CTCF-binding events within species-specific repeats in human

or macaque, nor did we discover recent activity of retrotransposon expansion of CTCF within these two species.

Nevertheless, CTCF binding has expanded via retrotransposition in multiple, independent, diverse mammalian lineages; therefore, this mechanism of regulatory evolution is a profoundly ancient strategy that must predate the mammalian radiation.

Molecular Paleontology of Fossilized, Repeat-Driven CTCF Expansions

If the repeat-driven mechanisms currently active in creating functional CTCF-binding events were also active in the common mammalian ancestor, then ancestral expansions would eventually become shared binding events in descendant species, such as our study species. Such a model would explain both the origin of shared CTCF-binding events as well as lineage-specific expansions via the same mechanism.

This hypothesis predicts that fossilized repeat sequences from ancient repeat expansions will be found around loci bound by CTCF in multiple mammals. However, tens of millions of years of evolution would likely have altered the genetic sequences surrounding the bound CTCF motif and so eliminated systematic evidence of associated repeat elements that could be obtained using a purely computational approach.

Taking a more targeted approach that exploited our six species' *in vivo* experimental data, we looked for evidence in any genome of repeat element survival within the set of partially—or fully—shared CTCF-binding events. We found just over 100 CTCF-binding events (Table S3), often very deeply conserved, which had fossilized repeat sequences surrounding them in one or more of the mammals we profiled (Figure 5).

In Figure 5, we show two representative examples of candidate CTCF-binding events carried by ancestral repeats. First, on HsChr13, we identified a partially, though deeply, shared CTCF-binding event located within an ancient amniote SINE element (Figure 5) (Hirakawa et al., 2009). Interestingly, this specific binding event was lost along the rodent lineage due to a motif disruption in the common rat-mouse ancestor. Second, on HsChr4, a highly conserved CTCF-binding event is found associated with a copy of mammalian repeat MamRep564, which is shared among all placentals but appears to have arisen subsequent to the placental-marsupial split.

These examples, along with the larger set of partially preserved repetitive elements associated with shared CTCF binding (Table S3), lend support to a model wherein repeat-carriage of CTCF binding created highly conserved CTCF-binding events throughout mammalian and most likely vertebrate evolution.

Newly Created, Repeat-Driven CTCF Expansion Events Demarcate Chromatin and Gene Expression Domains

To assess the functional impact of SINE-driven CTCF-binding events on chromatin, we explored CTCF's known role as a barrier element that divides chromatin domains (Cuddapah et al., 2009; Xie et al., 2007). We reasoned that genomic locations where CTCF plays a functional role in separating chromatin domains would show distinct changes in histone modifications to either side of the CTCF-binding event. We therefore profiled the genome-wide location of histone 2A lysine 5 acetylation

(H2AK5ac) (Cuddapah et al., 2009) and directly compared these data with matched CTCF occupancy data. This analysis identified hundreds of regions of abrupt changes in active chromatin demarcated by CTCF binding, consistent with CTCF's role as a barrier element and representing almost 5% of CTCF-binding events. Negative controls, such as unrelated TFs and random regions, showed only background level association with H2AK5ac in liver (Figure S6). In mouse, approximately 25% of CTCF chromatin boundaries were found to be associated with repetitive element expansion. For example, in mouse a CTCF-binding event found within a B2 SINE represented the boundary between the highly transcribed, liver-specific *ApoA* cluster of genes and the neighboring genes downstream on chromosome 9 (Figure 6A).

We asked whether newly expanded CTCF-binding events function as chromatin barriers as often as the five-way shared CTCF-binding events that predate the placental mammalian expansion. We exploited the recent expansion of B2 elements that have introduced thousands of novel, lineage-specific CTCF-binding events to the mouse genome. We categorized mouse CTCF binding by whether it was (1) conserved in all five placental mammals, (2) present in a mouse-specific SINE repeat, (3) present in a rodent-shared SINE repeat, and (4) all other binding events, as well as adding (5) random genomic regions as controls (Figure 6B). CTCF-binding events demarcate active and inactive chromatin at a similar frequency, regardless of whether the CTCF-binding events are shared between the eutherian mammals, rodent B2 associated, or mouse-specific B2 associated. Likewise, all CTCF boundaries are capable of demarcating transcriptionally active and inactive chromatin. Genes divided by CTCF-demarcated chromatin domains had higher transcriptional divergence (Figure 6C). In addition, we did not observe specific motif features associated with CTCF barrier elements, as CTCF binding generated from B2 transposons is equally likely to form CTCF barrier elements, as is non-repeat-associated CTCF binding.

To further assess the functional impact of SINE-driven CTCF-binding events on transcription and gene expression, we explored whether CTCF can act as a transcriptional insulator between tandem genes (Figure 7A). Tandem genes are transcribed by RNA polymerase in the same direction and have been shown to have more coherence in their relative gene expression than non-tandem-organized genes (Caron et al., 2001; Lercher et al., 2002). We collected mRNA sequencing data in livers of all studied species, identified the subset of tandem genes divided by at least one CTCF-binding event in each species, and further subdivided this set by whether the CTCF-binding event was shared, repeat associated, or neither. In all species, we observed statistically significant increases in gene expression differences between tandem genes divided by CTCF (Figure 7B). We did not find any significant effects of the presence or absence of M2 on transcriptional insulation (data not shown). Indeed, repeat-associated CTCF-binding events in mouse, rat, and dog serve to transcriptionally separate members of tandem gene pairs.

Our data thus indicate that newly arisen CTCF-binding events in multiple mammalian species functionally demarcate chromatin domains and influence transcription at a similar frequency as do ultra-conserved CTCF-binding events.

DISCUSSION

Understanding the structure, function, and origins of the genome is fundamental to understanding the mechanisms of mammalian evolution. By assaying CTCF binding in matched tissues of six diverse mammals, we generated high-resolution *in vivo* maps of CTCF evolution. This uncovered over 100,000 previously unidentified CTCF-binding events in multiple species. Our data reveal a highly conserved 33/34 bp motif consisting of a two-part profile for CTCF binding, confirm that CTCF binding is remarkably conserved compared to other TFs, and demonstrate that CTCF has a core set of over 5,000 bound regions shared among five representative placental mammals. Word-level analysis of the binding events revealed a conserved motif hierarchy, and that new CTCF-binding events are born in highly diverse mammalian lineages via the expansion of repetitive elements. Many of these newborn CTCF-binding locations function as barriers to both chromatin and gene expression. Finally, we provide compelling evidence that the same process that currently drives lineage-specific expansion of CTCF-binding events in diverse mammals ancestrally generated the core set of strong, deeply conserved CTCF-binding events.

