



dREAM co-operates with insulator-binding proteins and regulates expression at divergently paired genes

Citation

Korenjak, Michael, Eunjeong Kwon, Robert T. Morris, Endre Anderssen, Arnaud Amzallag, Sridhar Ramaswamy, and Nicholas J. Dyson. 2014. "dREAM co-operates with insulator-binding proteins and regulates expression at divergently paired genes." *Nucleic Acids Research* 42 (14): 8939-8953. doi:10.1093/nar/gku609. <http://dx.doi.org/10.1093/nar/gku609>.

Published version

<https://doi.org/10.1093/nar/gku609>

Link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:13581241>

Terms of use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material (LAA), as set forth at

<https://harvardwiki.atlassian.net/wiki/external/NGY5NDE4ZjgzNTc5NDQzMGIzZWZhMGFIOWI2M2EwYTg>

Accessibility

<https://accessibility.huit.harvard.edu/digital-accessibility-policy>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#)

dREAM co-operates with insulator-binding proteins and regulates expression at divergently paired genes

Michael Korenjak¹, Eunjeong Kwon², Robert T. Morris¹, Endre Anderssen¹,
Arnaud Amzallag¹, Sridhar Ramaswamy¹ and Nicholas J. Dyson^{1,*}

¹Massachusetts General Hospital Cancer Center and Harvard Medical School, Charlestown, MA 02129, USA and

²Massachusetts General Hospital, Cutaneous Biology Research Center, Charlestown, MA 02129, USA

Received March 6, 2014; Revised June 03, 2014; Accepted June 24, 2014

ABSTRACT

dREAM complexes represent the predominant form of E2F/RBF repressor complexes in *Drosophila*. dREAM associates with thousands of sites in the fly genome but its mechanism of action is unknown. To understand the genomic context in which dREAM acts we examined the distribution and localization of *Drosophila* E2F and dREAM proteins. Here we report a striking and unexpected overlap between dE2F2/dREAM sites and binding sites for the insulator-binding proteins CP190 and Beaf-32. Genetic assays show that these components functionally co-operate and chromatin immunoprecipitation experiments on mutant animals demonstrate that dE2F2 is important for association of CP190 with chromatin. dE2F2/dREAM binding sites are enriched at divergently transcribed genes, and the majority of genes upregulated by dE2F2 depletion represent the repressed half of a differentially expressed, divergently transcribed pair of genes. Analysis of mutant animals confirms that dREAM and CP190 are similarly required for transcriptional integrity at these gene pairs and suggest that dREAM functions in concert with CP190 to establish boundaries between repressed/activated genes. Consistent with the idea that dREAM co-operates with insulator-binding proteins, genomic regions bound by dREAM possess enhancer-blocking activity that depends on multiple dREAM components. These findings suggest that dREAM functions in the organization of transcriptional domains.

INTRODUCTION

The E2F family of transcription factors is a central regulatory hub for the proper control of key cellular processes such as cell cycle progression, differentiation and apopto-

sis. Discovered more than two decades ago, E2F has been shown to be of critical importance in a plethora of organisms, ranging from mammals, insects and worms to plants (1–3). E2F transcription factors are heterodimeric complexes consisting of an E2F and a DP subunit. Activator E2Fs mediate the expression of critical cell cycle-regulated genes, which in turn facilitate progression from the G1 to S phase of the cell cycle. pRB family proteins transiently inhibit activator E2Fs, providing temporal regulation of transcription. pRB family proteins also interact with repressor E2Fs to suppress transcription of E2F target genes. Repressor E2F complexes are more stable than activator complexes and occupy E2F target genes during quiescence and at the onset of cellular senescence.

Human cells contain at least three activator E2Fs and five repressor E2Fs. The roles of these proteins are highly interconnected and the complexity of the E2F network has become a roadblock to our understanding of E2F function. It has not yet been possible, for example, to generate a comprehensive map of E2F proteins on the chromatin of mammalian cells. The Rb–E2F pathway is functionally conserved in *Drosophila*, but the smaller number of family members makes this a much more tractable experimental system. The activity of the activator E2F (dE2F1) is transiently repressed by RBF1 (4–7), whereas the repressor E2F (dE2F2) resides in a multisubunit complex (dREAM/Myb-MuvB) with either RBF1 or 2, dMyb and a set of dMyb-interacting proteins (Mips) (8,9).

Over the past 10 years dREAM complexes have been characterized extensively in flies, worms and human cells (3,10). Biochemical purifications show that the composition of dREAM is highly conserved between these organisms (8,9,11–13). The *Drosophila* complex is best characterized for its role in the stable repression of developmentally regulated genes (8,9,14,15). In contrast, in human cells dREAM has mainly been implicated in the regulation of cell cycle genes (12,13,16,17). Its functions include the shutdown of cell cycle genes during quiescence and upon induction of senescence (12,18,19). dREAM lacks any known enzymatic activity and it does not bind to a well-characterized

*To whom correspondence should be addressed. Tel: +1 617 726 7804; Fax: +1 617 726 7808; Email: Dyson@helix.mgh.harvard.edu

chromatin mark. Although genetic experiments suggest that dREAM is involved in the epigenetic regulation of gene expression (20), it is currently unclear how dREAM promotes stable transcriptional repression.

ChIP experiments have identified thousands of dREAM binding sites in the *Drosophila* genome. However, the expression level of most dREAM-bound genes (~92%) was unchanged following knockdown of individual dREAM components (6,14). These observations may be explained, at least in part, by functional redundancy/compensation between dREAM subunits, or between dREAM and other chromatin-associated complexes. Alternatively, the varied importance of dREAM complexes may be due to other proteins. It is conceivable that transcriptional activators required for the expression of dREAM-bound genes are only expressed in specific developmental or environmental contexts, or the activity of dREAM may depend on substoichiometric dREAM components (9). In these models, dREAM proteins might remain bound at many potential targets even when they are not functional or needed for repression. These observations highlight some key unanswered questions about dREAM. What do dREAM complexes do? Are all dREAM complexes active and functional or only a few?

Several observations have suggested that pRB–E2F complexes may not simply direct transcriptional regulation at the nearest-bound promoter but may also have more extensive effects on chromosome structure. The *Caenorhabditis elegans* ortholog of pRB, LIN-35, exhibits extensive binding site overlap with HPL-2 (the ortholog of Hp1) in the intestine (21). These sites are characterized by an unusually low overlap with promoters of known genes and are significantly enriched on the X chromosome. Inactivation of pRB family members in mouse fibroblasts results in hypocondensed chromatin and decreased levels of H3K27me3 at repetitive sequences throughout the genome (22–25). Mutation of fly *rbf1* gives rise to hypocondensed chromosomes during mitosis. This may be due, at least in part, to an interaction between RBF1 and the Condensin II complex (26). Such changes in chromosome compaction may be the consequence of altered expression of critical regulatory factors but they may also reflect a direct role for RBF1 and the Condensin II subunit Cap-D3 in chromosome organization. The common set of genes altered by loss of RBF1 or Cap-D3 includes a significant fraction of gene clusters. Interestingly, one such cluster is bracketed by RBF1 and Cap-D3 binding sites upstream and downstream of the cluster, but lacks binding sites in the promoter regions (27), suggesting that RBF1 may influence gene expression through an effect on the overall architecture of the region.

Here, we describe an unexpected connection between dREAM and insulator-binding proteins that provides a new perspective on the action of dREAM complexes. Insulators are DNA elements that possess enhancer-blocking and/or barrier activity. Enhancer-blocking insulators have the ability to disrupt the communication between an enhancer and a promoter when placed between these two elements. Barrier insulators, in contrast, protect a flanked transgene from position-dependent silencing effects. In *Drosophila*, the study of insulator elements led to the identification of sequence-specific DNA-binding proteins that can associate

with these elements. Based on the DNA-binding factors, insulator elements have been classified into Su(Hw), dCTCF, Beaf-32 and GAF-dependent insulators (28). These factors are required for the enhancer-blocking and/or barrier function of insulator elements. CP190 and Mod(mdg4)2.2 are thought to get recruited to insulator complexes through interaction with the sequence-specific DNA-binding proteins and are also required for enhancer-blocking activity (29–33).

