The Drosophila homolog of the mammalian imprint regulator, CTCF, maintains the maternal genomic imprint in Drosophila melanogaster

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Abstract

Background: CTCF is a versatile zinc finger DNA-binding protein that functions as a highly conserved epigenetic transcriptional regulator. CTCF is known to act as a chromosomal insulator, bind promoter regions, and facilitate long-range chromatin interactions. In mammals, CTCF is active in the regulatory regions of some genes that exhibit genomic imprinting, acting as insulator on only one parental allele to facilitate parent-specific expression. In Drosophila, CTCF acts as a chromatin insulator and is thought to be actively involved in the global organization of the genome.

Results: To determine whether CTCF regulates imprinting in Drosophila, we generated CTCF mutant alleles and assayed gene expression from the imprinted Dp(1;f)LJ9 mini-X chromosome in the presence of reduced CTCF expression. We observed disruption of the maternal imprint when CTCF levels were reduced, but no effect was observed on the paternal imprint. The effect was restricted to maintenance of the imprint and was specific for the Dp(1;f)LJ9 mini-X chromosome.

Conclusions: CTCF in Drosophila functions in maintaining parent-specific expression from an imprinted domain as it does in mammals. We propose that Drosophila CTCF maintains an insulator boundary on the maternal X chromosome, shielding genes from the imprint-induced silencing that occurs on the paternally inherited X chromosome.

See commentary: http://www.biomedcentral.com/1741-7007/8/104

Background

The correct establishment and propagation of epigenetic states are essential for normal development, and disruption of these processes leads to disease. Genomic imprinting is a striking example of the effect of epigenetics on gene regulation. In genomic imprinting, a mark, the imprint, is imposed on the two parental genomes during gametogenesis. In the zygote, the imprint is maintained through each mitotic division and results in the parental alleles of a gene, or entire homologous chromosomes, adopting different epigenetic states. As a result of these different epigenetic states, one parental allele can be silenced while the allele from the other parent, although identical in DNA sequence, is active.

The CCCTC-binding factor, CTCF, is a key player in maintaining epigenetically distinct chromatin domains. CTCF is an evolutionarily conserved zinc finger-containing DNA-binding protein that can function both directly in gene regulation as a transcription factor and also indirectly by mediating long-range chromatin interactions. In this latter role, CTCF acts as a chromatin insulator by isolating enhancer and promoter regulatory units and as a barrier to the spread of heterochromatin [1]. CTCF binds at multiple sites throughout the genome [2-4], indicating a widespread role in generating chromatin domains. Epigenetic isolation is necessary for correct maintenance of genomic imprints as imprinted domains are often interspersed among nonimprinted domains [5,6], necessitating their isolation from flanking...
CTCF binding has been reported at multiple mammalian imprinted domains [5], illustrating the importance of insulator function in maintaining parent-specific expression. The role of CTCF in imprinting has been best characterized for the mammalian Igf2/H19 genes, in which only the maternal H19 allele and paternal Igf2 alleles are expressed [8-10]. On the maternal chromosome, CTCF binds to a differentially methylated domain (DMD) located between the Igf2 and H19 genes, preventing interaction of downstream enhancer sequences with the promoter of Igf2, effectively silencing the gene. Methylation of the paternal DMD effectively blocks CTCF binding, allowing activation of Igf2 expression while also initiating the silencing of the H19 gene. Binding of CTCF is necessary to maintain the epigenetic state of the imprinted alleles. Consequently, if the CTCF binding site in the Igf2/H19 DMD is mutated, the monoallelic expression arising from the imprint is lost [11,12]. An additional facet of CTCF binding appears to be the facilitation of higher-order chromatin structures through DNA looping, a property which fortifies the silencing of Igf2 and the activation of H19 on the maternal chromosome [13,14]. The details of CTCF binding and its consequences are less well studied at other imprinted loci; however, its insulator function and role in establishing higher-order chromatin function appear to be shared features of other mammalian imprinted loci which bind CTCF [5,15-17]. The KvDMR1 imprinted domain, which contains two CTCF binding sites, regulates the tissue-specific expression of the gene Cdkn1c. It has been suggested that the tissue-specific imprinting of Cdkn1c is due to tissue-specific binding of CTCF to the KvDMR1 imprint domain [18,19]. The imprinted domain Wsb1/Nfj also requires CTCF-mediated interchromosomal association with the Igf2/H19 imprinted domain for proper parent-specific expression [20].

