ments for the complex pattern of transcriptional regulation of the *py235* genes remain to be elucidated. Py235 proteins have previously been shown to be involved in red blood cell invasion. Because a subset of these proteins is expressed in the sporozoite and is the target of antibodies that inhibit hepatocyte invasion, these proteins may be important in the recognition and/or invasion of the mosquito salivary glands and the liver. Merozoites released from both the liver and the infected erythrocyte invade red blood cells, so the need to express a distinct set of *py235* genes in the infected hepatocyte is puzzling. This differential expression of *py235* in the hepatic schizont reinforces the idea that the obligatory passage of the parasite through the liver not only amplifies the number of parasites injected by the mosquito but also preadapts the parasite to invade red blood cells. The presence of distinct rhoptry proteins in the sporozoite and the liver-stage malaria parasite may form the basis of an efficient vaccination strategy to target these pre-erythrocytic–stage parasites, which are present in small numbers and are at their most vulnerable. Conserved regions of the rhoptry proteins that are the target of protective immune responses may also form the basis of a vaccine against both pre-erythrocytic– and erythrocytic-stage parasites.

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# CTCF, a Candidate *Trans*-Acting Factor for X-Inactivation Choice


**Wendy Chao, Khanh D. Huynh, Rebecca J. Spencer, Lance S. Davidow, Jeannie T. Lee***



**In mammals, X-inactivation silences one of two female X chromosomes. Silencing depends on the noncoding gene, *Xist* (inactive X-specific transcript), and is blocked by the antisense gene, *Tsix*. Deleting the choice/imprinting center in *Tsix* affects X-chromosome selection. Here, we identify the insulator and transcription factor, CTCF, as a candidate *trans*-acting factor for X-chromosome selection. The choice/imprinting center contains tandem CTCF binding sites that function in an enhancer-blocking assay. In vitro binding is reduced by CpG methylation and abolished by including non-CpG methylation. We postulate that *Tsix* and CTCF together establish a regulatable epigenetic switch for X-inactivation.**


Dosage compensation ensures equal expression of X-linked genes in XX females and XY males. In mammals, this process results in inactivation of one female X chromosome (XCI) (*1*) in a random or imprinted manner. In the random form (eutherian), a zygotic counting mechanism initiates dosage compensation and enables a choice mechanism to randomly designate one active (Xa) and one inactive (Xi) X [reviewed in (*2*)]. In the imprinted form, zygotic counting and choice are superseded by parental imprints that direct exclusive paternal X-silencing (*3, 4*). Imprinted XCI is found in ancestral marsupials (*3*) but vestiges remain in the extraembryonic tissues of eutherians such as mice (*4*).

An epigenetic mark for random and imprinted XCI has long been postulated (*2*). The marks are placed at the X-inactivation center (*Xic*) (*5*), which includes the *cis*-acting noncoding gene, *Xist* (*6, 7*), and its antisense counterpart, *Tsix* (*8*). *Xist* RNA accumulation along the Xi initiates the silencing step (*9, 10*), whereas *Tsix* represses silencing by blocking *Xist* RNA accumulation (*11, 12*). A *cis*-acting center for choice and imprinting lies at the 5′ end of *Tsix*, as its deletion abolishes random choice in epiblast-derived cells to favor inactivation of the mutated X (*11, 13*) and disrupts maternal *Xist* imprinting in extraembryonic tissues (*14, 15*). Thus, while imprinted XCI is parentally directed and random XCI is zygotically controlled, both work through *Tsix* to regulate *Xist*.

To date, only X-linked *cis*-elements have been identified as XCI regulators. Yet, virtually all models invoke *trans*-acting factors which interact with the X-linked sites. In one model for imprinted XCI, a maternal-specific *trans*-factor confers resistance to XCI (*16*). In models for random XCI, an autosomally expressed "blocking factor" protects a single X from silencing (*2*). We have proposed that *Tsix* is the *cis*-target of both *trans*-factors (*11, 14*).