Insulator-binding proteins associate with thousands of sites in the *Drosophila* genome (34–38) and localize extensively to topological domain boundaries identified in genome-wide chromosome conformation studies (39,40). Together with a role of insulator elements and proteins in long-range chromatin interactions (41–44), these findings raise the possibility of an important function for insulator-binding proteins in nuclear architecture.

The genomic distribution of insulator-binding proteins suggests that there are likely to be multiple types of sites where these factors serve distinct roles. For example, Beaf-32 is frequently bound close to transcription start sites (TSS), dCTCF is less commonly associated with promoters, and Su(Hw) binding sites are mostly found at intergenic regions and introns, whereas CP190 co-localizes extensively with all three sequence-specific DNA-binding factors. The genome-wide studies also demonstrate that distinct classes of insulator proteins frequently bind the same sites (34,37,45). Several additional proteins, including Topoisomerase II, the ubiquitin ligase dTopors, the Rm62 helicase and the RNAi-component Ago2, have been implicated in proper insulator function (42,46–48), suggesting that there may be extensive crosstalk between different classes of proteins at insulator elements.

We report a striking overlap between the genomic distribution of dREAM proteins and the insulator-binding proteins CP190 and Beaf-32. These components interact genetically and CP190 association with known binding sites depends, at least in part, on dE2F2. We show that dREAM and CP190 are important for the regulation of differentially expressed genes in divergent gene pairs, separating a stably repressed from a highly expressed gene. In addition, genomic fragments that are bound by dREAM-CP190-Beaf-32 possess strong enhancer-blocking activity and, in most cases, dREAM proteins are important for this activity. These results reveal that the role of dREAM complexes is intimately linked with insulator-binding proteins and provide new insight into the ‘function’ of dREAM binding sites.

MATERIALS AND METHODS

Antibodies

Anti-dE2F1 and anti-dE2F2 antibodies used for ChIP were described previously (6,49). An independently generated antibody to dE2F1 was a generous gift from P. Spierer (Geneva, Switzerland). Other antibodies included anti-CP190 (42,50,51), anti-Beaf-32 (52) and Mip130/Twt (8). Rabbit anti-H3 (ab1791) and mouse monoclonal H3K27me3 (ab6002) antibodies were purchased from Abcam.

Fly strains

w1118 or *yw* flies were used as wildtype control flies. The following null alleles were used in this work: *de2f2^{76Q1}* and *de2f2^{c03344}* (53) (Exelixis); *mip130¹⁻⁷²³* and *mip1301-36* (54); *cp190¹* and *cp190²* (55); *dp^{a3}* and *dp^{a4}* (56). Extra lethal mutations were removed from the *dp^{a3}* and *dp^{a4}* chromosomes by homologous recombination (57). All mutations were analyzed in *trans*-heterozygous combinations. AB-KO was used as a *beaf-32a* and *beaf-32b* mutant (58). Act-Gal4 and Nub-Gal4 in combination with UAS-Dcr-2 (X) were used to drive the ubiquitous and wing-specific expression, respectively, of hairpins. RNAi lines for the knock-down of dE2F2, Mip120, CP190 and Beaf-32 were from Bloomington (Transgenic RNAi Project, TRiP) or the Vienna *Drosophila* RNAi Center (VDRC).

Chromatin Immunoprecipitation and ChIP-chip

ChIP experiments from *Drosophila* tissue culture cells and third instar larvae were performed using a previously published method (59). For tissue culture cells, 1.5×10^8 S2 cells were harvested and processed, omitting the initial homogenization steps and instead directly resuspending the cells in cross-linking buffer. Precipitated DNA was used for quantitative real-time PCR (qRT-PCR) or whole genome amplification (WGA2 Kit, Sigma). The amplified DNA was purified using the Qiagen PCR purification kit and processed for microarray hybridization (2.1M *Drosophila* Whole-Genome Tiling Arrays, NimbleGen) according to the manufacturer's instructions (NimbleGen ChIP-chip User Manual).

ChIP-chip analysis

Each ChIP-chip experiment was carried out in duplicate, with pre-immune serum as a control. Raw hybridization signal data were generated using NimbleScan software (NimbleGen). Peak detection was done using Ringo (60). Briefly, the measured log₂ ratios were smoothed by a running median over chromosomal probe location. A peak was called in the smoothed curves if three or more probes in a row showed ratios higher than a cutoff. Cutoffs were determined using the pre-immune serum controls to estimate the null distribution of peaks and then adjusted to give a false discovery rate of 0.02. Only peaks called in both duplicates and not present in the pre-immune serum controls were considered real peaks and kept for further analysis.

ChIP Re-ChIP

Chromatin for ChIP Re-ChIP experiments was prepared as described in Negre *et al.* (59). Six separate ChIP reactions were set up for the first IP of a single ChIP Re-ChIP experiment. Shortly, protein A or A/G Sepharose (GE Healthcare) was pre-blocked with 1 mg/ml BSA and 1 mg/ml sheared salmon sperm DNA (Ambion). Antibodies were pre-bound to the beads at 4°C for at least 6 h in 900 µl Dilution IP buffer (16.7 mM Tris pH 8, 1.2 mM EDTA pH8, 167 mM NaCl, 0.01% SDS, 1.1% Triton X100), chromatin was added and rotated at 4°C overnight. Beads were washed with 1 ml of Dilution IP buffer (2x), TSE buffer (1x) (20

mM Tris pH8, 2 mM EDTA pH8, 500 mM NaCl, 1% Triton X100, 0.1% SDS), LiCl buffer (1x) (100 mM Tris pH8, 500 mM LiCl, 1% deoxycholic acid, 1% NP40) and TE buffer (2x). Six ChIP reactions were combined and eluted for 20 min at 65°C in elution buffer (50 mM NaHCO₃, 140 mM NaCl, 1% SDS). Eluted material was added to antibody pre-bound to beads in Dilution IP buffer (see above) and incubated at 4°C overnight. Beads were washed as described above, DNA was eluted and purified as described (59) and used in qRT-PCR experiments.

Analysis of dE2F binding site overlap with chromatin features

Binding sites for chromatin features and transcription factors in *Drosophila* tissue culture cells were extracted from the modENCODE database and studies investigating binding of comprehensive sets of chromatin proteins (34,61). To search for overlap, the coordinates of the peak centers of dE2F and control transcription factor sites (plus 500 bp upstream and downstream of the peak center, based on the width of dE2F peaks) were analyzed with respect to their overlap with the chromatin feature coordinates. The observed overlap frequencies were plotted against each other using hierarchical clustering.

Co-immunoprecipitation

S2 cells were washed and harvested in Lysis buffer (50 mM Tris pH 8, 0.2% NP-40, 300 mM NaCl, 10 mM MgCl₂, protease inhibitors), incubated on ice for 15 min and cleared by centrifugation. Lysates were diluted in the same buffer to a final concentration of 120 mM NaCl and 0.05% NP-40, pre-cleared with protein A or A/G sepharose beads for 1 h, incubated with antibody for 3 h and antibody was captured on beads for 1 h (all steps at 4°C). Beads were washed 5x with IP buffer and precipitated proteins were analyzed by western blot.

Co-regulation analysis of divergently paired genes

Pearson correlations were calculated for the expression of the two genes in each pair between different cell lines and developmental stages (modENCODE_3305). Correlation values were grouped in equally sized bins and frequencies calculated. These frequencies were used to draw a distribution for all divergently paired genes (DPGs), dE2F2- and dREAM-repressed DPGs.

RNA isolation, reverse transcription and qRT-PCR

RNA was isolated from S2 cells or third instar larvae using Trizol (Life Technologies) according to the manufacturer's instructions. Isolated RNA was quantified and 1 µg used for the reverse transcription reaction using the TaqMan Reverse Transcription Kit (Applied Biosystems). Typically 1/100 of the reaction was used for qRT-PCR.

