

CTCF is conserved from *Drosophila* to humans and confers enhancer blocking of the *Fab-8* insulator

Hanlim Moon¹, Galina Filippova², Dmitry Loukinov¹, Elena Pugacheva¹, Qi Chen³, Sheryl T. Smith³, Adam Munhall², Britta Grewe⁴, Marek Bartkuhn⁵, Rüdiger Arnold⁵, Les J. Burke⁵, Renate Renkawitz-Pohl⁴, Rolf Ohlsson⁶, Jumin Zhou³, Rainer Renkawitz⁵⁺ & Victor Lobanenkov¹⁺⁺

¹Section of Molecular Pathology, Laboratory of Immunopathology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland, USA, ²Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA, ³Molecular Genetics Division, The Wistar Institute, Philadelphia, Pennsylvania, USA, ⁴Philipps-Universität Marburg, Fachbereich Biologie, Zoologie/Entwicklungsbiologie, Marburg, Germany, ⁵Institute for Genetics, Justus-Liebig-Universität Giessen, Giessen, Germany, and ⁶Department of Development & Genetics, Evolution Biology Centre, Uppsala University, Uppsala, Sweden

Eukaryotic transcriptional regulation often involves regulatory elements separated from the cognate genes by long distances, whereas appropriately positioned insulator or enhancer-blocking elements shield promoters from illegitimate enhancer action. Four proteins have been identified in *Drosophila* mediating enhancer blocking—Su(Hw), Zw5, BEAF32 and GAGA factor. In vertebrates, the single protein CTCF, with 11 highly conserved zinc fingers, confers enhancer blocking in all known chromatin insulators. Here, we characterize an orthologous CTCF factor in *Drosophila* with a similar domain structure, binding site specificity and transcriptional repression activity as in vertebrates. In addition, we demonstrate that one of the insulators (*Fab-8*) in the *Drosophila Abdominal-B* locus mediates enhancer blocking by dCTCF. Therefore, the enhancer-blocking protein CTCF and, most probably, the mechanism of enhancer blocking mediated by this remarkably versatile factor are conserved from *Drosophila* to humans.

Keywords: *Drosophila*; CTCF; enhancer blocking; Fab-8; Abdominal-B

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INTRODUCTION

Expression of the eukaryotic genome is controlled by enhancer and silencer elements, both of which can mediate their function from a distance. Insulator elements with enhancer-blocking activity curb enhancer activity, such that only appropriate promoters are activated (Ishii & Laemmli, 2003). The proteins that mediate insulator function have been identified for only a few *Drosophila* insulator sequences. These are Zw5, BEAF-32 (Zhao *et al*, 1995; Gaszner *et al*, 1999), GAGA factor (Ohtsuki & Levine, 1998; Belozzerov *et al*, 2003) and Su(Hw) (Gerasimova *et al*, 1995).

Another perspective on the requirement of insulators comes from the fact that many genes are controlled by several regulatory elements needed for tissue- and cell-specific expression. For example, the *Drosophila* gene *Abdominal-B* (*Abd-B*) contains an extended 3' regulatory region that is functionally subdivided into distinct enhancer domains. Functional separation of the enhancer sequences is achieved by intervening insulators such as *Front-abdominal* (*Fab*)-7 and *Fab*-8 (Hagstrom *et al*, 1996; Barges *et al*, 2000). Although both elements have been shown to mediate enhancer-blocking function, the protein involved in this activity has not been described.

In sharp contrast to *Drosophila*, the genome of vertebrates is much more expanded, due primarily to larger distances between genes. Therefore, the need for insulators to separate genes may not seem as pronounced as it is in *Drosophila*. Indeed, until now, only a single protein, CTCF, has been identified to mediate enhancer-blocking activity (Ohlsson *et al*, 2001). Binding sites for CTCF have been shown to be involved in

¹Section of Molecular Pathology, Laboratory of Immunopathology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852, USA

²Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109, USA

³Molecular Genetics Division, The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104, USA

⁴Philipps-Universität Marburg, Fachbereich Biologie, Zoologie/Entwicklungsbiologie, Karl-von-Frisch-Strasse, D-35039 Marburg, Germany