To isolate candidate *trans*-factors, we now used computational analysis (Fig. 1) to identify mouse-to-human conserved elements within the 2- to 4-kilobase (kb) sequence implicated in choice and imprinting (*11, 13–15*), a region including *DXPas34* (*17*). We found that the region is composed almost entirely of 60- to 70-base pair (bp) repeats with striking resemblance to known binding sites for CTCF, a transcription factor with a 60-bp footprint and 11 zinc fingers that work in various combinations to generate a wide range of DNA-binding activities (*18*). CTCF functions as a boundary element at the globin locus (*19*), regulates enhancer access to the *H19-Igf2* imprinted genes (*20–23*), and associates with CTG/CAG repeats


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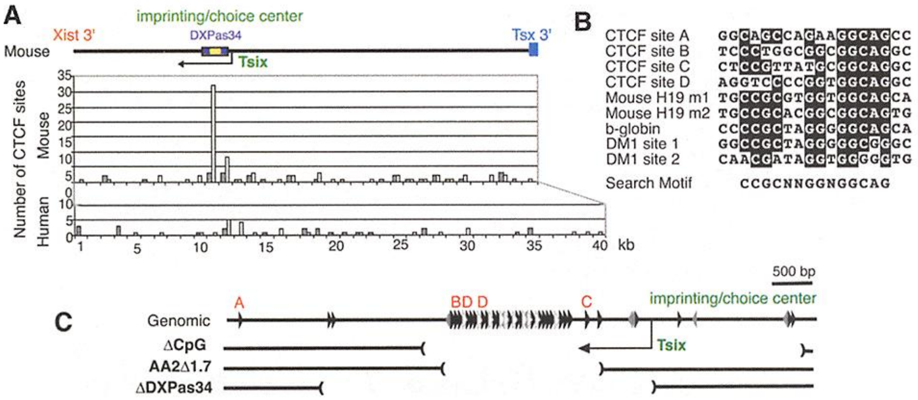



**Fig. 1.** Tandem CTCF-like binding sites in the *Tsix* imprinting/choice center. (**A**) Histogram of conserved human and mouse sites with 0 to 3 mismatches to the CTCF consensus (*20, 21*). Open and shaded bars represent two orientations. (**B**) Alignment of mouse *Tsix*, *H19*, *DM1*, and chicken β-globin sites. Shading indicates identity with the consensus. (**C**) Clustering of CTCF motifs. ΔCpG (*11, 14*), ΔDXPas34 (*13*), and AA2Δ1.7 (*15*). Filled triangles, sites with 0 to 3 mismatches. Open triangles, sites in the center with >3 mismatches. Forward sites, gray; reverse sites, black. Tested CTCF sites are indicated by red letters.

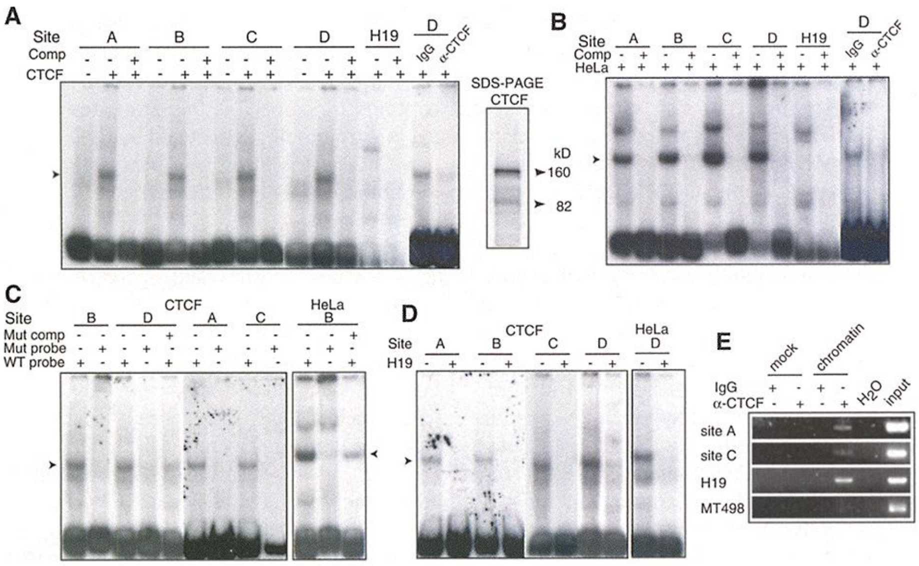
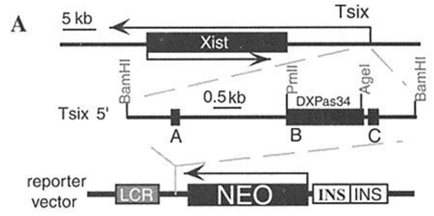