Drosophila tissue culture and Luciferase reporter assay

S2 cells were maintained in Shields and Sang M3 medium (Sigma) with 10% fetal bovine serum, BPYE and 1%

Penicillin/Streptomycin. For the enhancer-blocking assay, S2 cells were transfected with 100 ng of the enhancer-blocking reporter and 250 ng of the Renilla control plasmid, using the X-tremeGENE HP transfection reagent (Roche). Cells were harvested 48 h after transfection and Luciferase activity measured using the Dual-Luciferase reporter system (Promega) with a Centro LB 960 Microplate Lumimeter (Berthold).

dsRNA and Luciferase reporter assay

Cells were seeded at a concentration of 1×10^6 /ml and soaked in 15 μ g of dsRNA, synthesized using the T7 Ribomax RNA production system (Promega). dsRNA treated cells were cultured for 4 days, diluted to a concentration of 1×10^6 /ml and soaked again in 15 μ g of dsRNA. Following dsRNA treatment, the cells were transfected with the reporter plasmids and Luciferase activity was measured as described above.

RESULTS

We previously mapped genomic binding sites for dE2F1 and dE2F2 *in vivo* in third instar *Drosophila* larvae and found dE2F1 bound at a surprisingly small number of sites compared to dE2F2 (62). Although dE2F1 had been detected on promoters of genes important for mitosis in tissue culture cells (6), this binding was largely absent in larvae. The heterogeneity of larval tissues might cause the number of dE2F1 binding sites to be underestimated and we therefore generated genomic dE2F1 binding profiles in cultured *Drosophila* cells for comparison. We used antibodies specific for dE2F1 to perform ChIP on chromatin from S2 tissue culture cells. DNA isolated from the immunoprecipitation was hybridized to NimbleGen 2.1 M whole genome tiling arrays. Two independently generated antibodies against dE2F1 were used for ChIP and pre-immune serum was included as a negative control. Peaks detected by both dE2F1 antibodies but absent from the pre-immune serum were considered true dE2F1 binding sites (Supplementary Table S1). The vast majority of dE2F1 binding sites were located close to the TSS of known genes (Supplementary Figure S1). dE2F1 was found in the promoter regions of 1486 genes, many of which corresponded to previously characterized dE2F1-dependent transcripts (Figure 1A). The number of specific dE2F1 binding sites detected in S2 cells was 4-fold greater than had been evident in larvae. In agreement with previous studies, functional classification of dE2F1 target genes showed a strong enrichment of genes involved in cell cycle-related processes (Figure 1B).

We compared the pattern of dE2F1 binding sites to the previously identified profiles for dE2F2 and other components of the dREAM complex in tissue culture cells (14). Like dE2F1, dE2F2 also associates with the promoters of cell cycle genes (14, Figure 1B). In addition, dE2F2 is strongly enriched at genes involved in a subset of developmental processes, including genes involved in sexual reproduction (Figure 1B). Concordantly, dE2F2 and dREAM have been implicated in the stable transcriptional repression of several of those genes in proliferating somatic cells (8,9,63).

Even when the longer list of dE2F1-bound sites seen in S2 cells is considered, it is evident that the number of dE2F2 binding sites in the genome is almost four times higher than the number of dE2F1 binding sites (4434 versus 1178). The majority of dE2F1 binding sites (70%) were also associated with dE2F2, whereas the reverse was not the case (Figure 1C). The dREAM complex contains several proteins with DNA-binding activity, and these provide one potential explanation why dE2F2/dREAM binding sites are more numerous than dE2F1 binding sites. Since activator E2Fs are only transiently present at cell cycle regulated promoters, while repressors can be bound at multiple phases of the cycle, differences in the stability of dE2F1 and dE2F2 complexes might also affect our ability to detect these binding sites by ChIP.

These results highlight a second curious feature: the number of binding sites for dE2F1 and dE2F2 in the *Drosophila* genome is far higher than the number of genes that show a change in the level of expression when E2F regulation is disrupted by either the depletion of the dE2F1 activator or the co-repressor dE2F2. In the case of dE2F2 less than 10% of bound genes are de-regulated upon dE2F2 RNAi (6,14). This may reflect the fact that transcription regulation results from the integrated activity of multiple factors and that no single factor is likely to be rate limiting at all of its targets. It is also possible that the net effect of E2F regulation on transcription is generally smaller than the cut-off values that were used for analysis of expression data. Alternatively, this discrepancy could be interpreted as evidence that most of the E2F complexes on DNA are actually non-functional. To distinguish between these possibilities, we need a clearer understanding of the molecular activities of dE2F1- and dE2F2-containing complexes.

To better understand the context in which E2F proteins act, we compared the distribution of the dE2F proteins with histone modifications and other chromatin binding proteins. We gathered genomic binding data for chromatin proteins and histone modifications from *Drosophila* S2 and Kc cells and assessed whether dE2F1, dE2F2 and dREAM binding sites overlapped with these chromatin features (Figure 2). Co-localization was called if the dE2F peak center localized within the peak of the chromatin feature or within 500 bp of it (since this was the average width of dE2F peaks). For comparison, we also analyzed the overlap between the selected chromatin features and a panel of additional DNA-binding transcription regulators.

The tested chromatin features included a set of chromatin domains that were identified through combinatorial binding patterns of a large number of chromatin-associated proteins (61). The individual chromatin types (termed BLUE, GREEN, BLACK, YELLOW and RED chromatin) represent distinct classes of actively transcribed or inactive genomic regions and, together, account for the entire *Drosophila* genome. As a positive control, we saw that the Polycomb group protein Pho overlapped extensively with H3K27me3, a characteristic feature of Polycomb-regulated chromatin. Indeed, Pho was the only transcription regulator in our set that overlapped with Polycomb chromatin as defined by Filion *et al.* (BLUE chromatin) (61, Figure 2). None of the transcription regulators examined displayed extensive overlap with heterochromatic chro-