Insights from an Expanded CTCF-Binding Motif

A larger motif for CTCF binding explains ambiguous results from prior studies (see also Figure S2), which suggested that the regions bound by CTCF are larger than 20 bp. For instance, the 40 bp DNase I footprint at the APP gene promoter sequence (Quitschke et al., 2000) contains the full motif we have identified (Figure 2E). Earlier work described CTCF as a multivalent TF that binds to two different DNA sequences in human (CTCF human fragment A) and chicken (CTCF fragment V) (Filippova et al., 1996). Our results explain that chicken fragment V contains the previously known 20-mer CTCF motif (Figure 2F) and is thus readily bound *in vitro* by zinc fingers 2 to 7; in contrast, the human fragment A contains the full 33 bp motif and thus requires the additional four C-terminal zinc fingers to be bound *in vitro*. A 3 bp mutation within the critical DNA bases of the M2 motif abolished CTCF binding (Figures 2F and S2) (Filippova et al., 1996). The existence of a set of CTCF-binding events that require the M1 and M2 motifs over the sole presence of the constitutive M1 motif can also help explain the exceptional conservation of CTCF's 11 zinc finger DNA-binding domain (Klenova et al., 1993; Moon et al., 2005). From the interaction of the C-terminal zinc fingers with the M2 motif, we can also deduce CTCF's orientation relative to the binding sequence. The expanded CTCF-binding motif helps explain previous, somewhat conflicting results and supports recent reports describing a preferred orientation of CTCF binding relative to its target sequence (Quitschke et al., 2000; Renda et al., 2007).

The Genetic Architecture and Regulatory Implications of CTCF-Binding Conservation

The structural and regulatory organization of the mammalian genome is fundamentally dependent on CTCF (Phillips and Corces, 2009). Prior studies have revealed in the context of the rapid divergence of tissue-specific TF binding that CTCF binding is comparatively well conserved between human and mouse cell

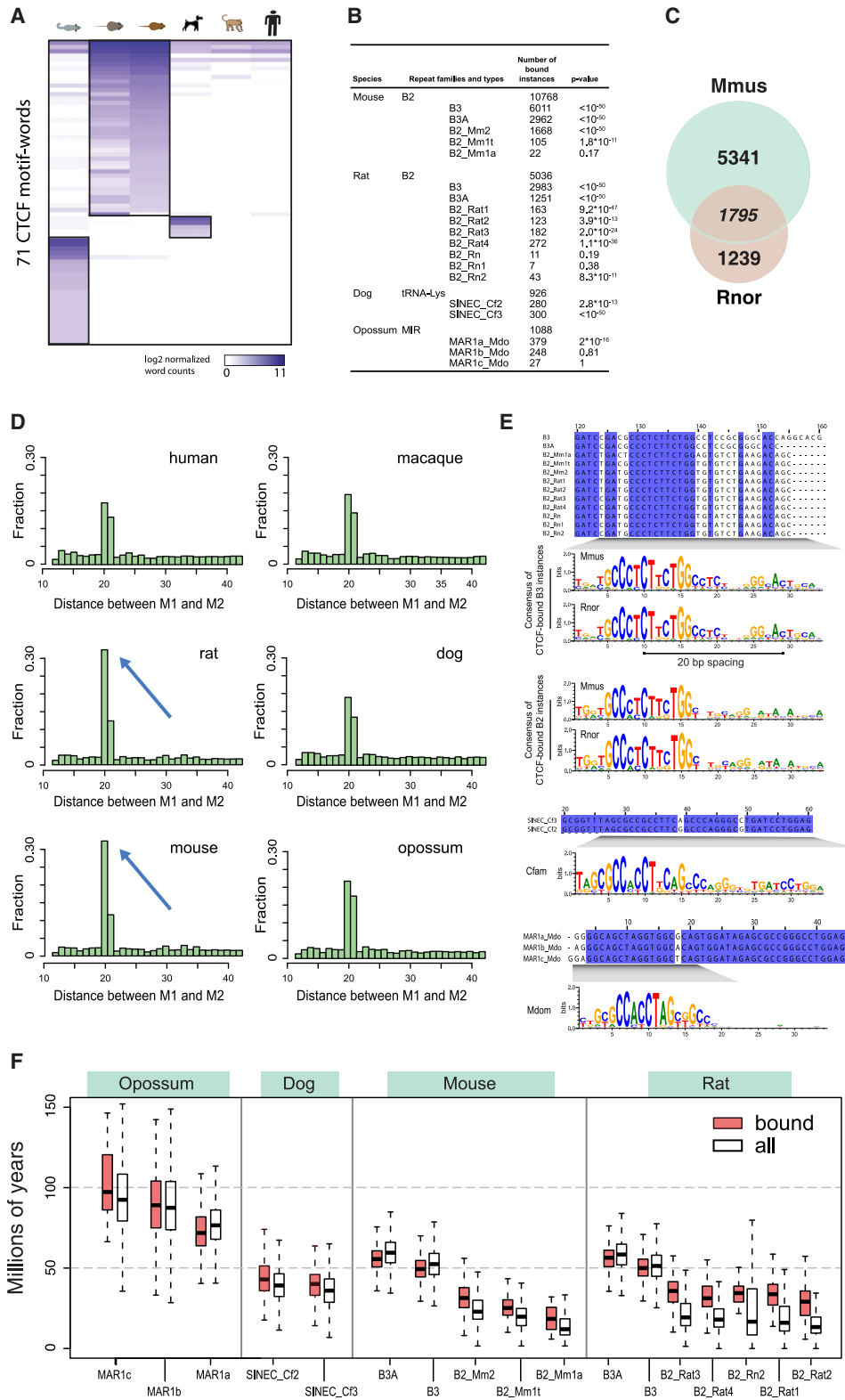


Figure 4. Repeat Expansions Remodeled CTCF Binding in Three Mammalian Lineages
 (A) Heatmap of 71 motif-words identified as highly enriched in mammalian lineages.
 (B) Lineage-specific repeats that are associated with the lineage-specific motif-words.
 (C) Venn diagram showing the number of B2 repeat-associated binding events shared between mouse and rat.