Although CTCF appears to be the major insulator protein in vertebrates, the more compact Drosophila genome uses a variety of insulator proteins, among which is the Drosophila CTCF homolog dCTCF [21-23]. The insulator activity of dCTCF has been well characterized in the bithorax complex, where it demarcates the chromatin domains that define separate regulatory regions [22,24,25], and, as in mammals, dCTCF is widely used as an insulator throughout the Drosophila genome and also acts directly as a transcription factor [26-28]. Though the role of CTCF in the formation of distinct chromatin domains is conserved from Drosophila to mammals, the roles of CTCF in epigenetic processes such as genomic imprinting have been assumed to differ [1]. To assess the effect of dCTCF on Drosophila imprinting, we used a well-characterized imprinting assay system, the Dp(1;f)LJ9 mini-X chromosome, in which a readily visible eye color gene, garnet (g), is juxtaposed to an imprint control region and so becomes a marker for imprinting [29]. Regulation of the Dp(1;f)LJ9 imprint previously has been shown to share properties of mammalian imprinting, including transcriptional silencing of gene clusters and differential chromatin states between homologues [30-32]. Here we present the first demonstration that dCTCF has a role in the regulation of genomic imprinting in Drosophila. As is the case in mammalian imprinting, dCTCF in Drosophila is involved in the regulation of the maternal imprint by maintaining parent-specific expression from the maternally inherited X chromosome.

**Results**

**Characterization of dCTCF alleles**

The CTCF^{EY15833} allele (FBrf0132177) was produced by insertion of the P[EPgy2] element into the +26 position relative to the transcription start site of the dCTCF gene by the Berkeley Drosophila Genome Project (BDGP) Gene Disruption Project [33]. Homozygous CTCF^{EY15833} adults appear healthy and are reasonably fertile. CTCF^{30}, created by a partial deletion of the P[EPgy2] element, is homozygous lethal. CTCF^{30} lacks the entire 5′ end of the P[EPgy2] element but retains 4860 bp of the 3′ end. Flanking dCTCF sequences and the quemao (qm) gene remain intact in CTCF^{30}. The reduced severity of CTCF^{EY15833}, with an intact P[EPgy2] element, suggests that a promoter in the 5′ end of P[EPgy2] may partially rescue dCTCF expression. To test this idea, we measured the dCTCF transcript levels by quantitative real-time PCR (qRT-PCR) of third instar larvae. Expression in homozygous CTCF^{EY15833} larvae is 20% ± 4% of that in wild-type (y w) controls. This is consistent with previous studies showing that CTCF^{EY15833} homozygotes produce ~50% of wild-type dCTCF protein levels [22]. Homozygous CTCF^{30} larvae cannot be recovered in sufficient numbers for qRT-PCR, but heterozygous CTCF^{30}/+ larvae display 68 ± 9% of wild-type transcript levels, consistent with a severe reduction in expression by this mutation. Taken together, the phenotypic and expression analysis of dCTCF alleles indicates that both CTCF^{EY15833} and CTCF^{30} alleles have reduced dCTCF expression and that the 5′ end of P[EPgy2] may drive sufficient expression to allow recovery of CTCF^{EY15833} adults.

**Drosophila CTCF maintains the maternal imprint of the garnet gene on the Dp(1;f)LJ9 mini-X chromosome**

To test the effect of the dCTCF alleles on Drosophila imprinting, we used the Dp(1;f)LJ9 mini-X chromosome.
Maternal inheritance of the $Dp(1;f)LJ9$ mini-X chromosomes ($Dp(1;f)LJ9^{MAT}$) generate full expression of the marker gene garnet, whereas paternal inheritance ($Dp(1;f)LJ9^{PAT}$) generates variegated garnet expression (Figures 1a and 1b, control). The variegated garnet gene phenotype arising from paternal transmission is mitotically stable and so results in distinct clonal regions exhibiting garnet expression in an eye devoid of garnet expression. Expression of garnet affects both red (pteridine) and brown (omochrome) eye pigments, which makes this mini-X chromosome an easily assayed system in which to assess the effect of $dCTCF$ alleles on imprinting in Drosophila.

Transmission of the $Dp(1;f)LJ9$ mini-X chromosome through the female results in $y^{1}z^{a}g^{53d}/Dp(1;f)LJ9^{+}$;/+ mini-X chromosome-bearing male progeny with essentially wild-type expression of the garnet imprint marker gene. Eyes are phenotypically wild type, with 85.3 ± 1.4% wild-type red pigment levels and 85.7 ± 3.4% wild-type brown pigment levels (Figure 1a, control). To determine the effects of $dCTCF$ on the maternal maintenance of imprinted garnet expression, the $CTCF^{X}$ alleles ($X$ represents $CTCF^{30}$ or $CTCF^{EY15833}$) were crossed to females with the $Dp(1;f)LJ9$ mini-X chromosome: $y^1z^a g^{53d}/Y; CTCF^X/TM3, Sb Ser$ males x $X^+X/Dp\ (1;f)LJ9$ females. This cross-generated progeny with a mutant $dCTCF$ allele and a maternally imprinted mini-X chromosome ($y^1z^a g^{53d}/Dp(1;f)LJ9^{MAT}; CTCF^X/+)$, which were compared with progeny similarly carrying a maternally imprinted chromosome, but wild type for $dCTCF$.