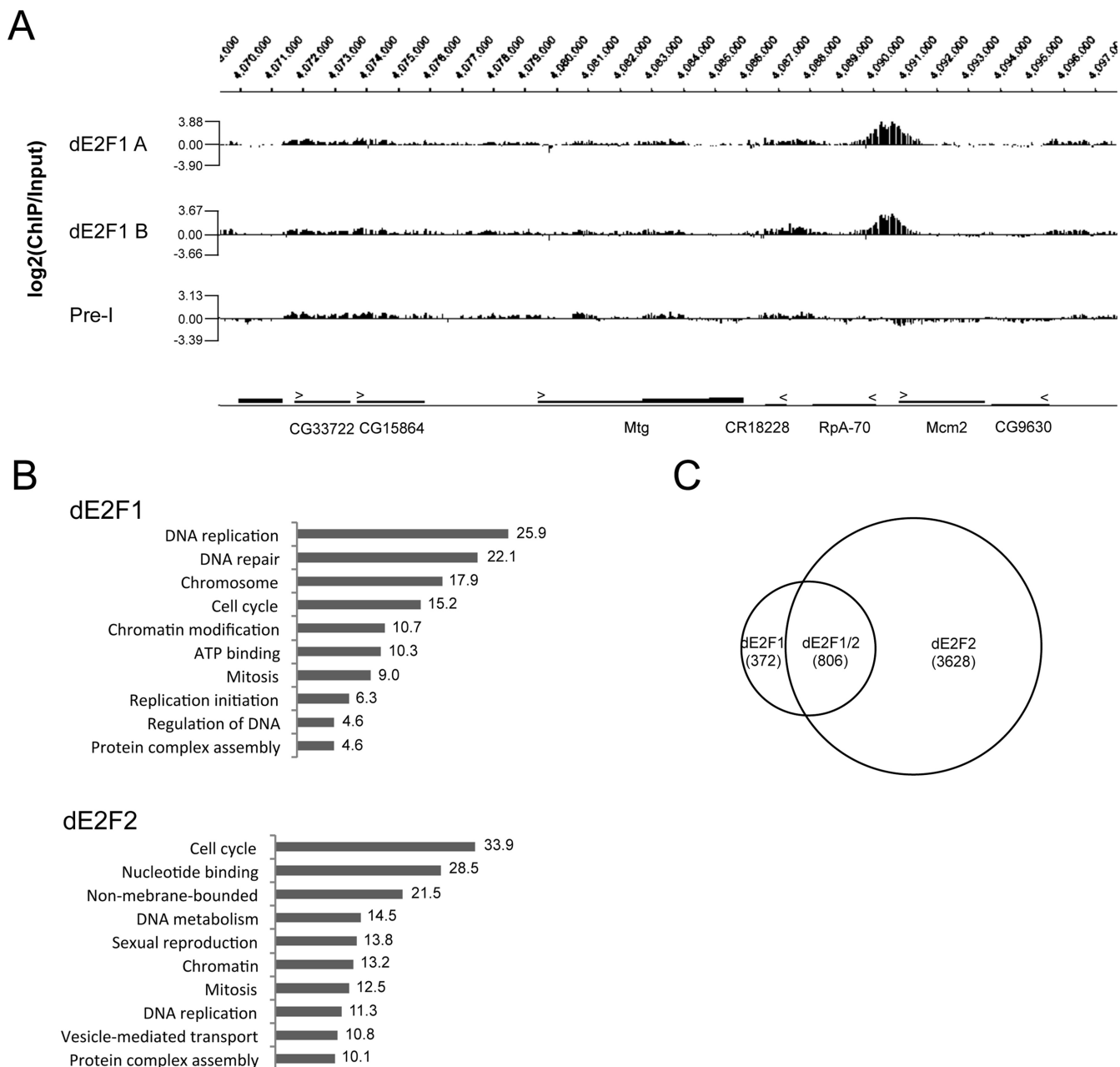


Figure 1. dE2F1 and dE2F2 associate with thousands of sites in *Drosophila* tissue culture cells. (A) dE2F1 binds specifically to the Mcm2 promoter. Specific dE2F1 binding is represented by prominent peaks at the TSS of the Mcm2 gene. Genes are depicted as black bars and the direction of transcription is indicated by arrows. dE2F1a and dE2F1b represent two independent antibodies against dE2F1. Pre-I, preimmune serum. (B) dE2F1 and dE2F2 bound genes are highly enriched in cell cycle genes. Bound genes were annotated using the DAVID database (<http://david.abcc.ncifcrf.gov/>). Values indicate enrichment scores. (C) dE2F1 and dE2F2 binding sites overlap extensively. dE2F1/2, overlapping binding sites. Numbers in brackets indicate the number of binding sites.

matin regions (GREEN and BLACK chromatin). Likewise, none of these factors showed overlap with H3K9 methylation, a mark that is enriched in heterochromatin. In contrast, the binding of dE2F1, dE2F2 and dREAM (as well as the other transcription factors) overlapped extensively with active histone marks, suggesting that these DNA-binding factors associate almost exclusively with euchromatin. Interestingly, and in contrast to the other transcription regulators analyzed, dE2F1, dE2F2 and dREAM localized al-

most exclusively to YELLOW chromatin (Figure 2, green rectangle) (Supplementary Figure S2A), a form of euchromatin that is enriched in H3K36me3 and Mrg15, a protein that specifically binds to H3K36me2 and 3 (61).

A small subset of chromatin features showed strong overlap with dE2F and dREAM binding sites, but overlapped only weakly or not at all with the other transcription regulators (Figure 2, green rectangle). These features include binding sites for the insulator-binding proteins Beaf-32 and

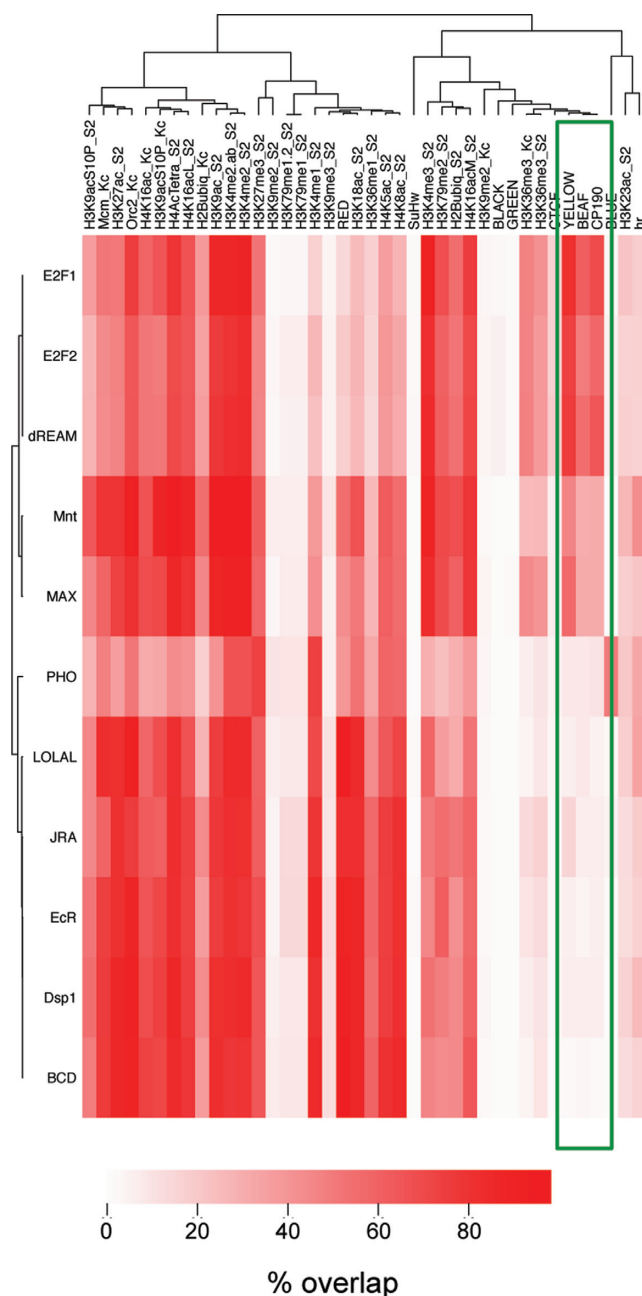


Figure 2. Overlap of dE2F binding sites with general chromatin features. Coordinates of the peak centers of dE2F and control transcription factor sites were analyzed with respect to their overlap with chromatin feature coordinates extracted from previous studies (see Materials and Methods). The observed overlaps were plotted against each other using hierarchical clustering. Red intensity indicates the frequency of overlap. Extensive overlap with YELLOW chromatin, CP190 and Beaf-32 is highlighted with a green box. Lolal, Lola-like; Jra, Jun-related antigen; EcR, Ecdysone Receptor; Bcd, Bicoid.

CP190. The overlap of dE2F and insulator-binding proteins was specific for CP190 and Beaf-32, as the observed overlap with Su(Hw) and dCTCF was much lower (Supplementary Figure S2B). Because dE2F2 binding sites greatly outnumber dE2F1 binding sites, the bulk of the overlap between Beaf-32 and E2F occurs at loci that are bound by

dE2F2/dREAM. In these comparisons it is striking that the overlap between dE2F2 and either CP190 or Beaf-32 is higher than the co-localization seen between the two insulator-binding proteins (Figure 3A). Moreover, the overlap of dREAM-bound genes with both CP190- and Beaf-32-bound genes is extremely statistically significant ($P = 7.05E-96$ (CP190); $P = 9.66E-88$ (Beaf-32)).