⁵Institute for Genetics, Justus-Liebig-Universität Giessen, Heinrich-Buff-Ring 58–62, D-35392 Giessen, Germany

⁶Department of Development & Genetics, Evolution Biology Centre, Uppsala University, Norbyvägen 18A, S-752 36 Uppsala, Sweden

*Corresponding author. Tel: +49 641 993 5460; Fax: +49 641 993 5469; E-mail: renkawitz@gen.bio.uni-giessen.de

++Corresponding author. Tel: +1 301 435 1690; Fax: +1 301 402 0077; E-mail: vlobanenkov@niaid.nih.gov

gene activation (Vostrov & Quitschke, 1997), gene repression (Baniahmad *et al*, 1990; Lobanenkov *et al*, 1990) and enhancer blocking (Bell *et al*, 1999; Hark *et al*, 2000; Kanduri *et al*, 2000; Szabo *et al*, 2000; Filippova *et al*, 2001; Lutz *et al*, 2003; Tanimoto *et al*, 2003). Furthermore, vertebrate- and mammalian-specific functions, such as X-chromosome inactivation and control of the epigenetic DNA methylation state, seem to involve CTCF (Lee, 2003).

Obviously, the function of enhancer blocking has developed during evolution such that *Drosophila* uses several proteins and mechanisms for enhancer blocking and insulation (Kuhn *et al*, 2003). However, none of the known *Drosophila* insulator proteins has a counterpart found to be conserved in vertebrates. Rather, vertebrates use CTCF, which has not yet been found in *Drosophila*. Here, we characterize a *Drosophila* orthologue of CTCF with similarities to many of the features identified for vertebrate CTCF. Furthermore, we show that a previously characterized *Drosophila* insulator, *Fab-8*, mediates enhancer blocking by CTCF in *Drosophila* as well as in vertebrate cells. Thus, the enhancer-blocking protein CTCF and, probably, the mechanisms of CTCF-driven enhancer blocking are both conserved from *Drosophila* to humans.

RESULTS AND DISCUSSION

Vertebrate and *Drosophila* CTCF are similar

FlyBase data entries and cDNA sequence analysis (see supplementary information online) revealed an open reading frame (ORF) coding for a protein similar to vertebrate CTCF with respect to the overall structure. dCTCF contains all of the expected 11 zinc fingers (Zn-fingers), separated by both standard and noncanonical inter-finger linkers (Fig 1A). Furthermore, most of the crucial DNA base recognition residues at positions -1, 2, 3 and 6 are identical. Variation in position 6 for fingers #6 and #9 generate a change from alanine or serine to methionine, which is of no consequence for the DNA-binding specificity, as the recognition code is not changed (Suzuki *et al*, 1994).

Similarities in Zn-fingers do not necessarily imply similarities in function. Therefore, we examined whether dCTCF can act as a transcriptional repressor, as has been demonstrated previously for vertebrate CTCF (Burcin *et al*, 1997). The strongest repressive function has been shown to reside within the combined carboxy-terminal plus Zn-finger domains (Lutz *et al*, 2000). Equivalent regions of *Drosophila* and chicken CTCF (chCTCF) were fused to the yeast GAL4 transcription factor DNA-binding domain. Both *Drosophila* and chicken GAL4-CTCF fusions repressed reporter gene activity to a similar extent in two different cell lines (Fig 1E) and in a way comparable with the previously characterized strong repressor GAL4-*v-erbA*362 (Baniahmad *et al*, 1992). These results clearly indicate that dCTCF, like its vertebrate counterpart, has transcriptional repressor activity.

In vertebrates, CTCF is ubiquitously expressed (Burke *et al*, 2002), apparently functioning as a global transcriptional regulator in all cell types (Ohlsson *et al*, 2001). In comparison, we monitored *dCTCF* RNA expression levels at various stages of fly development. Using *in situ* hybridization, we found that *dCTCF* RNA is present in the cytoplasm of the nurse cells within the fly egg chamber (Fig 1B), transported into and distributed uniformly in the developing oocyte and in 0–24h embryos as a maternal