For each $dCTCF$ allele tested, the mutant allele substantially reduced expression of the maternally transmitted imprint marker gene (Figure 1a). Progeny with a maternally inherited mini-X chromosome ($Dp(1;f)LJ9^{MAT}$) with $CTCF^{EY15833}$ reduced pigment levels to 67.2 ± 3.1% ($P < 0.001$) and 68.4 ± 4.2% ($P < 0.001$) for red and brown pigments, respectively. $Dp(1;f)LJ9^{MAT}$ progeny coupled with $CTCF^{30}$ resulted in an even greater reduction of pigment levels: 58.9 ± 3.1% ($P < 0.001$) and 56.9 ± 4.7% ($P < 0.001$) for red and brown pigments, respectively. No variegated garnet
expression was observed in flies with \textit{Dp(1)fLJ9} and wild type for \textit{dCTCF} (Figures 2a and 2b). However, when \textit{Dp(1)fLJ9} was inherited along with mutant \textit{dCTCF} alleles, variegated \textit{garnet} expression was observed (Figures 2a and 2b). These results demonstrate that the maintenance of the maternal imprint is highly sensitive to \textit{dCTCF} dosage.

This cross also produced sibling progeny that have a maternally inherited \textit{Dp(1)fLJ9} and \textit{dCTCF} alleles, ranging from 0% to 100% pigmentation; control \( n = 300 \), \textit{CTCF}\textsubscript{EY15833} \( n = 300 \), \textit{CTCF}\textsubscript{30} \( n = 300 \). Each eye was scored depending on its phenotypic class, and the prevalence of each phenotypic class is expressed as a percentage versus the total number of eyes scored (n).

\textbf{Drosophila CTCF does not regulate the paternal imprint of the \textit{Dp(1)fLJ9} mini-X chromosome}

Variegated silencing of \textit{garnet} from the paternally inherited \textit{Dp(1)fLJ9\textsuperscript{MAT}} is a consequence of the spreading of heterochromatin from the imprinted region [29,32]. In
Figure 3 Absence of maternal or paternal effects from mutant dCTCF on Dp(1;f)LJ9 garnet expression. External control progeny (no modifier allele) have the same genotype as internal control progeny (y w y y z/y Dp(1;f)LJ9, TM3, So; Ser +) but are generated from a separate cross with parents that have never encountered a mutant CTCF allele. (a) Maternally inherited Dp(1;f)LJ9 CTCF internal control eye pigment levels; no significant difference in pigment levels was observed between external control progeny (no modifier allele) and internal control progeny from fathers carrying CTCF internal, demonstrating that no paternal effect occurs. (b) Phenotypes of maternally inherited Dp(1;f)LJ9 ranging from 0% to 100% pigmentation; No modifier allele n = 300, CTCF internal n = 300, CTCF internal n = 300. No garnet variegation was detected from the internal controls. (c) Paternally inherited Dp(1;f)LJ9 CTCF internal control eye pigment levels; no significant difference in pigment levels was observed between the external control progeny (no modifier allele) and internal control progeny from mothers carrying CTCF internal, demonstrating that no maternal effect occurs. (d) Phenotypes of paternally inherited Dp(1;f)LJ9 ranging from 0% to 100% pigmentation; no modifier allele n = 300, CTCF internal n = 300, CTCF internal n = 300. No significant change in garnet variegation was detected from the internal controls. Red eye pigment levels are measured by absorbance at 480 nm, and pigment quantification mean values for each group are based on n = 5 samples (40 heads total); error bars represent standard deviation.
contrast to the effects observed when the Dp(1;f)LJ9 is maternally imprinted, dCTCF mutants had no effect on the paternal expression of the garnet imprint marker gene. Paternal inheritance of the mini-X chromosome (X,Y)/Dp(1;f)LJ9 males crossed to y¹z²g³d/y¹z²g³d; TM3, Sb Ser/+ females) results in y¹z²g³d/Dp(1;f)LJ9PAT; +/+ progeny with variated garnet expression and a marked reduction in eye pigment levels (35.5 ± 1.5% red and 52.5 ± 0.2% brown wild-type pigment levels; Figure 1b, control). The introduction of dCTCF mutant alleles (y¹z²g³d; CTCFX/TM3, Sb Ser females to X,Y/Dp(1;f)LJ9 males) to generate progeny with either mutant CTCF<sup>EX15833</sup> or CTCF<sup>30</sup> alleles and a paternally imprinted Dp(1;f)LJ9PAT mini-X chromosome (y¹z²g³d/Dp(1;f)LJ9; CTCF<sup>30</sup>+) yielded no significant change in either red or brown eye pigment levels or phenotype (Figures 1b and 3d).