In order to address whether dREAM and CP190/Beaf-32 are in fact simultaneously associated with a common genomic region, we performed ChIP Re-ChIP experiments. Using the genomic binding data of the factors, we identified regions bound by both (Trc8, Neu3, Cks30A), none or only one of the proteins (control regions) that were subsequently tested in sequential ChIP experiments (Figure 3B, top panel). dE2F2(first IP)–Mip130(second IP) and dE2F2–Beaf-32 chromatin IPs show clear enrichment over dE2F2–IgG control ChIPs for the common binding sites (Figure 3B, bottom panel). In contrast, we detected no enrichment at a control region that is not bound by the factors (Act88F). As an additional control we tested for simultaneous binding at a region associated with dE2F2 and Mip130, but devoid of Beaf-32-binding (CG4679). As predicted, we detected co-binding of dE2F2 with Mip130, but not with Beaf-32. Analogously, in CP190–dE2F2 and CP190–Beaf-32 ChIP Re-ChIP experiments, we found co-binding of these proteins at the common binding sites, but not at the control regions (Supplementary Figure S3). These findings suggest that dREAM and CP190/Beaf-32 associate simultaneously with common binding sites and indicate a strong connection between these factors that has previously been unappreciated.

dREAM genetically interacts with Beaf-32 and CP190

To determine whether dE2F2/dREAM synergize with Beaf-32 and CP190, or whether they act antagonistically, we used the *Drosophila* wing as an assay system. We took advantage of transgenic RNAi lines and crossed them to a wing-specific driver (Nub-G4) to achieve tissue-specific knockdown of the desired proteins. The individual knockdown of CP190 and Beaf-32 resulted in mild (+, extra vein tissue) or severe (++, blistering, wing structure changes) wing malformation, respectively, in a small percentage of wings (Figure 4). These phenotypes provided a useful sensitized background, and we asked whether the simultaneous inactivation of dREAM subunits would enhance or suppress these phenotypes. RNAi transgenes that reduced the levels of dE2F2 or Mip120 had no visible effect on adult wing morphology when examined alone (Supplementary Figure S4). However, the co-depletion of either Mip120 or dE2F2 strongly enhanced the penetrance of the Beaf-32 wing phenotype (Figure 4B). Likewise, simultaneous knockdown of dREAM subunits strongly enhanced the penetrance of the CP190 RNAi phenotype in the wing (Figure 4C). In agreement with this, we noted that the Beaf-32-targeting hairpin reduced viability when combined with Act-Gal4, a driver that gives ubiquitous expression, and simultaneous knockdown of Mip120 and dE2F2 led to a further decrease in viability (data not shown). These genetic interactions show that dREAM and insulator-binding pro-

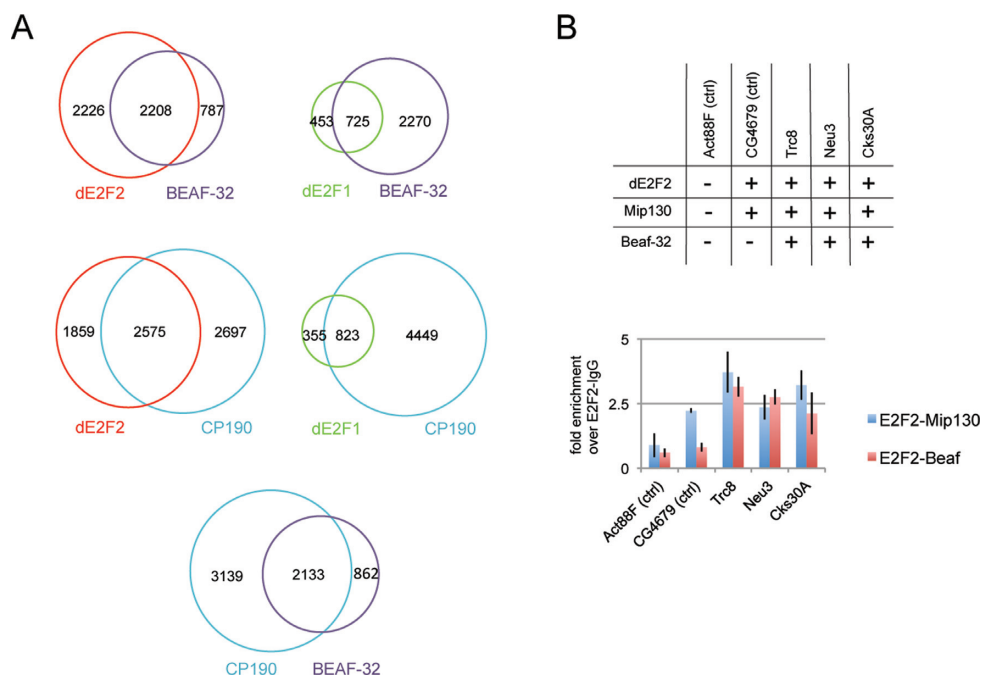


Figure 3. dE2F binding sites overlap extensively with insulator proteins CP190 and Beaf-32. (A) Binding site overlap of dE2F proteins with CP190 and Beaf-32. Numbers represent unique or overlapping binding sites, respectively. (B) dE2F2 and Beaf-32 simultaneously associate with common binding sites. Top panel shows binding of the indicated proteins at a set of five genes based on genomic association data. +, binding; -, no binding. ChIP Re-ChIP experiments were performed using chromatin from *Drosophila* third instar larvae. First IP: dE2F2; second IP: IgG, Mip130 or Beaf-32. Fold enrichment of dE2F2-Mip130 and dE2F2-Beaf-32 over dE2F2-IgG is shown for the indicated sites.

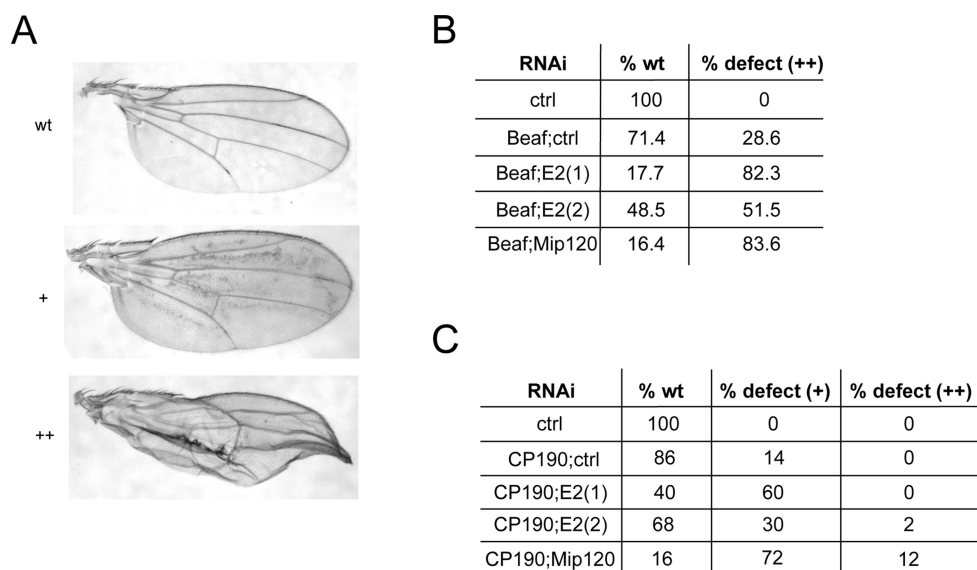


Figure 4. CP190 and Beaf-32 genetically interact with dREAM subunits. (A) Co-depletion of dREAM subunits and CP190 or Beaf-32 during wing development. RNAi lines were crossed to UAS-Dcr;Nub-Gal4 for expression in the larval wing disc and phenotypes were scored based on the severity of the wing defect. wt, wildtype; +, mild defects (extra vein tissue); ++, severe defects (blistering, wing structure changes). dREAM components genetically interact with Beaf-32 (B) and CP190 (C). Numbers represent the percentage of flies with the observed phenotype. Crosses were repeated three times and a representative outcome is shown in the table. ctrl, Luciferase RNAi line. E2(1) and E2(2) represent independent RNAi lines targeting dE2F2 with different efficiency [E2(1) > E2(2)].

teins do functionally interact, and suggest that they act cooperatively rather than antagonistically.