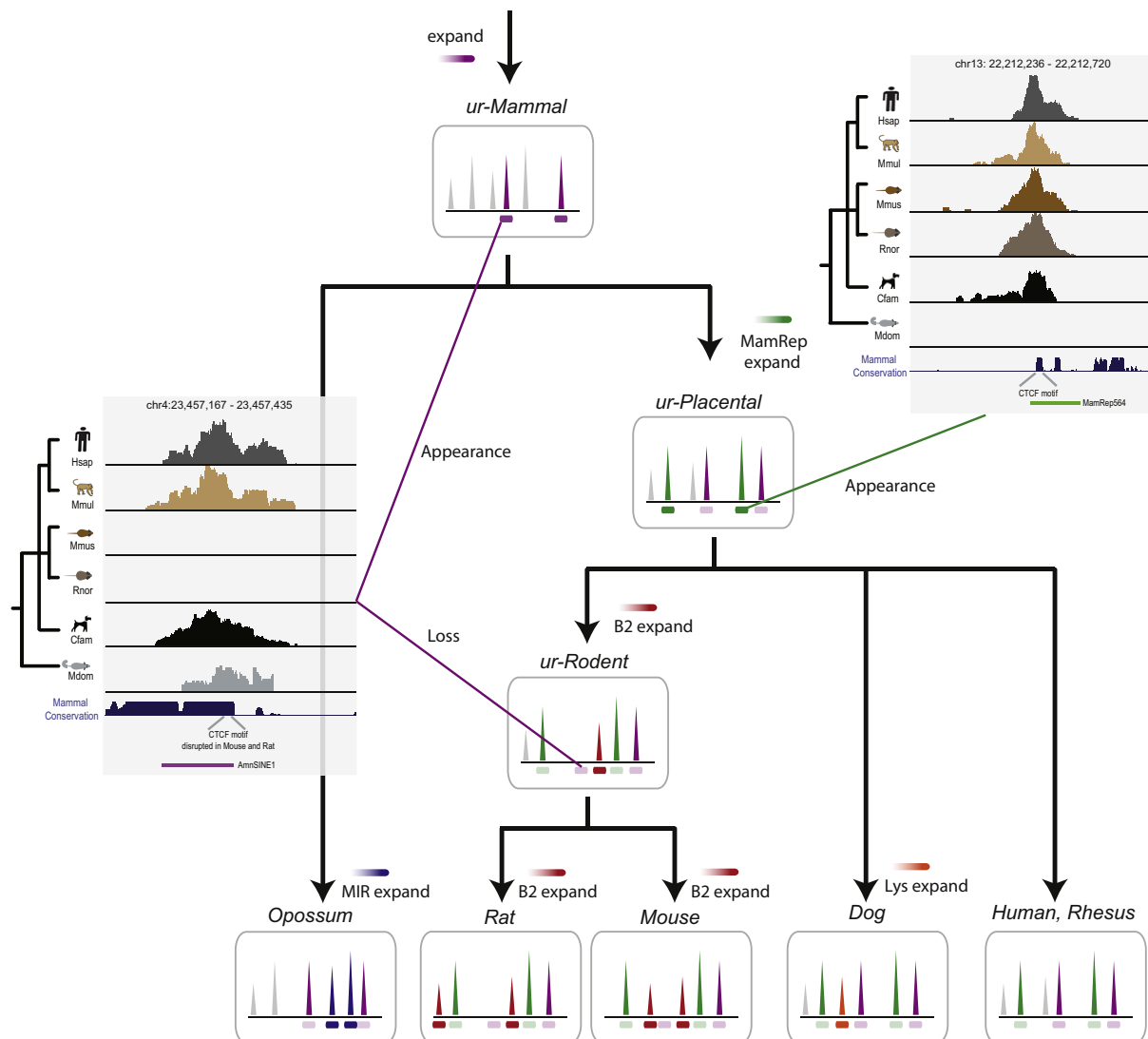


Figure 5. Intermittent Repeat Expansions Can Lead to Conserved, Lineage-Specific, and Species-Specific CTCF Binding in Mammals

A CTCF-binding site found within an ancient transposon shows conserved binding in placental and nonplacental mammals (left data inset) and must have been present in the mammalian ancestor (*ur-Mammal*). In contrast, a CTCF-binding site generated in the eutherian ancestor (*ur-Placental*) shows conserved binding across placental mammals but is absent in marsupials (right data inset). More recent CTCF-binding expansions lead to increasingly lineage- and species-specific CTCF binding. For example, the expansions of B2 repeats in the mouse and rat ancestor (*ur-Rodent*) created CTCF binding that is highly shared between mouse and rat, whereas the continued B2 expansions along both lineages also generated species-specific CTCF-binding sites (see Figure 4C). See also Table S3.

lines (Bourque et al., 2008; Kunarso et al., 2010). Reflecting the organizational role of CTCF, one of the few hundred CTCF-bound regions reported as shared among human, mouse, and chicken cells has been shown to serve as a genomic barrier to redirect EVI5 intron-located enhancers to regulate the distal GF11 gene (Martin et al., 2011).