Sibling progeny with a paternally inherited Dp(1;f) LJ9PAT along with the balancer chromosome are wild type for dCTCF (described in “Methods,” genotype: y¹z²g³d/Dp(1;f)LJ9; TM3, Sb Ser+) and can be used to determine whether there is a maternal effect from mothers mutant for CTCF<sup>X</sup>. No maternal effect was detected; progeny wild type for dCTCF from mothers with either CTCF<sup>EX15833</sup> or CTCF<sup>30</sup> showed no significant change in either red or brown eye pigment levels or phenotype (Figures 1b and 3d).

**Drosophila CTCF does not regulate the establishment of the maternal or paternal imprint of the Dp(1;f)LJ9 mini-X chromosome**

To determine whether the effect of dCTCF was on the somatic maintenance of the imprint or its establishment in the germline of the parents, we examined the phenotype of progeny from male or female parents with both the Dp(1;f)LJ9 mini-X chromosome and a mutant CTCF<sup>30</sup> allele. If dCTCF affects the establishment of the imprint, the imprint should be disrupted in the progeny of mutant CTCF<sup>30</sup> parents, but not wild-type (CTCF<sup>+</sup>) parents. When we compared the phenotype of progeny wild type for dCTCF but differing in their parental genotype, no significant alternation in garnet expression levels resulted between Dp(1;f)LJ9<sup>MAT</sup> progeny from mothers carrying CTCF<sup>30</sup> (Figure 4a; Mat-Est. CTCF<sup>30</sup>) and either of the external or internal controls wild type for dCTCF (Figure 4a; Ex. Control and Mat-Est. CTCF<sup>+</sup>), respectively. This was reflected in the unchanged phenotype of progeny from mothers mutant or wild type for dCTCF (Figure 4b; Mat-Est. CTCF<sup>30</sup> and Mat-Est. CTCF<sup>+</sup>, respectively). Likewise, mutant dCTCF did not affect the establishment of the paternal imprint. Dp(1;f) LJ9<sup>PAT</sup> progeny from fathers carrying Dp(1;f)LJ9 and CTCF<sup>30</sup> (Figure 4c; Pat-Est. CTCF<sup>30</sup>) had no significant change in garnet expression compared with either the external or internal controls (Figure 4c; Ex. Control and Pat-Est. CTCF<sup>+</sup>, respectively). Again, these Dp(1;f)LJ9<sup>PAT</sup> progeny also had no observable change in phenotype between fathers mutant or wild type for dCTCF (Figure 4d; Pat-Est. CTCF<sup>30</sup> and Pat-Est. CTCF<sup>+</sup>, respectively). These findings distinguish the function of dCTCF in the maintenance versus the establishment of the imprint on the Dp(1;f)LJ9 mini-X chromosome; dCTCF is involved in the maintenance of the imprint in the soma of progeny as its reduction disrupts the maternal imprint. However, dCTCF is not involved in the establishment of the imprint as the presence of mutant CTCF<sup>30</sup> in either the maternal or paternal germline during establishment of the imprint does not affect regulation of the imprint.

**Drosophila CTCF is not a general modifier of position-effect variegation**

To determine the effect of dCTCF mutant alleles on the Dp(1;f)LJ9<sup>MAT</sup> imprint, we tested the effect of CTCF<sup>30</sup> on In(1)wm<sup>4</sup>, a classical variegating rearrangement [34], and two fourth chromosome transgenic constructs [35] in which the white (w) gene is variegated. Like the Dp(1; f)J9 mini-X chromosome, the variegated silencing in In(1)wm<sup>4</sup> is induced by the centric heterochromatin of the X chromosome [35] and the fourth chromosome has been proposed to be evolutionarily related to the X chromosome [36]. We found that the CTCF<sup>30</sup> allele decreased silencing of white in In(1)wm<sup>4</sup>;CTCF<sup>30</sup>/+ females while having no significant effect on white expression levels in In(1)wm<sup>4</sup>;CTCF<sup>30</sup>/+ males compared with sibling In(1)wm<sup>4</sup>;Tb/+ controls (Figure 5a). Similarly, the 6-M193 strain responded to CTCF<sup>30</sup> with a modest decrease in white reporter silencing in females only (Figure 5b), whereas the 39C-33 strain showed no significant change in white reporter silencing from CTCF<sup>30</sup> (Figure 5c). These results demonstrate that CTCF<sup>30</sup> is not a ubiquitous modifier of variegated heterochromatic silencing in *Drosophila*, consistent with the absence of an effect on silencing of the paternally inherited Dp(1;f)LJ9 mini-X chromosome. Furthermore, the decreased silencing of the nonimprinted variegators is opposite to the effect of CTCF<sup>30</sup> on Dp(1;f)LJ9<sup>MAT</sup> silencing. Thus, the role of dCTCF in the maintenance of the maternal Dp(1;f)LJ9 imprint represents a distinct parent-specific function for dCTCF on the imprinted Dp(1;f)LJ9 mini-X chromosome.