CP190 DNA binding is impaired in *de2f2* mutant animals

Both dE2F2/dREAM and CP190/Beaf-32 associate with specific DNA regions. Like dE2F2, Beaf-32 can bind directly to DNA through the recognition of sequence motifs, whereas CP190 is thought to associate indirectly with DNA and to be recruited via interaction with sequence-specific DNA-binding factors (such as Beaf-32). Conceivably, these proteins might affect the DNA-binding activities of one another, either positively or negatively. To examine this, we first confirmed that the co-localization of dE2F2/dREAM and insulator-binding proteins was not restricted to *Drosophila* cell lines, but also occurred *in vivo*. ChIP was performed on chromatin from *Drosophila* third instar larvae using antibodies against dE2F2, CP190 and Beaf-32. We analyzed binding at predicted sites of co-localization from the cell line data by qRT-PCR, a subset of which are shown in Figure 5A–C. dE2F2, CP190 and Beaf-32 were all enriched at the tested sites compared to a control region (Act) (Figure 5A–C). dE2F2 and CP190 were both strongly enriched at all the tested regions, but the level of enrichment of Beaf-32 was slightly more variable.

Next, we used a *trans*-heterozygous combination of *de2f2* alleles (76Q1 and c03344) to ask whether the absence of dE2F2 influenced chromatin binding by CP190 or Beaf-32 (Figure 5D). As expected, dE2F2 binding to a set of known binding sites was almost completely absent in mutant larvae compared to wildtype animals (blue bars, 1 = level of binding in wildtype animals). Interestingly, CP190 binding to each of the tested sites was strongly reduced (red bars), although not to the extent seen for dE2F2. In contrast, Beaf-32 binding was largely unchanged in the *de2f2* mutant larvae (green bars). A few sites even showed an increase in Beaf-32 association, but this increase is not specific since it was mirrored by a similar change in a control ChIP using an antibody to histone H3 (compare purple and green bars). In reciprocal experiments, we tested whether the loss of CP190 or Beaf-32 had an effect on the chromatin association of dE2F2 and Mip130, but failed to detect any defects in the binding of dREAM subunits in mutant larvae (data not shown). The ChIP data suggest that dE2F2/dREAM, either directly or indirectly, promotes the chromatin association of CP190, whereas Beaf-32 binding to chromatin appears to be regulated independently of dE2F2.

Although CP190 has not been identified in the original purifications of the dREAM complex (8,9), it remains possible that these proteins interact with each other in a transient manner. Therefore, we performed immunoprecipitation experiments from *Drosophila* S2 cells using antibodies against CP190 and dDP along with IgG as a control (Figure 5E). As shown in Figure 5E, we detected physical association of several dREAM subunits with CP190 and, in reciprocal IP experiments, we also found CP190 in the dDP immunoprecipitation. This suggests that chromatin binding of CP190 is, at least in part, mediated through a physical interaction with dREAM.

dREAM is needed for transcriptional regulation at differentially expressed, divergently paired genes

Neighboring genes can be arranged in three potential ways depending on their direction of expression (Figure 6A). In *Drosophila* a large percentage (32%) of genes are transcribed in opposite direction, with their TSS separated by less than 1000 bp (64). These genes are referred to as divergently paired genes (DPGs). Binding of the insulator-binding proteins CP190, Beaf-32 and dCTCF is significantly enriched at DPGs (36,52), suggesting that they have a critical structural function at these sites. We analyzed dREAM-bound genes and found that the fraction of DPGs was almost doubled among dREAM-bound genes compared to their expected random occurrence in the genome (Figure 6A, light blue bar; dREAM b). We also analyzed genes occupied by several unrelated DNA-binding transcription regulators. In contrast to dREAM targets, the percentage of DPGs was either similar to or reduced compared to the background level. Similar to dREAM-bound genes, genes de-repressed concertedly upon inactivation of a set of core dREAM subunits or dE2F2 were significantly enriched in DPGs, with almost 70% of dREAM-repressed genes being divergently transcribed (Figure 6A, dark blue bars; dREAM r, dE2F2 r).

The majority of genes within individual DPGs are co-regulated among different cell types and developmental stages in *Drosophila* (65, Figure 6B; red graph, major peak around 0.7). However, as pointed out by Yang *et al.*, a significant subset of gene pairs does not show co-regulation (Figure 6B, red graph, shoulder around 0). In stark contrast to the bulk of DPGs, dREAM- and dE2F2-repressed divergent gene pairs are strongly skewed toward independent regulation (Figure 6B; green and blue graph). These findings raise the possibility that an activity related to insulators might be critical at those DPGs to allow for independent regulation of the two genes.

To test this, we took advantage of the finding that dE2F2/dREAM-regulated genes include almost entirely gene pairs that are differentially expressed, with one gene being stably repressed and its partner actively transcribed. We isolated RNA from third instar larvae carrying *trans*-heterozygous combinations of *de2f2*, *mip130* and *cp190* mutant alleles along with wildtype controls, and performed qRT-PCR analysis for differentially expressed DPGs. As expected, inactivation of dE2F2 and Mip130 resulted in de-repression of the stably repressed gene (Figure 6C, left). In support of the previously described relationship between CP190 and dREAM, loss of CP190 also caused de-repression of those genes. Importantly, expression of the divergently paired, actively transcribed genes was reduced by 15–35%, depending on the gene pair (Figure 6C, right). A similar pattern of inverse de-regulation of gene pairs was also observed upon RNAi-mediated knockdown using the ubiquitous Act-Gal4 driver (data not shown). These results suggest that dREAM is important for the independent regulation of differentially expressed DPGs and, hence, transcriptional integrity at these loci.

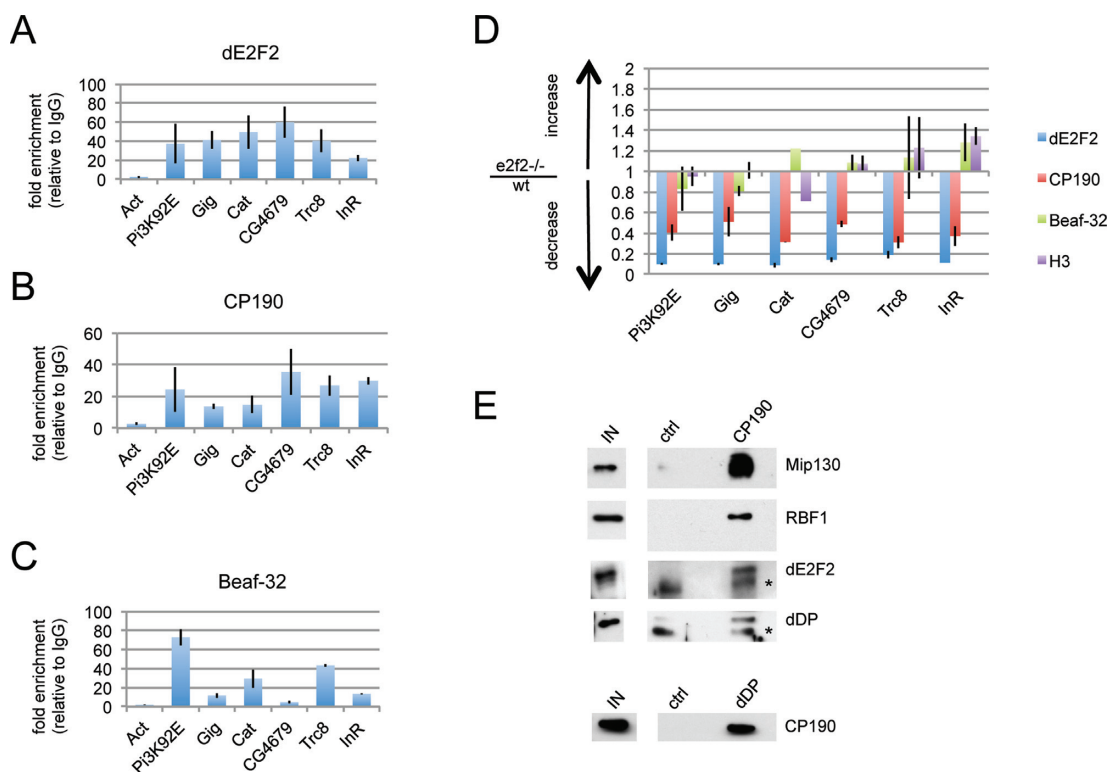


Figure 5. dE2F2 is required for efficient chromatin binding of CP190. ChIP-qPCR in *Drosophila* third instar larvae using antibodies against Beaf-32 (A), CP190 (B) and dE2F2 (C) for a set of randomly chosen sites confirmed co-occupancy of the proteins *in vivo*. Fold enrichment relative to IgG is shown. The average of three independent experiments is shown. (D) Reduced CP190 chromatin binding in *e2f2* mutant animals. Bargraphs show the change in chromatin binding of the indicated proteins in *e2f2* mutant relative to wildtype animals. dE2F2 (blue), CP190 (red), Beaf-32 (green), H3 (purple). The average of three independent experiments is shown. (E) dREAM and CP190 physically interact. Immunoprecipitation using antibodies against CP190 (top panel) or dDP (bottom panel) from S2 cells. Co-immunoprecipitation was confirmed by western blotting for the indicated proteins. ctrl, IgG. IN, input. 0.5% input is shown on the left. *, unspecific cross-reacting band.