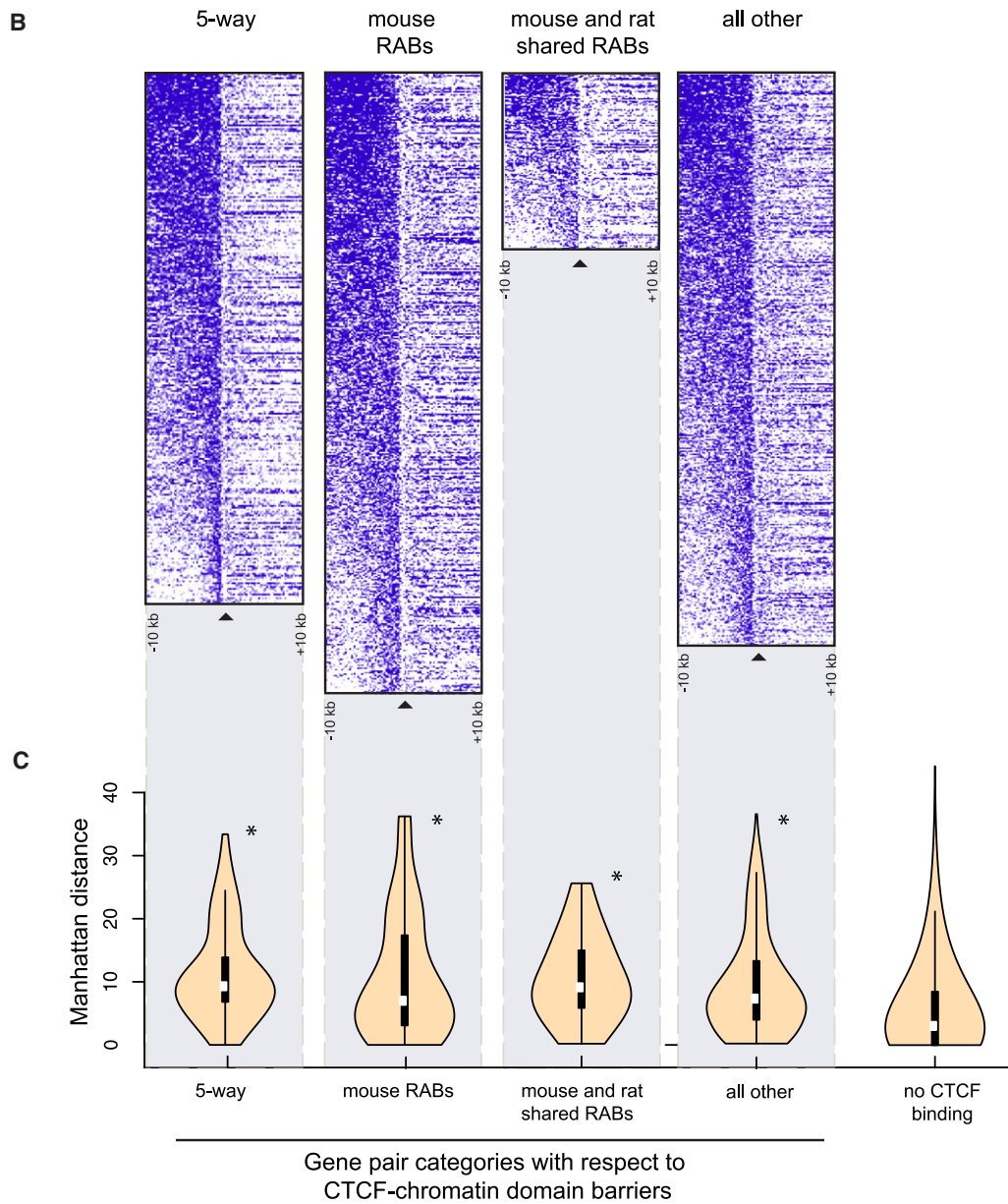
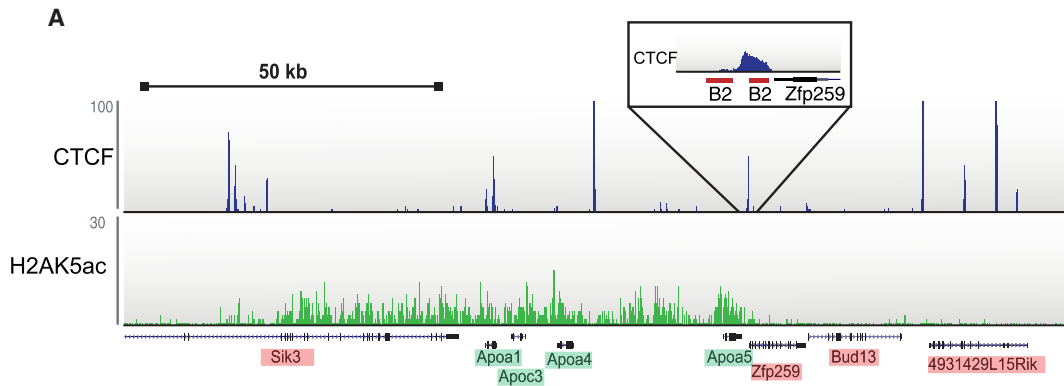
Our global data extend these studies, exploring CTCF-binding evolution in matched tissues from six mammals. This comparison revealed that conserved CTCF binding often shows a number of specific features, including the following: (1) tissue invariance, (2) a specific and conserved motif-word composition, (3) high ChIP enrichment, and (4) high-affinity

(D) Frequencies of distances between the centers of M1 and M2 in all six studied species. There is a smaller spacing between M1 and M2 in mouse and rat (blue arrow), due to the B2 repeat expansion.

(E) Sections of the aligned consensus sequences from CTCF-carrying retrotransposons in mouse, rat, dog, and opossum; rat and mouse contain the M1+M2 motif, dog and opossum only contain M1. Consensus motifs for CTCF binding solely based on bound repeat instances are shown below each alignment.

(F) Estimated ages of lineage-specific repeats that expanded CTCF binding. White box plots are all instances of the indicated repeat; red box plots are only those bound by CTCF.

See also Figure S4.



protein-DNA interactions, as shown by strong resistance to RNAi-mediated knockdown. In contrast, most tissue-specific mammalian TFs not only evolve rapidly in their genomic binding but also differ from CTCF in most other features as well (Kunarse et al., 2010; Mikkelsen et al., 2010; Odom et al., 2007; Schmidt et al., 2010b). The conserved set of CTCF-binding events, therefore, represent an organizational pattern present in all mammalian cells, regardless of the developmental stage and tissue, and delineate chromatin structures required for conserved genome functions (as explored at one genomic locus; Martin et al., 2011).