**Discussion**

CTCF is essential for insulator function in vertebrates, where it plays an active role in regulating imprinted gene expression. In *Drosophila*, dCTCF has likewise been shown to be involved in the insulator function of boundary elements [28,37]. Our results show that parent-specific expression from an imprinted domain in
Drosophila is dependent on dCTCF function. Maintenance of expression from maternally inherited Dp(1;f)LJ9 mini-X chromosome is highly sensitive to dCTCF; even a modest decrease in dCTCF mRNA alters the maternal imprint so that it resembles the paternal imprint.

The effect of dCTCF on maternal-specific expression is limited to the maintenance of imprint. The presence of mutant dCTCF in either the maternal or paternal parents, when the imprint is being established, does not affect the imprint in the progeny. These results are strikingly similar to the role of CTCF in mammalian imprinting, where CTCF assists in the postfertilization formation of an imprinted region, but is dispensable for the establishment of an imprint [11,12,38].

Furthermore, the requirement for dCTCF for maintenance of the maternal Dp(1;f)LJ9 imprint is specific and does not represent a ubiquitous role for dCTCF in regulating heterochromatic silencing. Not only is the paternal Dp(1;f)LJ9 imprint unaffected by mutant dCTCF, but other variegating Drosophila reporter genes respond differently to mutant dCTCF. Thus, the association of dCTCF expression with the maintenance of the maternal Dp(1;f)LJ9 imprint boundary demonstrates a distinct function for dCTCF in imprinted gene expression.

In mammals, maternally imprinted regions that bind CTCF rely critically on this binding to insulate the imprinted loci and establish distinct chromatin domains. Our results show that a reduction in dCTCF levels disrupts the maternal imprint boundary on the Drosophila Dp(1;f)LJ9 mini-X chromosome, and consequently the marker gene, garnet, is silenced. Variegated silencing of garnet from Dp(1;f)LJ9PAT inheritance is a consequence of heterochromatin formation, nucleated from the paternal imprint control region, spreading in cis [29,32]. The
absence of an effect upon the introduction of dCTCF mutant alleles to Dp(1f)IJ9\textsuperscript{PAT} suggests that dCTCF binding and boundary function occurs only on the maternal chromosome. Thus, it is conceivable that a reduction in dCTCF levels enables the spreading of heterochromatin on the maternal Dp(1f)IJ9\textsuperscript{MAT} in a manner similar to that of the paternal Dp(1f)IJ9\textsuperscript{PAT}. This would suggest that dCTCF defines the boundary of a distinct maternal-specific imprinted chromatin domain required to maintain maternal-specific gene expression on the X chromosome.

The model organism Encyclopedia of DNA Elements (mod\textsuperscript{ENCODE} project) provides detailed mapping of regulatory elements throughout the Drosophila genome [39]. Large-scale profiling of dCTCF insulator sites from early embryo mod\textsuperscript{ENCODE} data reveals several candidate dCTCF insulator sites present proximal to the predicted heterochromatic breakpoint of the Dp(1f)IJ9 mini-X chromosome. These dCTCF insulator sites, located between the centric heterochromatin imprinting center and the imprint marker gene garnet, could account for the sensitivity of the maternal imprint to dCTCF expression. If dCTCF were bound only when the X chromosome was transmitted maternally, mutations to dCTCF would disrupt insulator function and lead to maternal silencing of the imprint marker gene. Although such binding remains to be tested, it is similar to the function of CTCF at mammalian imprinted regions.

That the structure of CTCF and its role as an insulator, barrier, and transcriptional regulator is conserved between mammals and insects have been well established [23,27,28]. However, the finding that CTCF maintains its function in regulating the imprinting of diverse genes in such phylogenetically distinct organisms is remarkable. CTCF is a versatile DNA binding factor; subsets of its zinc fingers are adept at binding diverse DNA sequences, and the rest of the protein is able to maintain common regulator interactions and insulator function [40]. This feature may explain how CTCF can regulate imprinting in organisms as diverse as insects and mammals, in which the imprinted target sequences are different.