dREAM binding sites possess enhancer-blocking activity

The boundary-like activity of dREAM at DPGs prompted us to test whether bound regions would share other characteristics with insulator protein binding sites. An activity attributed to insulator elements and proteins is their ability to block the activity of enhancers. Enhancers are regulatory DNA elements that positively regulate the promoter of a gene. Insulator elements can inhibit these regulatory interactions if placed between an enhancer and a promoter (66). We took advantage of a cell-based enhancer-blocking assay in which the enhancer of *copia* is positioned upstream of a firefly *Luciferase* reporter under the control of the *Hsp70* promoter (67). We cloned a set of genomic fragments that bind both, dREAM and CP190, placed them between the enhancer and promoter (Supplementary Figure S5), and transfected the reporter constructs into S2 cells.

The Fab-8 insulator was used as a positive control and, as expected, it displayed substantial enhancer-blocking activity (Figure 7). Interestingly, each of the fragments that contained dREAM and CP190 binding sites also exhibited enhancer-blocking activity to various degrees. Indeed, some of these fragments had enhancer-blocking activity that was comparable to, or even stronger than, the Fab-8 element. In contrast, a control genomic fragment of comparable length (ctrl), that lacked dREAM or CP190 binding sites, had no effect on the activity of the enhancer. As described for

other insulators in this experimental system, the enhancer-blocking activity of the assayed fragments was observed regardless of whether these regions were inserted in the forward or reverse orientation (data not shown) (see also (67)).

dREAM is required for efficient enhancer blocking at a subset of bound sites

To test whether dREAM was required for efficient enhancer blocking at the identified sites, we treated S2 cells with dsRNAs targeting various subunits of the dREAM complex prior to transfection with the constructs for the enhancer-blocking assay. As a positive control, we knocked down dCTCF and monitored the effect on enhancer-blocking activity of Fab-8 (Figure 8A). As expected, enhancer blocking by Fab-8 was impaired when the levels of dCTCF protein were reduced. Next, we knocked down the dREAM complex subunits Mip130 and Mip120 (Figure 8B and C). None of these treatments affected the enhancer-blocking activity of Fab-8. However, depletion of both Mip130 and Mip120 significantly reduced the enhancer-blocking activity of most, but not all, of the fragments containing dREAM/CP190 binding sites. The strongest effects were seen on fragments adjacent to Trc8, Ent1, Ance-4 and Lrrk (Figure 8B and C).

Many dREAM, CP190 and Beaf-32 sites lie within the promoter region of genes, raising a potential concern that

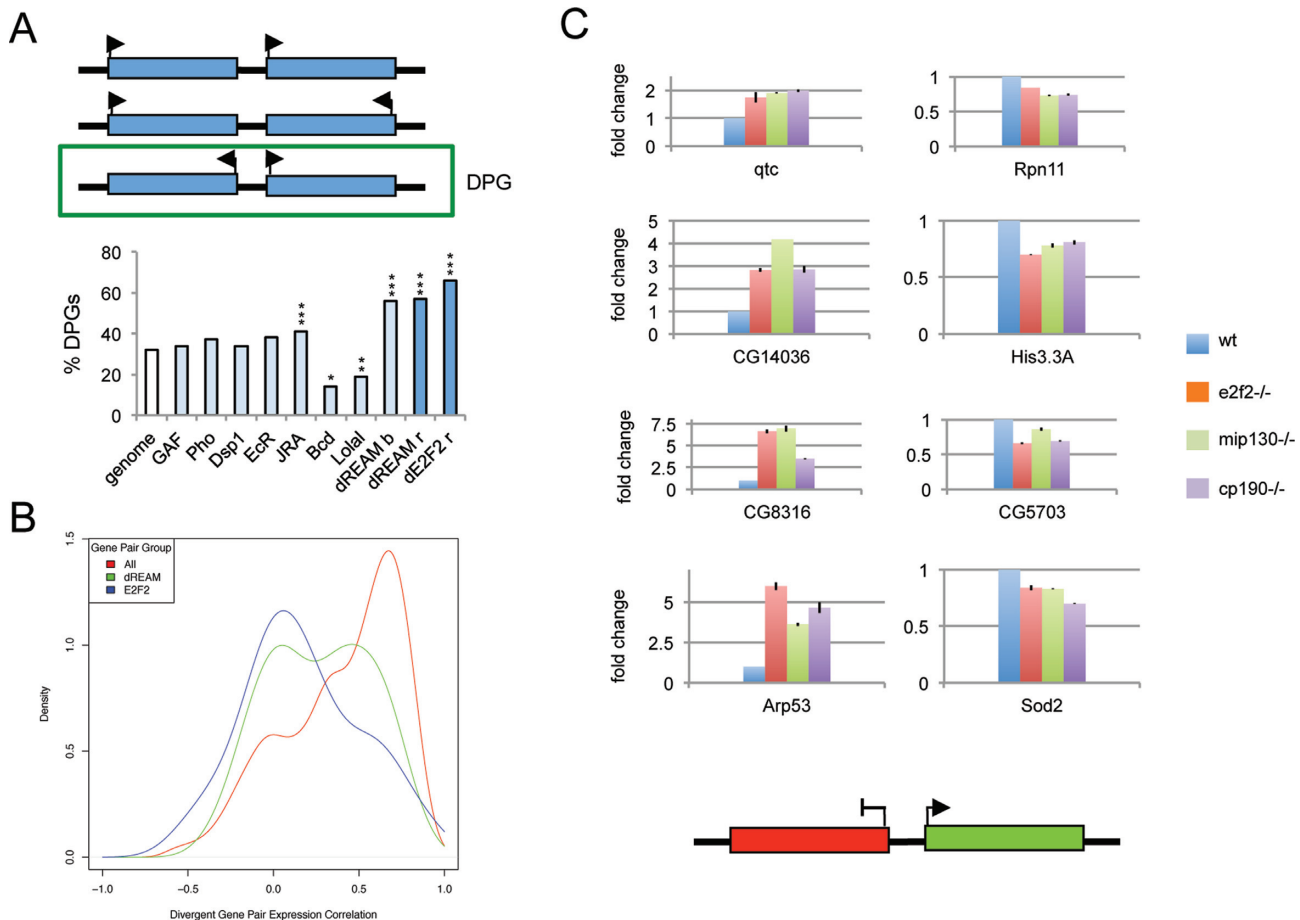


Figure 6. dREAM and CP190 are important for transcriptional integrity at differentially expressed, DPGs. (A) dREAM is enriched at DPGs. The diagram in the upper panel depicts the possible orientation of neighboring genes. Green box highlights DPGs. Lower panel shows the percentage of DPGs among genes bound by the indicated transcription factors, including dREAM (light blue bars), compared to the entire genome (white bar). The dark blue bars represent the percentage of DPGs among dREAM- and dE2F2-regulated genes, respectively (14,6). b, bound; r, repressed. $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$ (Chi-Square Test). (B) dREAM-repressed DPGs are not co-regulated. The distributions of expression correlation for the two genes in all divergent gene pairs (red), dE2F2- (blue) and dREAM-repressed DPGs (green) were plotted. (C) Differentially expressed DPGs are de-regulated in dREAM and cp190 mutants. qRT-PCR with primers for the indicated genes was carried out using RNA isolated from wildtype, e2f2, mip130 and cp190 mutant third instar larvae. Fold change of gene expression in mutant compared to wildtype animals is shown. Stably repressed genes are shown on the left, their highly expressed divergent pairs are displayed on the right. Experiments were performed in triplicate.