**Fig. 2.** *Tsix* elements bind CTCF in vitro and in vivo. (**A**) Gel-shift assay of P32-labeled *Tsix* oligos and CTCF protein. Reactions were carried out for 30 min at room temperature with 0.5 to 5.0 μl in vitro–synthesized CTCF protein (see SDS-PAGE) and 10 fmol double-stranded DNA probes in 20 mM HEPES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.3 mg/ml BSA, 5% glycerol, 0.5% Triton X-100, and 1 μg poly-dI:dC before resolution in 5% acrylamide, 0.5× TBE gels at 4°C. Cold competitors here and below (comp) were added at 200× molar excess. Supershifts were carried out using normal IgG or COOH-terminal CTCF antibodies (*19*). Site A, 5′-TGGAGCCTAAACCTGTCTGTCTCTTTACCAGACGCAGGGCAGCCAGAAGGCAGCCATTCACAATCCAGGAAGACAGGAAGGG-3′; site B, GGGGTTGGTTATAAGGCAGGGATTTTAGCGATCTCCCCAGGTCCCTGGCGGCGGCAGGCATTTTAGTGATAGCCCAGGTCCCCG; site C, ATTTTGGCTCCAGGACCCAGCAGACATTTTAGTTATTCCTCCGTTATGCGGCAGGCATTTTAACTATCGGTTCGGGACTACGCAGG; site D, CAGATCCCCAGTGGCAGACATTTTAGTGATAGCCCAGGTCCCCGGTGGCAGGCATTTTAGTGATAGCCCAGGTCCCCGGTGGCA. H19, MS1 (*20*). Arrowhead, *Tsix* DNA-protein complex. (**B**) An activity in HeLa nuclear extract (1 to 2 μg/reaction) also binds *Tsix* sites. (**C**) Mutated CTCF sites show reduced binding. Mut, mutated; WT, wild type. MutA, 5′-TGGAGCCTAAACCTGTCTGTCTCTTTACCAGTAATAGAATTCATGTAATATATCCATTCACAATCCAGGAAGACAGGAAGGG-3′; MutB, GGGGTTGGTTATAAGGCAGGGATTTTAGCGATCTCCCCAGGTCTAATAGAATTCATGGCATTTTAGTGATAGCCCAGGTCCCCG; MutC, ATTTTGGCTCCAGGACCCAGCAGACATTTTAGTTATTCCTTAATAGAATTCATGGCATTTTAACTATCGGTTCGGGACTACGCAGG; MutD, CAGATCCCCAGTGGCAGACATTTTAGTGATAGCCCAGTAATAGAATTCATGGCATTTTAGTGATAGCCCAGGTCCCCGGTGGCA. (**D**) Unlabeled *H19* sites compete against *Tsix* sites for CTCF. (**E**) CTCF binds *Tsix* in vivo (female fibroblasts) using ChIP analysis as described (*28*). Immunoprecipitations were performed overnight at 4°C with anti-CTCF antibodies (Upstate) or normal IgG. Primers pairs GTGTGTCATAGCTCAAGAGG, GGAGCCTAAACCTGTCTGTC (site A); AATGCTTGCCAGCTATGCGG, TAACCACCTGTAAGGGACAG (site C).




| | Mean number of colonies | S.D. | P-value |
|---|---|---|---|
| **B** | | | |
| No insulator | 100 | 0.00 | -- |
| 2.3 kb λ DNA | 96.9 | 4.04 | 0.07 |
| Globin insulators x2 | 14.1 | 4.76 | <0.0001 |
| BamHI-BamHI (F) | 90.2 | 32.4 | 0.45 |
| BamHI-BamHI (R) | 18.2 | 7.25 | <0.0001 |
| AgeI-PmlI (F) | 86.4 | 3.53 | <0.0001 |
| AgeI-PmlI (R) | 56.1 | 20.9 | <0.0001 |
| **C** | | | |
| Mutated site B | 100 | 0.00 | -- |
| Globin insulators x2 | 18.9 | 12.3 | <0.0001 |
| site A | 64.7 | 26.8 | <0.05 |
| site B | 65.9 | 18.8 | <0.05 |
| site C | 62.5 | 20.5 | <0.05 |