Previously, the evolutionary origin of imprinting has been extrapolated from the conservation of imprinting among specific genes. Such studies have led to the proposal that mammalian imprinting is of relatively recent origin and restricted to eutherian mammals [41,42]. However, studies showing that the molecular mechanism of imprinting is highly conserved have suggested a much more ancient origin [7,30,43]. Mammalian imprint control elements inserted into transgenic Drosophila act as discrete silencing elements [44,45] and can retain posttranscriptional silencing mechanisms involving
noncoding RNA [46]. Whereas these transgenic imprinting elements lose their parent-specific functions, the retention of epigenetic silencing mechanisms suggests an ancient and conserved origin of imprinting mechanisms. Our finding that CTCF has a role in the maintenance of maternal imprints in insects, as it does in mammals, supports the possibility of evolutionary conservation for both CTCF function and the mechanisms of genomic imprinting.

Conclusions

CTCF is a multifunctional protein with a conserved role as a chromosomal insulator in both mammals and Drosophila. To determine whether dCTCF is involved in imprinted regulation in Drosophila as it is in mammals, we generated a dCTCF mutant allele with severe reduction in dCTCF expression and tested its effects on the expression of the imprint marker gene, garnet, on the Dp(1;f)LJ9 mini-X chromosome. Full garnet gene expression, which occurs when the Dp(1;f)LJ9 mini-X chromosome is maternally inherited, was disrupted when dCTCF expression levels were reduced. No effect of reduced dCTCF expression was observed on the Dp(1;f)LJ9 mini-X chromosome when it was inherited paternally. The effect of dCTCF mutations is on the maintenance rather than on the establishment of the imprint, and is specific to the Dp(1;f)LJ9 mini-X chromosome. These results demonstrate that dCTCF is involved in maintaining parent-specific expression from the maternally inherited X chromosome in Drosophila, a role paralleling its involvement in mammalian imprinting.

Methods

Drosophila culture

All crosses were maintained at 22°C and cultured on standard cornmeal-molasses Drosophila media with methyl benzoate (0.15%) as a mold inhibitor. Each set of crosses was performed in 55-ml shell vials and contained 10-15 virgin females and 10-15 males. Each of the crosses was subcultured three or four times at 3-day intervals before the parents were discarded. Each cross was replicated four to six times, and the progeny were pooled. All stocks were obtained from the Bloomington Drosophila stock center, with the exception of the CTCF30 allele and the variegating fourth chromosome transgene strains. The CTCF30 allele was created by insertion of P[EPgy2] 27 bp downstream of the dCTCF transcription start site. The homozygous lethal CTCF30 allele was generated by imprecise excision of CTCF30. The CTCF30 deletion was characterized by amplification across the break and sequencing of the PCR product. CTCF30 is deleted for all 5′ transposon sequences but retains 4860 bp at the 3′ end. No genomic sequence was removed by the CTCF30 deletion. The fourth chromosome variegating strains were generated using the transposable P element P [hsp26-pt, hsp70-w], which contains the hsp70-driven white gene that is susceptible to silencing caused by heterochromatin formation [47]. The 6-MI93 strain has the construct inserted within a 1360 transposon and inside the Syt7 gene (fourth chromosome coordinate: 323400), whereas the 39C-33 strain is generated from the construct being inserted into gene of the RNA binding protein gawky (fourth chromosome coordinate: 680211), which is in close proximity to a 1360 transposon [47].

To determine the effect of mutant dCTCF on imprint maintenance, the CTCF30 and CTCFEY15833 alleles were crossed into y1 z5 g3d background to yield stable stocks of y1 z5 g3d/Dp(1;f)LJ9; CTCF30/TM3, Sb Ser (where CTCF30 is the CTCF30 or CTCF30 allele). To test the effect of a dCTCF allele on the paternal imprinting of garnet, Dp(1;f)LJ9, y1 g5/Y male flies were crossed to y1 z5 g3d/y1 z5 g3d; CTCF30/Y; TM3, Sb Ser females, and the reciprocal cross with Dp(1;f)LJ9, y1 g5/Y male flies was performed to test the effect on the maternal imprinting of garnet (Figure 6). y1 z5 g3d/Dp(1;f)LJ9; CTCF30/Y male progeny were collected on the basis of wild-type yellow (y1) body color, which independently confirms the presence of the Dp(1;f)LJ9 chromosome, whereas the zeste allele (z5) reduces background eye color of the g allele (g3d). The y1 z5 g3d/Y; Dp(1;f)LJ9; y1 z5 g3d male flies were used as internal controls (Figure 6). The “no modifier” control test cross for paternal garnet imprinting consisted of Dp(1;f)LJ9, y1 g5/Y male flies crossed to y1 z5 g3d/y1 z5 g3d; TM3, Sb Ser/+; females, with the reciprocal cross serving as the maternal control: y1 z5 g3d/Y; Dp(1;f)LJ9; +/+ and y1 z5 g3d/Y; Dp(1;f)LJ9; TM3, Sb Ser/+ male progeny were collected as controls.