Repeat-Driven Expansions of CTCF Binding Are an Ancient and Ongoing Source of Genome and Regulatory Evolution

Due to CTCF's long, high information content motif, new CTCF-binding events are dramatically less likely to be generated by random mutations than binding events for TFs targeting much shorter motifs. However, the copy and paste mechanism of transposable elements is blind to size. Therefore, once a CTCF motif has been acquired by a transposon, it can spread across the genome by generating carbon copies of the originally gained motif sequence. Our experiments revealed that repeat-associated binding expansion carried functional CTCF-binding events throughout the muridae, canidae, and didelphidae genomes, suggesting that most mammalian lineages are subject to similar CTCF expansions. Interestingly (and perhaps surprisingly), our data in human and macaque show no evidence of such events. It is possible, however, that primate lineages that we have not yet studied have indeed been subject to repeat-driven expansion of CTCF binding, as other primate SINEs such as Alu elements have been active recently.

Expansions via transposable elements are increasingly recognized as a general mechanism for the generation of new binding sites of TFs with complex binding motifs (Johnson et al., 2006, 2009; Mortazavi et al., 2006). In addition, recent reports provide evidence that transposable elements contain sequences for larger regulatory assemblies that restructure tissue-specific transcriptomes (Lynch et al., 2011; Kunarse et al., 2010). For example, many binding events of the neuronal repressor NRSF/REST have been generated across vertebrate genomes by means of lineage-specific transposons (Johnson et al., 2006, 2009; Mortazavi et al., 2006), and the composite OCT-SOX motif has been expanded in humans (Kunarse et al., 2010). Similar expansions of retrotransposons that carry CTCF binding might, in fact, have an evolutionary advantage over those that do not: it has been shown that CTCF binding can prevent DNA methylation and the establishment of repressive chromatin modifications

(Lee et al., 2010; Rand et al., 2004). Consequently, CTCF binding might provide transposable elements with a survival strategy, by protecting them against repressive chromatin and DNA modifications. Alternatively, CTCF and similar factors may have been part of genomic defense strategies against specific transposable element invasions.

Taken together, our data support a model in which lineage-specific repeat expansions have been propelling distinct CTCF motif-words and their associated binding events across the genome many times throughout mammalian evolution (Figure 7). The same mechanisms creating lineage-specific CTCF binding in extant species are almost certainly responsible for creating the ancient CTCF events found across all mammals. Despite the gradual divergence of genetic sequences surrounding the core CTCF sequence motif, we found evidence that multiple repeat sequences have carried CTCF binding in common ancestors. Indeed, deliberate molecular paleontology across our data revealed over a hundred such repeat fossils associated with conserved CTCF binding.

How repeat elements can globally contribute toward organismal phenotypes, from tissue-specific gene expression (Kunarse et al., 2010) to coat color (Blewitt et al., 2006) to lactation (Lynch et al., 2011), has only begun to be explored. Here, we have described how mammalian repeat elements are a major mechanism by which CTCF binding is born, thus revealing how complex eukaryotic regulatory structures and the repetitive sequences they control can collaborate to drive genome evolution.

EXPERIMENTAL PROCEDURES

We performed chromatin immunoprecipitation experiments followed by high-throughput sequencing (ChIP-seq) (Schmidt et al., 2009) using liver material isolated from six mammalian species: human (Hsap; primate), macaque (Mmul; primate), dog (Cfam; carnivora), mouse (Mmus; rodent), rat (Rnor; rodent), and short-tailed opossum (Mdom; didelphimorphia). For each ChIP experiment, at least two independent biological replicates from different animals, generally two young adult males, were performed (see Extended Experimental Procedures). ChIP-seq experiments were performed as recently described (Schmidt et al., 2009), and most interspecies analyses were performed as previously reported (Schmidt et al., 2010b).

The CTCF antibody 07-729 (Millipore) was used for all experiments except the opossum ones, which were performed using a custom antibody as described and validated in Figure S5. The custom opossum CTCF antibody is available upon request. The STAG1 antibody used for validation of the opossum results and the H2AK5ac antibody were both purchased from abcam, ab4457 and ab1764, respectively.

ACCESSION NUMBERS

Data have been deposited under ArrayExpress accession numbers E-MTAB-437 and E-MTAB-424.

Figure 6. Chromatin Boundaries Separated by Repeat-Associated CTCF Binding in Rodents

(A) A B2-associated CTCF-binding event separates the ApoA cluster from downstream genes on mouse chromosome 9 (top blue track). Active transcription is reflected both by H2AK5ac occupancy in mouse liver (bottom green track) and in direct sequencing of mouse liver mRNA by gene name shading (red is silent; green is active) (Mortazavi et al., 2008).

(B) Heat map representation of H2AK5ac chromatin domains flanked by CTCF binding that is shared between all five species (five-way), mouse unique and repeat-associated (mouse RABs), repeat-associated and shared between mouse and rat (mouse and rat shared RABs), and not within the previous categories (all other).

(C) Violin plots represent gene expression differences (Manhattan distances) between H2AK5ac and CTCF defined chromatin domains for different gene pair categories.

See also Figure S6.