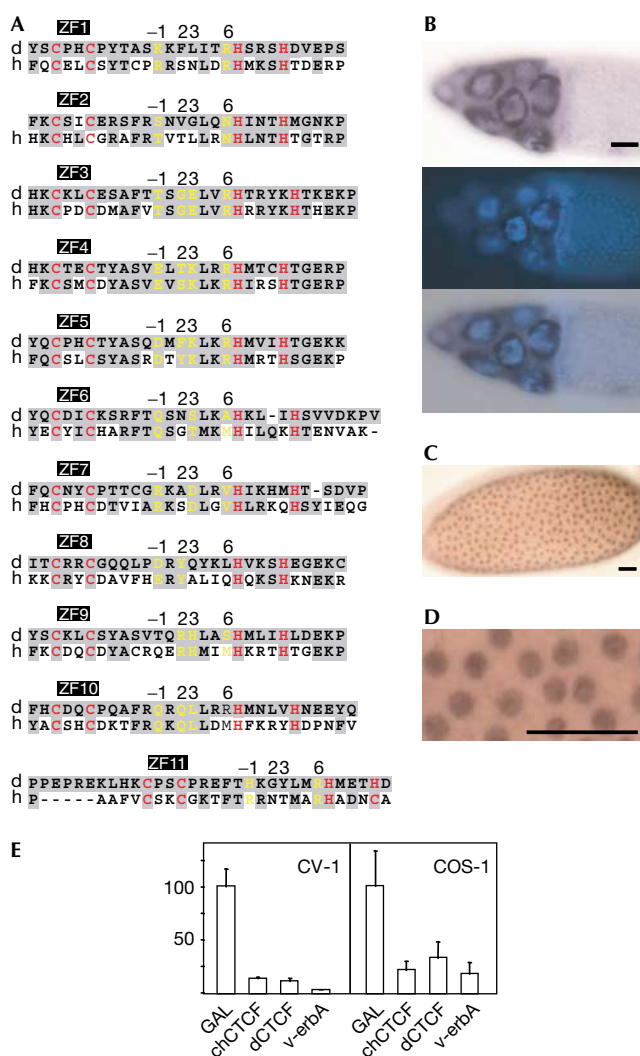


Fig 1 | *Drosophila* CTCF encodes a protein similar to human CTCF. (A) Sequence alignment of the Zn-finger domain of dCTCF and hCTCF. Similar and identical residues (shading), zinc-coordinating amino acids (red) and amino acids with identical binding site recognition (yellow) are indicated. (B) *In situ* hybridization of *Drosophila* egg chamber with DIG-labelled antisense dCTCF RNA. Polyploid nurse cell nuclei are surrounded by dark staining of cytoplasmic dCTCF RNA (top), nuclei are identified by Hoechst staining (middle) and in the overlay (bottom). The egg chamber to the right is surrounded by follicle cells (Hoechst-stained nuclei). (C,D) dCTCF detection in syncytial blastoderm stage 3 with the rabbit anti-dCTCF-C antibody and visualization by avidin-peroxidase at two different magnifications (scale bars (B–D), 40 µm). (E) CV-1 and COS-1 cells were transfected with DNA constructs expressing the GAL DNA-binding domain (GAL), a fusion of the Zn-finger domain plus the C-terminus of chicken CTCF, a similar fusion of dCTCF or of *v-erbA*. Relative CAT activity is calculated with the GAL transfection defined as 100%.

RNA (not shown). Later stages show expression in all tissues and stages (see supplementary information online), revealing that dCTCF is a ubiquitous factor as in vertebrates. Location of dCTCF protein is clearly nuclear, exemplified by the nuclear

staining of syncytial blastoderm embryos with dCTCF-specific antibodies (Fig 1C,D).

To extend the comparison of vertebrate and *Drosophila* CTCF, we tested *in vitro*-translated *Drosophila* and vertebrate CTCF for binding to several previously characterized vertebrate CTCF targets (CTS). The sequences tested included the CTS of the FII insulator element of the β -globin gene, the *APP* gene, the *myc* genes and the mouse *ARF* promoter (Fig 2A–D). With the exception of the two *myc* FPV and A sites, all the other sequences bound chicken and *Drosophila* CTCF similarly. Binding and enhancer-blocking results with additional CTS are summarized in supplementary Table 1 online.