To test for the effects of CTCF on germline imprint establishment, mutant CTCF must be present in parents carrying the Dp(1;f)LJ9 mini-X chromosome. To detect the effect of CTCF30 on the establishment of the imprint, Dp(1;f)LJ9; e/e flies were balanced over X3; CTCF30/e for maternal establishment (Figure 7), or X; Y; CTCF30/e for paternal establishment (Figure 8). X3; Dp(1;f)LJ9; CTCF30/e females were crossed to y1 z5 g3d/Y male flies to test maternal imprint establishment (Mat-Est. CTCF30), and the reciprocal cross tested for paternal imprint establishment (Pat-Est. CTCF30). Maternal establishment controls (Mat-Est. CTCF30) consisted of X3; Dp(1;f)LJ9; e/e females crossed to y1 z5 g3d/Y males, and paternal establishment controls (Pat-Est. CTCF30) were X3;Y/Dp(1;f)LJ9; e/e males crossed to y1 z5 g3d/Y; y1 z5 g3d females. External controls were also produced by crossing F1 generation X3; Dp(1;f)LJ9; e/e females to y1 z5 g3d/Y males for maternal establishment, and the reciprocal cross for paternal establishment.
Figure 6 Mating schematic for testing the effect of dCTCF on the maintenance of the Dp(1;f)LJ9 imprint. Two sets of progeny are generated from this cross: progeny that have independently inherited the Dp(1;f)LJ9 mini-X chromosome and a CTCF<sup>x</sup> mutant allele (modifier progeny) and progeny that have inherited Dp(1;f)LJ9 and the TM3, Sb Ser balancer, but had a parent carrying a CTCF<sup>x</sup> mutant allele (maternal and paternal effect test progeny).
To assess the effect of CTCF on other variegating strains, CTCF/TM6, Tb flies were crossed to In(1)wm4 and two variegating fourth chromosome (6-M193 or 39C-33) strains. For the In(1)wm4 crosses, In(1)wm4 females were crossed to w1118; CTCF30 males and the red pigment levels of the In(1)wm4/Y; CTCF30/+ and In (1)wm4/w1118; CTCF30/+ progeny were compared with that of their In(1)wm4; TM6, Tb siblings. Reciprocal crosses were performed with the variegating fourth chromosome strains to control for both the maternal and paternal inheritance of the variegating transgene. The maternal cross consisted of w-/w-; +/+; +/-; var/var females crossed to y/w-; CTCF30 /TM6, Tb; +/-; +/- males, and paternal inheritance used y/w-; +/-; +/-; var/var males crossed to w-/w-; CTCF30 /+; +/-; +/- females, where var represents the variegating fourth chromosome transgene. The resulting progeny were separated by sex (y/w-; CTCF30; +/-; var/var males and w-/w-; CTCF30; +/-; +/-; var/var females) and compared with the balancer controls (y/w-; TM6, Tb/+; +/-; var/var males and w-/w-; TM6, Tb/+; +/-; var/var females).

**Measurement of dCTCF expression**

qRT-PCR was used to measure dCTCF expression. Total RNA was prepared from three groups of 50 larvae for each genotype. One microgram of total RNA was
reverse transcribed using random hexamers and ImProm-II reverse transcriptase (Promega). Quantitative PCR was performed as previously described [48].

dCTCF primers (CTCF F2400, ACGAGGAGGTGTTGGTCAAG and CTCF R2485, ATCATCTCGTCCTCGAAC) were used at 300 nM. Two technical replicates from each sample were amplified. Expression was normalized to Dmn, a gene that has proved reliable for this purpose [48].

Quantification of eye pigment levels
Expression of the imprint marker gene garnet was quantified both visually and through the use a spectrophotometric assay of extracted eye pigments. The visual assay assigns each eye a score in relation to its variegation class as described by Joanis and Lloyd [32]: 0-25% pigmentation, 25-50% pigmentation, 50-75% pigmentation, and 75-100% pigmentation. The prevalence of each variegation class is expressed as a percentage of all eyes assayed. As the variegated phenotype of maternally inherited Dp(1;f)LJ9 in the presence of mutant dCTCF is skewed toward fully pigmented eyes, a second assay with the following variegation classes was performed: >80% pigmentation, 80-90% pigmentation, 90-100% pigmentation, and 100% pigmentation.