**Fig. 3.** The 5′ end of *Tsix* contains enhancer-blocking activity. (**A**) The enhancer-blocking assay (*26*) for *Tsix* sites in K562 cells. Sites A, B, and C are indicated by black boxes. Fragments in both forward (F) and reverse (R) orientations ("F," *Tsix* and *Neo* transcription in same direction) were inserted between the β-globin LCR and a neomycin-resistance reporter (*Neo*). Flanking globin insulators (Ins) protects against position effects (*26*). + control, globin insulators (pJC13-1) (*26*). (**B**) Results of enhancer-blocking assay. We transfected 1.5 pmol each of test plasmid and pTK-Hygromycin (transfection efficiency control). Neo-resistant colonies were counted 2 to 3 weeks after transfection and normalized to hygromycin-resistant colonies. Three to four experiments were averaged. *P*-values, unpaired one-tailed Student's *t* test in pairwise comparisons against the no-insulator control. (**C**) Enhancer-blocking activities for sites A, B, C, and mutated B. Constructs contained 1.5 kb of spacer to maintain equal distance. *P*-values, unpaired one-tailed Student's *t* test in pairwise comparisons against mutated B.

at *DM1* (*24*). Murine *Tsix* contains >40 CTCF motifs and the human sequence has >10 (Fig. 1A). Dotplot analysis indicated a contiguous head-to-tail arrangement of highly homologous *DXPas34* repeats (*25*). This clustering is rare, with only three other loci of comparable density (40 sites per 1629 bp) occurring in 40.4 Mb of available sequence (ScanACE, http://twod.med.harvard.edu). The clustering of nine human elements is not above genome average (test of 933 random 100-kb fragments; random sequence selection program, J. Aach). CTCF function, however, does not require a clustering of sites (*20–23*).

To determine if the sites could bind CTCF in vitro, we performed gel retardation analysis of representative sites A, B, C, and D (Fig. 1, B and C). Using in vitro–translated murine CTCF, we observed a protein-DNA complex at all sites that was eliminated by unlabeled self-competitor DNA (Fig. 2A). The complex migrated more rapidly than that formed by *H19*, possibly due to differential binding of CTCF

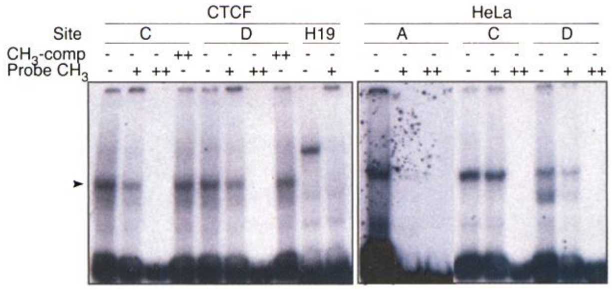



**Fig. 4.** CTCF binding is sensitive to DNA methylation in vitro. Gel-retardation analysis using *Tsix* probes which were unmethylated (–), methylated at CpGs only (+), or methylated at all C-nucleotides (++). Cold competitor ($CH_3$-comp) at 200× was methylated at all Cs. CpG methylation, achieved by SssI methylase and confirmed by insensitivity to HpaII or AciI digestion. Non-CpG methylation, achieved by direct synthesis. Arrow, *Tsix* DNA-protein complex.

isoforms (Fig. 2A; SDS-PAGE) or differential DNA bending induced by CTCF (*22*). Unprogrammed lysates did not shift the probe, indicating that the activity was specific to CTCF. HeLa extracts yielded two bands (Fig. 2B), one similar to that seen with in vitro–synthesized CTCF and one of lower intensity with a mobility similar to that for *H19* (this band was not always seen, e.g., Fig. 2D). Preincubation with polyclonal anti-CTCF antibodies blocked complex formation (Fig. 2, A and B). Mutating the 14-bp consensus (*20, 21*) within the 70-bp sites reduced binding (Fig. 2C) and unlabeled *H19* DNA effectively competed against *Tsix* for CTCF binding (Fig. 2D). Thus, CTCF specifically binds *Tsix* in vitro.