We used a methylation-interference assay to determine whether both proteins contact the same guanidine nucleotides on a given target DNA site (Fig 2E). Both *Drosophila* and human CTCF were found to contact the same nucleotides on the β -globin FII insulator fragment. These results indicate that, despite considerable overall sequence divergence, fly and human CTCF show a striking degree of functional conservation with respect to DNA binding.

Enhancer blocking of the *Fab-8* insulator by CTCF

To identify potential *Drosophila* CTCF regulatory targets, we performed an *in vitro* screen for CTCF-binding sites (supplementary information online) and found the *Fab-8* element for which

enhancer-blocking and boundary function have been shown (Barges *et al*, 2000). This sequence is situated in the *Abd-B* locus, separating and insulating enhancer domains *infraabdominal-7* (*iab-7*) from *iab-8*. As the protein involved in this mechanism was unknown, and as vertebrate CTCF mediates enhancer-blocking activity (Ohlsson *et al*, 2001), we tested whether dCTCF might have a similar role in the context of the *Fab-8* element. *In vitro* binding of dCTCF to *Fab-8*, as determined by methylation interference (Fig 3A), suggested two binding sites for CTCF. We generated binding site mutations resulting in single-site mutations (mut1 or mut2) and in a double-site mutation (mut1 + 2) used for electrophoretic mobility shift assay (EMSA; Fig 3B). The wild-type *Fab-8* element generates two retarded bands corresponding to a different mobility of the same DNA molecule occupied by CTCF at one of the two closely spaced CTS sequences. These different mobilities are probably caused by a site-specific DNA bending (Arnold *et al*, 1996), which has also been observed on other dual binding sites, such as the H19 locus (Kanduri *et al*, 2000). Excess protein generated a slow mobility complex only resolved after a long run of the gel (Fig 3B), reflecting binding of CTCF to both sites.

To test *in vivo* dCTCF binding to this important element, we prepared crosslinked chromatin from *Drosophila* embryos and precipitated CTCF-occupied sites with the anti-dCTCF-C antibody. PCR primers for the *Fab-8* sequence identified specifically