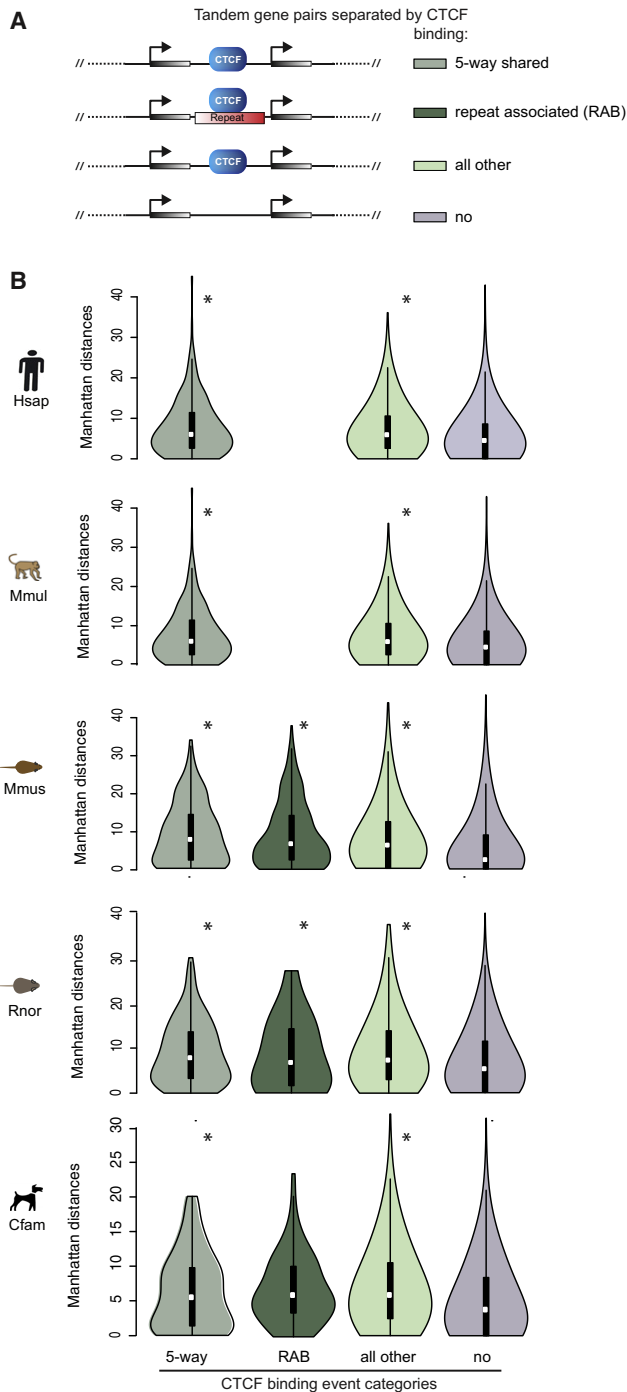


Figure 7. Tandem Gene Pairs Separated by CTCF Differ More in Their Expression than Gene Pairs that Are Not Separated by CTCF (A) Exemplified tandem gene pairs that are separated by CTCF binding or not separated by CTCF (no). The CTCF-separated tandem gene pairs are further distinguished into the following three groups: (1) shared between the five mammals shown in (B) (five-way shared), (2) associated with lineage-specific repeats (repeat-associated, RAB), (3) all other CTCF-separated gene pairs (all other). (B) Violin plots represent gene expression difference distributions (Manhattan distance) per tandem gene pair group as explained in (A). Stars (*) indicate p values compared to the no CTCF binding category that are smaller than 0.001 (wilcoxon rank-sum test).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and three tables and can be found with this article online at doi:10.1016/j.cell.2011.11.058.

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REFERENCES

Awad, T.A., Bigler, J., Ulmer, J.E., Hu, Y.J., Moore, J.M., Lutz, M., Neiman, P.E., Collins, S.J., Renkawitz, R., Lobanenko, V.V., and Filippova, G.N. (1999). Negative transcriptional regulation mediated by thyroid hormone response element 144 requires binding of the multivalent factor CTCF to a novel target DNA sequence. *J. Biol. Chem.* *274*, 27092–27098.

Baniahmad, A., Steiner, C., Köhne, A.C., and Renkawitz, R. (1990). Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. *Cell* *61*, 505–514.

Bejerano, G., Lowe, C.B., Ahituv, N., King, B., Siepel, A., Salama, S.R., Rubin, E.M., Kent, W.J., and Haussler, D. (2006). A distal enhancer and an ultraconserved exon are derived from a novel retroposon. *Nature* *441*, 87–90.

Bell, A.C., West, A.G., and Felsenfeld, G. (1999). The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell* *98*, 387–396.

Blewitt, M.E., Vickaryous, N.K., Paldi, A., Koseki, H., and Whitelaw, E. (2006). Dynamic reprogramming of DNA methylation at an epigenetically sensitive allele in mice. *PLoS Genet.* *2*, e49.

Borneman, A.R., Gianoulis, T.A., Zhang, Z.D., Yu, H., Rozowsky, J., Seringhaus, M.R., Wang, L.Y., Gerstein, M., and Snyder, M. (2007). Divergence of transcription factor binding sites across related yeast species. *Science* *317*, 815–819.

Bourque, G., Leong, B., Vega, V.B., Chen, X., Lee, Y.L., Srinivasan, K.G., Chew, J.-L., Ruan, Y., Wei, C.-L., Ng, H.H., and Liu, E.T. (2008). Evolution of the mammalian transcription factor binding repertoire via transposable elements. *Genome Res.* *18*, 1752–1762.

Boyle, A.P., Song, L., Lee, B.-K., London, D., Keefe, D., Birney, E., Iyer, V.R., Crawford, G.E., and Furey, T.S. (2011). High-resolution genome-wide in vivo footprinting of diverse transcription factors in human cells. *Genome Res.* *21*, 456–464.

Britten, R.J. (1997). Mobile elements inserted in the distant past have taken on important functions. *Gene* *205*, 177–182.

Burcin, M., Arnold, R., Lutz, M., Kaiser, B., Runge, D., Lottspeich, F., Filippova, G.N., Lobanenko, V.V., and Renkawitz, R. (1997). Negative protein 1, which is required for function of the chicken lysozyme gene silencer in conjunction with hormone receptors, is identical to the multivalent zinc finger repressor CTCF. *Mol. Cell. Biol.* *17*, 1281–1288.

Caron, H., van Schaik, B., van der Mee, M., Baas, F., Riggins, G., van Sluis, P., Hermus, M.C., van Asperen, R., Boon, K., Voûte, P.A., et al. (2001). The human transcriptome map: clustering of highly expressed genes in chromosomal domains. *Science* *291*, 1289–1292.