The spectrophotometric assay was adapted from Real et al. [49]. For red (pteridine) pigment, flies of each test genotype were aged for 4 days and then placed in

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**Figure 8** Mating schematic for testing dCTCF for an effect on the paternal establishment of the Dp(1;f)LJ9 imprint. Two primary sets of progeny and an external control were generated from this cross: progeny with a paternally-transmitted Dp(1;f)LJ9 mini-X chromosome from fathers with the CTCF30 mutation (Pat-Est. CTCF30) and progeny that also have a paternally imprinted Dp(1;f)LJ9 mini-X chromosome but from fathers wild type for CTCF (Pat-Est. CTCF+). External control crosses were produced by crossing F1 generation X/Y/Dp(1;f)LJ9, e/e males to y1z^a^g^53d/y^1^Z^a^g^53d females (not depicted).
1.5-ml Eppendorf microtubes and stored at -30°C. For each sample set, eight heads were placed into a 0.6-ml microtube containing 400 μl of acidified ethanol (30% EtOH, acidified to pH 2 with HCl). Pigment was extracted on an orbital shaker at 150 rpm in the dark for 48 hours. Absorbance of the extracted pigments was measured at 480 nm. Each 400-μl sample of extracted pigment was split into two 200-μl volumes, independently measured, and the values were averaged. Five tubes were run per sample set, with the values averaged and expressed as a percentage of wild-type pigment levels ± standard deviation. For brown (ommatochrome) pigment, flies of each test genotype were aged for 4 days and then placed in 1.5-ml Eppendorf microtubes and stored at -80°C. Ten heads were placed in a 1.5-ml Eppendorf tube and homogenized with 150 μl of 2 M HCl and 0.66% sodium metabisulfite (wt/vol). A total of 200 μl of 1-butanol was added, and the mixture was placed on an orbital shaker at 150 rpm for 30 min before being centrifuged at 9000 g for 5 min. The organic layer was removed, washed with 150 μl of 0.66% sodium metabisulfite in dH2O and placed back on the orbital shaker for a further 30 min, and then this step was repeated for a second wash. The organic layer was removed and measured for absorbance at 492 nm. Five tubes were run per sample set, with the values averaged and expressed as a percentage of wild-type (O. R) pigment levels ± standard deviation. Absorbance was determined with a Pharmacia Biotech Ultrospec 2000 spectrophotometer. Representative eye pictures were photographed with a Zeiss AxioCam MRc5 mounted on a Zeiss Stemi 2000-C dissecting microscope.

Spectrophotometric assay for quantifying the expression of the white transgene on the fourth chromosome variegating strains and In(1)wm4 followed the same procedure for fly aging and head collection. Heads were split into groups of five and placed into 150 μl of 30% EtOH acidified to pH 2 with HCl. Pigment extraction consisted of sonicating samples for 5 seconds at 50 MHz (Sonic Dismembrator Sonicator) prior to soaking samples at room temperature for 24 hours in the dark. For each sample, 90 μl of extracted pigment was loaded into 96-well microtiter trays and quantified with a Microplate Reader (Benchmark Bio-Rad) at a wavelength of 480 nm. The results from each sample group were pooled for a final mean pigment value. Pigment levels for imprint establishment crosses and assays, GES performed the imprint establishment crosses and assays and VR produced the CTCF transgene on the fourth chromosome strain crosses and assays, GES performed the regulation of imprinting in clusters: noncoding RNAs versus insulators. Adv Genet 2008, 61:207-223.

Acknowledgements
This study was enabled by strains created by the BDGP and supplied by the Bloomington Drosophila Stock Center. We would like to thank S. Elgin for the generous donation of the 6-M193 and 39C-33 strains and Elizabeth Whitfield for technical assistance. This work was supported by a Natural Sciences and Engineering Council grant to VKL, an ESCALATE award and Wayne State University Research Award to VM. WAM acknowledges the support of the Dalhousie Patrick Lett fund.

Authors’ contributions
WAM performed the imprinting experiments, provided analysis, and wrote the manuscript. DM characterized the CTCF alleles, NB performed the variegating 4th chromosome strain crosses and assays, GES performed the imprint establishment crosses and assays and VR produced the CTCF allele. VKL and VM participated in the conceptualization and design of the experiments, performed analysis, and participated in writing the manuscript. All authors read and approved the final manuscript.

Received: 10 December 2009 Accepted: 30 July 2010
Published: 30 July 2010

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