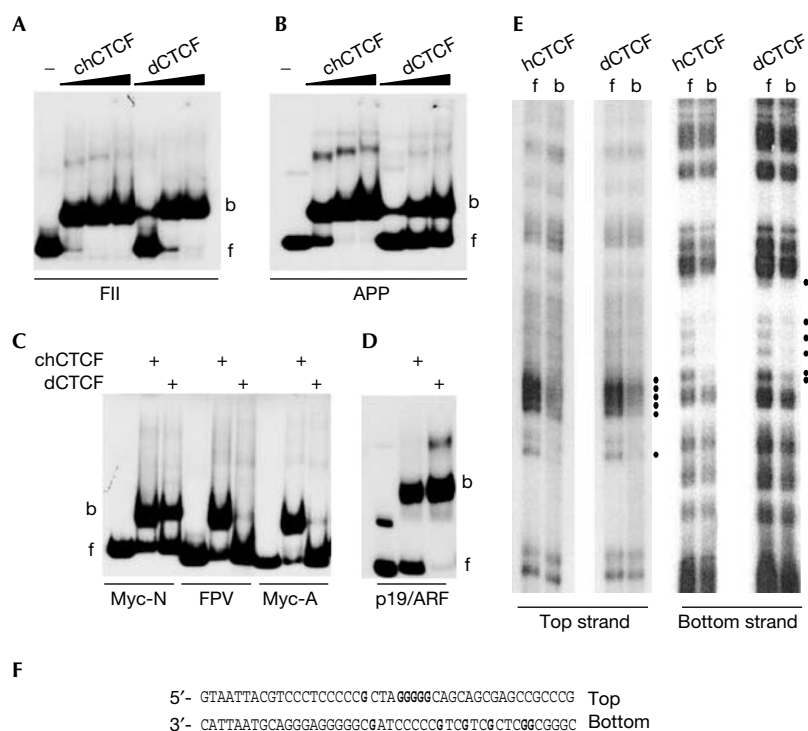


Fig 2 | Similar binding site specificity on vertebrate target sites. Zn-finger domains of chCTCF and dCTCF were *in vitro* translated and incubated with DNA. (A–D) Indicated DNA fragments were tested in electrophoretic mobility shift assay (EMSA), the β -globin FII insulator sequence (Bell *et al*, 1999), the CTCF-binding site in the *APP* gene (Vostrov & Quitschke, 1997), the sites N, FPV and A of the *myc* genes (Filippova *et al*, 1996; Lutz *et al*, 2003) and in the mouse *ARF* promoter (Filippova *et al*, 2002). (E) DMS methylation interference analysis was carried out with the β -globin FII insulator fragment labelled on the top or the bottom strand. After separation of bound (b) and unbound fragments (f), the DNA was analysed on a sequencing gel. The G nucleotides interfering with binding after methylation are indicated by dots, and in the sequence (F) by bold lettering.

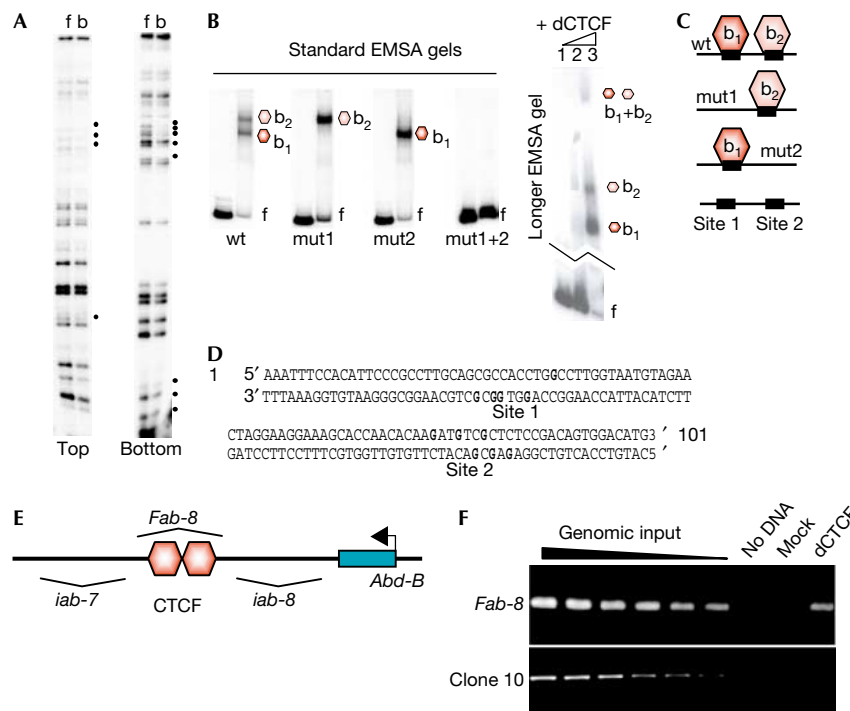


Fig 3 | *In vitro* and *in vivo* binding of dCTCF to the *Fab-8* element. (A) DMS methylation interference was determined with the *in vitro*-translated dCTCF Zn-finger domain. Bound (b) and unbound fragments (f) labelled on the top or the bottom strand were analysed on a sequencing gel. Two groups of interfering nucleotides (marked with dots) are indicated (sites 1 and 2). (B) EMSA analysis of dCTCF binding. *In vitro*-translated, full-length dCTCF was incubated with the *Fab-8* wild-type fragment and with the mutants mut1, mut2 and mut1 + 2. Interpretation of the single and double occupancy of dCTCF on the respective fragments is shown in (C), double occupancy can only be visualized with excess of protein (long gel, lane 3, (B)). The migration difference of b₁ and b₂ complexes is probably caused by site-specific DNA bending (see text). (D) Contact G nucleotides in the *Fab-8* sequence (bold) are converted to AT in mutants mut1 and mut2 (see also supplementary information online). (E) Diagram (not to scale) of the *Fab-8* location downstream of the *Abdominal-B* gene, flanked by the *iab-7* and *iab-8* regulator sequences. (F) *In vivo* binding of dCTCF to *Fab-8*. Chromatin from *Drosophila* embryos was precipitated with the rabbit anti-dCTCF-C antibody (dCTCF) and tested by PCR for the presence of *Fab-8* sequences. Increasing amounts of genomic input are included for standardization. A sequence (clone 10) without dCTCF binding, mock precipitation and no DNA are included as negative controls with no signal.

precipitated chromatin, whereas primers against a different non-dCTCF-binding site (clone 10) and mock-precipitated chromatin resulted in no signal (Fig 3F).