- Chen, X., Xu, H., Yuan, P., Fang, F., Huss, M., Vega, V.B., Wong, E., Orlov, Y.L., Zhang, W., Jiang, J., et al. (2008). Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell* 133, 1106–1117.
- Cooper, G.M., Stone, E.A., Asimenos, G., Green, E.D., Batzoglu, S., and Sidow, A.; NISC Comparative Sequencing Program. (2005). Distribution and intensity of constraint in mammalian genomic sequence. *Genome Res.* 15, 901–913.
- Cuddapah, S., Jothi, R., Schones, D.E., Roh, T.-Y., Cui, K., and Zhao, K. (2009). Global analysis of the insulator binding protein CTCF in chromatin barrier regions reveals demarcation of active and repressive domains. *Genome Res.* 19, 24–32.
- Dermitzakis, E.T., and Clark, A.G. (2002). Evolution of transcription factor binding sites in Mammalian gene regulatory regions: conservation and turnover. *Mol. Biol. Evol.* 19, 1114–1121.
- Dunn, K.L., and Davie, J.R. (2003). The many roles of the transcriptional regulator CTCF. *Biochem. Cell Biol.* 81, 161–167.
- ENCODE Project Consortium. (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* 447, 799–816.
- ENCODE Project Consortium. (2011). A user's guide to the encyclopedia of DNA elements (ENCODE). *PLoS Biol.* 9, e1001046.
- Fedoriw, A.M., Stein, P., Svoboda, P., Schultz, R.M., and Bartolomei, M.S. (2004). Transgenic RNAi reveals essential function for CTCF in H19 gene imprinting. *Science* 303, 238–240.
- Filippova, G.N., Fagerlie, S., Klenova, E.M., Myers, C., Dehner, Y., Goodwin, G., Neiman, P.E., Collins, S.J., and Lobanenko, V.V. (1996). An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. *Mol. Cell. Biol.* 16, 2802–2813.
- Han, J.S., Szak, S.T., and Boeke, J.D. (2004). Transcriptional disruption by the L1 retrotransposon and implications for mammalian transcriptomes. *Nature* 429, 268–274.
- Heath, H., Ribeiro de Almeida, C., Sleutels, F., Dingjan, G., van de Nobelen, S., Jonkers, I., Ling, K.-W., Gribnau, J., Renkawitz, R., Grosveld, F., et al. (2008). CTCF regulates cell cycle progression of alphabeta T cells in the thymus. *EMBO J.* 27, 2839–2850.
- Heintzman, N.D., Hon, G.C., Hawkins, R.D., Kheradpour, P., Stark, A., Harp, L.F., Ye, Z., Lee, L.K., Stuart, R.K., Ching, C.W., et al. (2009). Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature* 459, 108–112.
- Hirakawa, M., Nishihara, H., Kanehisa, M., and Okada, N. (2009). Characterization and evolutionary landscape of AmnSINE1 in Amniota genomes. *Gene* 441, 100–110.
- Johnson, R., Gamblin, R.J., Ooi, L., Bruce, A.W., Donaldson, I.J., Westhead, D.R., Wood, I.C., Jackson, R.M., and Buckley, N.J. (2006). Identification of the REST regulon reveals extensive transposable element-mediated binding site duplication. *Nucleic Acids Res.* 34, 3862–3877.
- Johnson, R., Samuel, J., Ng, C.K., Jauch, R., Stanton, L.W., and Wood, I.C. (2009). Evolution of the vertebrate gene regulatory network controlled by the transcriptional repressor REST. *Mol. Biol. Evol.* 26, 1491–1507.
- Jothi, R., Cuddapah, S., Barski, A., Cui, K., and Zhao, K. (2008). Genome-wide identification of *in vivo* protein-DNA binding sites from ChIP-Seq data. *Nucleic Acids Res.* 36, 5221–5231.
- Kass, D.H., Kim, J., Rao, A., and Deininger, P.L. (1997). Evolution of B2 repeats: the muroid explosion. *Genetica* 99, 1–13.
- Kim, T.H., Abdullaev, Z.K., Smith, A.D., Ching, K.A., Loukinov, D.I., Green, R.D., Zhang, M.Q., Lobanenko, V.V., and Ren, B. (2007). Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. *Cell* 128, 1231–1245.
- Klenova, E.M., Nicolas, R.H., Paterson, H.F., Carne, A.F., Heath, C.M., Goodwin, G.H., Neiman, P.E., and Lobanenko, V.V. (1993). CTCF, a conserved nuclear factor required for optimal transcriptional activity of the chicken c-myc gene, is an 11-Zn-finger protein differentially expressed in multiple forms. *Mol. Cell. Biol.* 13, 7612–7624.
- Kuniarso, G., Chia, N.-Y., Jeyakani, J., Hwang, C., Lu, X., Chan, Y.-S., Ng, H.-H., and Bourque, G. (2010). Transposable elements have rewired the core regulatory network of human embryonic stem cells. *Nat. Genet.* 42, 631–634.
- Lee, D.H., Singh, P., Tsai, S.Y., Oates, N., Spalla, A., Spalla, C., Brown, L., Rivas, G., Larson, G., Rauch, T.A., et al. (2010). CTCF-dependent chromatin bias constitutes transient epigenetic memory of the mother at the H19-Igf2 imprinting control region in prospermatogonia. *PLoS Genet.* 6, e1001224.
- Lercher, M.J., Urrutia, A.O., and Hurst, L.D. (2002). Clustering of housekeeping genes provides a unified model of gene order in the human genome. *Nat. Genet.* 31, 180–183.
- Lobanenko, V.V., Nicolas, R.H., Adler, V.V., Paterson, H., Klenova, E.M., Polotskaja, A.V., and Goodwin, G.H. (1990). A novel sequence-specific DNA binding protein which interacts with three regularly spaced direct repeats of the CCCTC-motif in the 5'-flanking sequence of the chicken c-myc gene. *Oncogene* 5, 1743–1753.
- Lynch, V.J., Leclerc, R.D., May, G., and Wagner, G.P. (2011). Transposon-mediated rewiring of gene regulatory networks contributed to the evolution of pregnancy in mammals. *Nat. Genet.* 43, 1154–1159.
- MacArthur, S., and Brookfield, J.F.Y. (2004). Expected rates and modes of evolution of enhancer sequences. *Mol. Biol. Evol.* 21, 1064–1073.
- Markljung, E., Jiang, L., Jaffe, J.D., Mikkelsen, T.S., Wallerman, O., Larhammar, M., Zhang, X., Wang, L., Saenz-Vash, V., Gnirke, A., et al. (2009). ZBED6, a novel transcription factor derived from a domesticated DNA transposon regulates IGF2 expression and muscle growth. *PLoS Biol.* 7, e1000256.
- Martin, D., Pantoja, C., Fernández Miñán, A., Valdes-Quezada, C., Moltó, E., Matesanz, F., Bogdanović, O., de la Calle-Mustienes, E., Domínguez, O., Taher, L., et al. (2011). Genome-wide CTCF distribution in vertebrates defines equivalent sites that aid the identification of disease-associated genes. *Nat. Struct. Mol. Biol.* 18, 708–714.
- McClintock, B. (1950). The origin and behavior of mutable loci in maize. *Proc. Natl. Acad. Sci. USA* 36, 344–355.
- Mikkelsen, T.S., Wakefield, M.J., Aken, B., Amemiya, C.T., Chang, J.L., Duke, S., Garber, M., Gentles, A.J., Goodstadt, L., Heger, A., et al; Broad Institute Genome Sequencing Platform; Broad Institute Whole Genome Assembly Team. (2007). Genome of the marsupial *Monodelphis domestica* reveals innovation in non-coding sequences. *Nature* 447, 167–177.
- Mikkelsen, T.S., Xu, Z., Zhang, X., Wang, L., Gimble, J.M., Lander, E.S., and Rosen, E.D. (2010). Comparative epigenomic analysis of murine and human adipogenesis. *Cell* 143, 156–169.
- Moon, H., Filippova, G., Loukinov, D., Pugacheva, E., Chen, Q., Smith, S.T., Munhall, A., Grewe, B., Bartkuhn, M., Arnold, R., et al. (2005). CTCF is conserved from *Drosophila* to humans and confers enhancer blocking of the Fab-8 insulator. *EMBO Rep.* 6, 165–170.
- Mortazavi, A., Leeper Thompson, E.C., Garcia, S.T., Myers, R.M., and Wold, B. (2006). Comparative genomics modeling of the NRSF/REST repressor network: from single conserved sites to genome-wide repertoire. *Genome Res.* 16, 1208–1221.
- Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., and Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods* 5, 621–628.
- Odom, D.T., Dowell, R.D., Jacobsen, E.S., Gordon, W., Danford, T.W., MacIsaac, K.D., Rolfe, P.A., Conboy, C.M., Gifford, D.K., and Fraenkel, E. (2007). Tissue-specific transcriptional regulation has diverged significantly between human and mouse. *Nat. Genet.* 39, 730–732.
- Ohlsson, R., Renkawitz, R., and Lobanenko, V. (2001). CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. *Trends Genet.* 17, 520–527.
- Phillips, J.E., and Corces, V.G. (2009). CTCF: master weaver of the genome. *Cell* 137, 1194–1211.
- Quitschke, W.W., Taheny, M.J., Fochtmann, L.J., and Vostrov, A.A. (2000). Differential effect of zinc finger deletions on the binding of CTCF to the