To test if CTCF binds *Tsix* in vivo, we carried out chromatin immunoprecipitation (ChIP) using anti-CTCF antibodies followed by *Tsix*-specific polymerase chain reaction in female mouse fibroblasts. Because the CTCF sites are tandemly repetitive, only sites A and C could be tested. Like the *H19* site [MS2 (*20*)], both sites were specifically coimmunoprecipitated with CTCF (Fig. 2E). In contrast, random loci on mouse chromosome 12 (MT498; www.jax.org) and in *Xist* (cDNA bp 13,177 to 13,428) did not coimmunoprecipitate (MT498 shown). Thus, CTCF complexes with *Tsix* DNA in vivo.

At some loci, CTCF sites act as chromatin insulators (*19–21*). In the established assay, insertion of these sites between the globin LCR and a neomycin (neo)–resistance reporter results in fewer neo-resistant K562 colonies (*26*). When a 4.3-kb Bam HI–Bam HI fragment containing all the *Tsix* sites was tested, we observed a dramatic reduction in colony number which was stronger in the R-orientation (Fig. 3, A and B). A 1.1-kb Pml–Age I fragment containing only sites B, D, and *DXPas34* also reduced colony number more strongly in the R-orientation (Student's $t$ test, $P < 0.0001$; ANOVA, $P < 0.0001$). This modest orientation-dependent effect is consistent with published reports (*19–23*). The greater activity in the Bam HI–Bam HI fragment might be attributable to additional CTCF sites outside of *DXPas34* or to possible unmapped *Tsix* promoter activity in the Bam HI–Bam HI fragment that would be antisense to *Neo*. Individual sites A, B, and C each exhibited fewer colonies relative to mutated site B (Fig. 3C; $t$ test, $P < 0.05$; ANOVA, $P < 0.05$). Thus, *Tsix* can block enhancer-promoter interaction and insulating activity correlates with CTCF binding in vitro.

Since CTCF responds to CpG methylation at some loci (*20–22*), we tested methylation-sensitivity at *Tsix* using gel retardation analysis. Unexpectedly, CTCF binding was only partially blocked by CpG methylation but was abolished when non-CpG methylation was included (Fig. 4). This contrasted with total inhibition at *H19* by CpG methylation alone. Relevant to this, *H19* sites contain three to four CpG's (*20, 21*), whereas many *Tsix* sites contain zero or one CpG in the consensus despite being strongly C-rich (Fig. 1B). These findings raised the possibility that non-CpG- together with CpG-methylation might regulate CTCF binding to *Tsix*. Notably, recent bisulfite sequencing has not uncovered differential CpG methylation in *DXPas34* (*27*). In light of our findings, the methylation status of non-CpG sites in the CTCF array will be critical in future work.

In summary, we have identified CTCF as a binding protein for the *cis*-acting choice/imprinting center in *Tsix*. We propose that CTCF and *Tsix* coordinately establish the epigenetic switch for *Xist* (Fig. 5). Because knocking out the CTCF array (choice/imprinting center) results in inactivation of the mutated X (*11, 13–15*), we favor a model in which binding of CTCF designates the future Xa. In this model, the zygotic blocking factor and the maternal protective factor work through CTCF to promote *Tsix* expression on the Xa. CTCF could directly stimulate *Tsix* transcription or do so by default through blocking *Xist*'s access to unidentified shared enhancers (*20–23*). *Tsix* transcription would in turn block *Xist* RNA accumulation (*12*). On the Xi, CTCF binding is excluded from *Tsix*, possibly by methylation ($CH_3$) of the CTCF array, thereby allowing the up-regulation of *Xist*. In the future, finer mutational analysis and the identification of differentially methylated regions will be required to test details of the model. Because CTCF is ubiquitous, developmental specificity must be achieved combinatorially with stage- and locus-specific factors. Identification of these protein-protein interactions will be instrumental in defining the long-postulated zygotic and maternal factors.

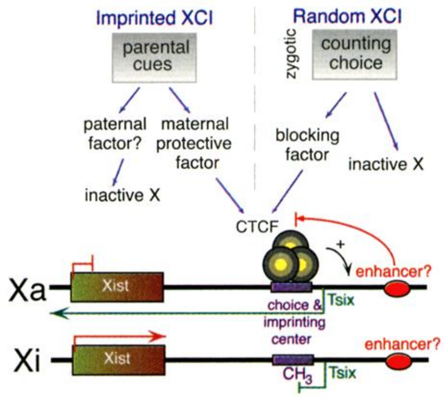



**Fig. 5.** Model of a regulatable epigenetic switch created by CTCF and *Tsix*.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