To test the functional similarity between dCTCF and vertebrate CTCF, we analysed enhancer blocking of *Fab-8* in vertebrate K562 cells. In comparison to the known enhancer-blocking effect mediated by the *FII* sequence (Bell *et al*, 1999), a similar reduction in colony numbers mediated by *Fab-8* was seen (Fig 4A). More importantly, specific abrogation of CTCF binding by the double mutation, mut1 + 2, resulted in loss of enhancer blocking.

The crucial test for enhancer-blocking activity of dCTCF had to be carried out in flies. Therefore, we used a vector with two regulatory regions containing the *iab-5* enhancer from the *Abd-B* locus and two copies of the minimal twist enhancer, PE, directing an additive pattern of expression when placed between divergently transcribed *white* and *lacZ* reporter genes (Lin *et al*, 2003). As shown in Fig 4C,D, the *iab-5* enhancer directs expression in the posterior one-third of the blastoderm stage embryo, whereas the 2 × PE enhancer activates transcription in the ventral-most region where twist is normally expressed. Enhancer elements are enhancing both the *white* gene as well as the *lacZ* gene. Altered

patterns of transcription were observed when the 1 kb spacer sequence was replaced by the 680 bp *Fab-8* element. On the *white* promoter, the *iab-5* activity was completely abolished (shown as the lack of staining in the *iab-5* activity region), while the 2 × PE enhancer was still activating the *white* gene (Fig 4E). The *lacZ* promoter, conversely, could be activated only by the proximal *iab-5* but not by the distal 2 × PE (Fig 4F). This result suggests that the *Fab-8* fragment blocks the respective distal enhancer for both the *white* and the *lacZ* promoters. When the CTCF sites were mutagenized (mut1 + 2 as in Fig 3), the *iab-5* activity on *white* was partly restored (Fig 4G). Similarly, the 2 × PE element again directed the transcription of the *lacZ* gene (Fig 4H). Chromatin/CTCF immunoprecipitation revealed specific CTCF binding to the *Fab-8* element of the enhancer-blocking vector, whereas binding to the *Fab-8* mut element was clearly reduced (Fig 4I). This correlation between strong CTCF binding and full enhancer-blocking function (Fig 4E,F) indicates that the activity of *Fab-8* is at least partly mediated by CTCF and that dCTCF, similar to vertebrate CTCF, confers enhancer blocking.

Thus, at least one enhancer-blocking protein (CTCF) in *Drosophila* and vertebrates is conserved with a similar enhancer-blocking