- promoter of the amyloid precursor protein gene. *Nucleic Acids Res.* **28**, 3370–3378.
- Rand, E., Ben-Porath, I., Keshet, I., and Cedar, H. (2004). CTCF elements direct allele-specific undermethylation at the imprinted H19 locus. *Curr. Biol.* **14**, 1007–1012.
- Renda, M., Baglivo, I., Burgess-Beusse, B., Esposito, S., Fattorusso, R., Felsenfeld, G., and Pedone, P.V. (2007). Critical DNA binding interactions of the insulator protein CTCF: a small number of zinc fingers mediate strong binding, and a single finger-DNA interaction controls binding at imprinted loci. *J. Biol. Chem.* **282**, 33336–33345.
- Schmidt, D., Wilson, M.D., Spyrou, C., Brown, G.D., Hadfield, J., and Odom, D.T. (2009). ChIP-seq: using high-throughput sequencing to discover protein-DNA interactions. *Methods* **48**, 240–248.
- Schmidt, D., Schwalie, P.C., Ross-Innes, C.S., Hurtado, A., Brown, G.D., Carroll, J.S., Flicek, P., and Odom, D.T. (2010a). A CTCF-independent role for cohesin in tissue-specific transcription. *Genome Res.* **20**, 578–588.
- Schmidt, D., Wilson, M.D., Ballester, B., Schwalie, P.C., Brown, G.D., Marshall, A., Kutter, C., Watt, S., Martinez-Jimenez, C.P., Mackay, S., et al. (2010b). Five-vertebrate ChIP-seq reveals the evolutionary dynamics of transcription factor binding. *Science* **328**, 1036–1040.
- Splinter, E., Heath, H., Kooren, J., Palstra, R.-J., Klous, P., Grosveld, F., Galjart, N., and de Laat, W. (2006). CTCF mediates long-range chromatin looping and local histone modification in the beta-globin locus. *Genes Dev.* **20**, 2349–2354.
- Wang, T., Zeng, J., Lowe, C.B., Sellers, R.G., Salama, S.R., Yang, M., Burgess, S.M., Brachmann, R.K., and Haussler, D. (2007). Species-specific endogenous retroviruses shape the transcriptional network of the human tumor suppressor protein p53. *Proc. Natl. Acad. Sci. USA* **104**, 18613–18618.
- Wilson, M.D., Barbosa-Morais, N.L., Schmidt, D., Conboy, C.M., Vanes, L., Tybulewicz, V.L.J., Fisher, E.M.C., Tavaré, S., and Odom, D.T. (2008). Species-specific transcription in mice carrying human chromosome 21. *Science* **322**, 434–438.
- Xie, X., Mikkelsen, T.S., Gnirke, A., Lindblad-Toh, K., Kellis, M., and Lander, E.S. (2007). Systematic discovery of regulatory motifs in conserved regions of the human genome, including thousands of CTCF insulator sites. *Proc. Natl. Acad. Sci. USA* **104**, 7145–7150.

Note Added in Proof

The presence of the full M1+M2 motif in a large subset of human CTCF-binding data was recently reported using a novel exonuclease digestion strategy.

Rhee, H.S., and Pugh, B.F. (2011). Comprehensive genome-wide protein-DNA interactions detected at single-nucleotide resolution. *Cell* **147**, 1408–1419.