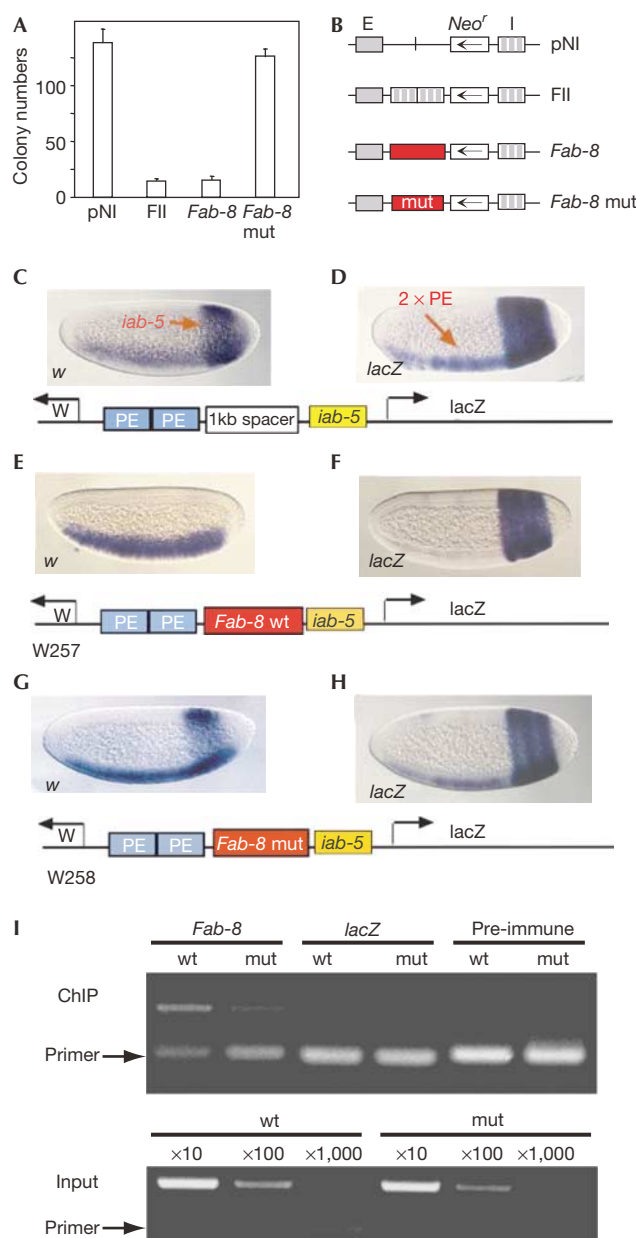


Fig 4 | CTCF-dependent enhancer blocking of *Fab-8* in vertebrate cells and in *Drosophila*. (A) K562 cells were transfected with the indicated DNA constructs (B) and colony number was determined after neomycin selection. The pNI and FII constructs (Chung *et al*, 1993) were used as controls. Transcription of the neomycin gene (*Neo*) is driven by the β-globin enhancer (E). In the FII construct, two copies of the β-globin insulator (I) separate the enhancer from *Neo*. The *Fab-8* fragment mediates enhancer blocking, whereas mutation of both CTCF-binding sites (*Fab-8* mut) does not. (C–H) *Fab-8* enhancer blocking in *Drosophila* depends on dCTCF-binding sites. Transgenic embryos with the indicated vectors were hybridized with either digoxigenin-labelled *white* (*w*) in (C,E,G) or *lacZ* antisense RNA probes (D,F,H). (C,D) *white* and *lacZ* expression is visualized when a 1.6 kb λ DNA fragment was inserted between the 2 × PE and *iab-5* enhancers (see text). (E,F) The *Fab-8* DNA insert between 2 × PE and *iab-5* enhancer was analysed in 13 independent lines. Expression of the *white* reporter gene is restricted to the ventral mesoderm and of the *lacZ* gene to the abdomen. With the CTCF sites mutagenized, 22 independent lines were generated: *white* gene activity in the abdomen (G) and *lacZ* expression in the ventral mesoderm (H) are seen. (I) Chromatin was prepared from embryos carrying transgenes with either *Fab-8* wt or the mut1 + 2 CTCF-binding sites (mut). Primers that specifically recognize transgenic (not genomic) sequences were used. ChIP using anti-CTCF-C antibody shows an increased occupancy at wild-type versus mutant sequences, in contrast to pre-immune serum and to *lacZ* sequences. For standardization, input dilutions were subjected to semiquantitative PCR as well.

Repression and enhancer blocking. Repression analysis in COS-1 and CV-1 cells was performed as described earlier (Lutz *et al*, 2000). The colony formation assay in K562 cells has been carried out essentially as in Chung *et al* (1993) and Lutz *et al* (2003). The *Drosophila* enhancer-blocking assay was carried out as described (Lin *et al*, 2003).

For information regarding primers for methylation interference, site-directed mutagenesis, plasmid construction, peptides for antibody production, immunocytology, western and chromatin immunoprecipitation, as well as *Drosophila* target-site library construction, see supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboports.org>).

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function. In addition to enhancer blocking, mammalian CTCF has gained functions involving the control of epigenetic states in the context of imprinted genes and X-chromosome inactivation (Lee, 2003; Lewis & Murrell, 2004).

METHODS

EMSA. Full-length CTCF and 11 Zn-finger fragments were subcloned into pET 16B vector by PCR-designed primers. *Drosophila* CTCF proteins were produced using the TnT *in vitro* coupled transcription–translation kit (Promega Co., Madison, WI, USA). Probes for EMSA were obtained by PCR, gel purified, end-labelled by T4 kinase and used in DNA binding as described (Filipova *et al*, 1996).

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