the enhancer-blocking assay might simply be an indirect measure of the ability of these regions to repress transcription. There are several lines of evidence against this interpretation. First, our panel of dREAM/CP190-bound regions includes a fragment containing sequences 3' to the Ance-4 gene that is not in the vicinity of any known promoters and would not be expected to regulate transcription. Second, the fragments that show the most significant abrogation of enhancer-blocking activity when dREAM proteins are depleted contain sequences from the promoter regions of the *Trc8* and *Ent1* genes, respectively. When we measured the effect of dREAM inactivation on the endogenous expression level of these genes in S2 cells, neither *Trc8* nor *Ent1* were de-repressed upon knockdown of Mip130 or dE2F2 (Figure 8D). Thus neither of these promoters appears to be regulated by dREAM-mediated transcriptional repression in S2 cells. Third, our panel of genomic fragments includes two previously described dREAM target genes (Arp53D and CG8316) that are strongly de-repressed when dREAM

proteins are depleted (Figure 8D). However, the enhancer-blocking activities of these fragments were largely unaffected by dREAM knockdown (Figure 8B and C). Collectively, these results show that multiple genomic fragments that bind dREAM have enhancer-blocking activity, and that in most cases this activity is impaired when dREAM proteins are depleted. However, the activity of these fragments in the enhancer-blocking assay is clearly separable from and distinct to the role of dREAM in transcription regulation.

DISCUSSION

The large number of E2F proteins has precluded all attempts to generate a comprehensive set of E2F binding sites in the human genome. Here, we have taken advantage of the simplicity of the *Drosophila* E2F family to examine the genome-wide distribution of E2F proteins. An unexpected finding from this analysis is that dE2F proteins strongly colocalize with insulator-binding proteins. The extent of over-

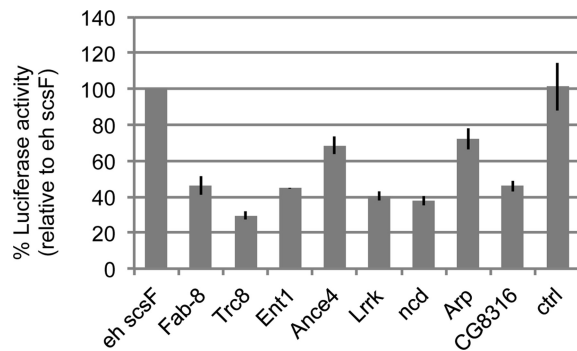


Figure 7. Regions bound by dREAM and CP190/Beaf-32 possess enhancer-blocking activity. A construct harboring the Luciferase reporter gene under the control of the Hsp70 promoter regulated by an upstream enhancer element (eh scsF) was transfected into S2 cells and used to assess baseline Luciferase activity. Controls: Fab-8, positive control; ctrl, negative control and dREAM, CP190/Beaf-32 bound fragments were inserted between enhancer and promoter. Luciferase activity is shown relative to baseline (eh scsF) activity. Enhancer-blocking assays were performed in triplicate.

lap between dE2F2/dREAM and CP190 binding sites is comparable to the co-localization previously described for dCTCF and CP190 (34), which are both required for insulator function at common binding sites (31–33,68). Furthermore, the co-localization between dREAM and Beaf-32 is even greater than that observed for Beaf-32 and CP190. This striking overlap of dREAM binding sites with proteins involved in nuclear architecture has exciting new implications for the function of dREAM complexes.

A role for dREAM in maintaining transcriptional integrity at DPGs

An interesting feature of dREAM bound genes is their strong enrichment in DPGs. Moreover, the set of genes de-regulated upon loss of dE2F2/dREAM include mostly DPGs that are differentially expressed, with one gene of the pair being stably repressed whereas its partner is actively transcribed. Inactivation of dREAM complex subunits or CP190 results in the loss of transcriptional integrity at these differentially expressed DPGs (Figure 6C).

Several different models could account for the observed transcriptional up-regulation of the stably repressed and down-regulation of the actively expressed gene. First, dREAM/CP190/Beaf-32 sites might act as boundary elements at DPGs, separating an active from a repressed chromatin domain (Figure 9A). Genome-wide binding maps for insulator-binding proteins revealed enriched binding to DPGs (36,52). In addition, these studies have shown that CP190 and Beaf-32 binding are significantly enriched at differentially expressed DPGs (36). The exact role of insulator-binding proteins at differentially expressed DPGs is still unclear. A recent study has shown that, upon inactivation of the SOX14 transcription regulator, DPGs that lack Beaf-32 binding show a significantly higher likelihood of concerted de-regulation of the two genes within a pair (up or down) than when Beaf-32 is present at the DPG (65). This suggests a role for Beaf-32 in the maintenance of independent regulation of gene expression at DPGs, consistent with a

function as boundary factor. Moreover, CP190 binding sites are commonly found at the borders of large H3K27me3 domains, which are a hallmark of Polycomb-mediated silencing (35,37). These studies further show that inactivation of CP190 can, at a subset of regions, result in local spreading of H3K27me3 beyond the CP190 binding site. Although the repressive mechanisms might vary at different dREAM-regulated DPGs, several of the stably repressed genes display H3K27me3 over the length of the gene body (data not shown). We tested the possibility of dREAM and CP190 being important for the physical separation of distinct chromatin domains by assessing the distribution of H3K27me3 over selected gene pairs (Supplementary Figure S6). In agreement with the observed de-repression of the inactive gene, mutant animals displayed loss of H3K27me3 in the gene body. However, spreading of the mark into the active gene was not observed, suggesting that the down-regulation is achieved by a different mechanism or the level of reduction in gene expression is below the detection limit of our H3K27me3 ChIP.

Second, dREAM, together with CP190 and possibly Beaf-32, may be involved in the silencing of stably repressed genes (Figure 9B). The repressive mechanism might include specific activities provided by CP190/Beaf-32. Both CP190 and Beaf-32 have been shown to be critical for the establishment of long-range chromatin interactions through looping mechanisms (43,44,69), which could be utilized to physically separate a stably repressed gene from the surrounding transcriptionally active chromatin environment. It is intriguing to speculate that dREAM, either by facilitating chromatin association of CP190 or even more directly, could be involved in the formation of these chromatin loops. Interestingly, the CP190 and Beaf-32 binding sites involved in long-range chromatin interactions are also prominent dREAM binding sites. Alternatively, Beaf-32 is known to compete for DNA binding with the transcriptional activator DNA replication-related element factor (DREF) (70). It has further been shown that, upon inactivation of Beaf-32, bound genes are specifically de-repressed when they also contain a DREF consensus site (71). In this scenario, loss of the repressive mechanism by inactivation of dREAM or CP190/Beaf-32 might either generate a vacant binding site for a transcription activator or result in the re-distribution of the general transcription machinery or a specific transcription activator from the actively expressed to the repressed gene.

Third, we cannot formally rule out the possibility that dREAM acts on both components of differentially expressed DPGs, serving as a repressor for one and as an activator for the other gene. dREAM has been implicated in transcriptional repression as well as activation (14). However, dREAM complexes containing dE2F2 do not appear to be involved in gene activation and, based on genome-wide binding studies for dREAM subunits, we have no evidence for the presence of two independent dREAM peaks at differentially expressed DPGs (14,62) (data not shown).

dREAM/CP190 sites display enhancer-blocking activity

Genome-wide association studies have identified a large number of binding sites for insulator-binding proteins, but

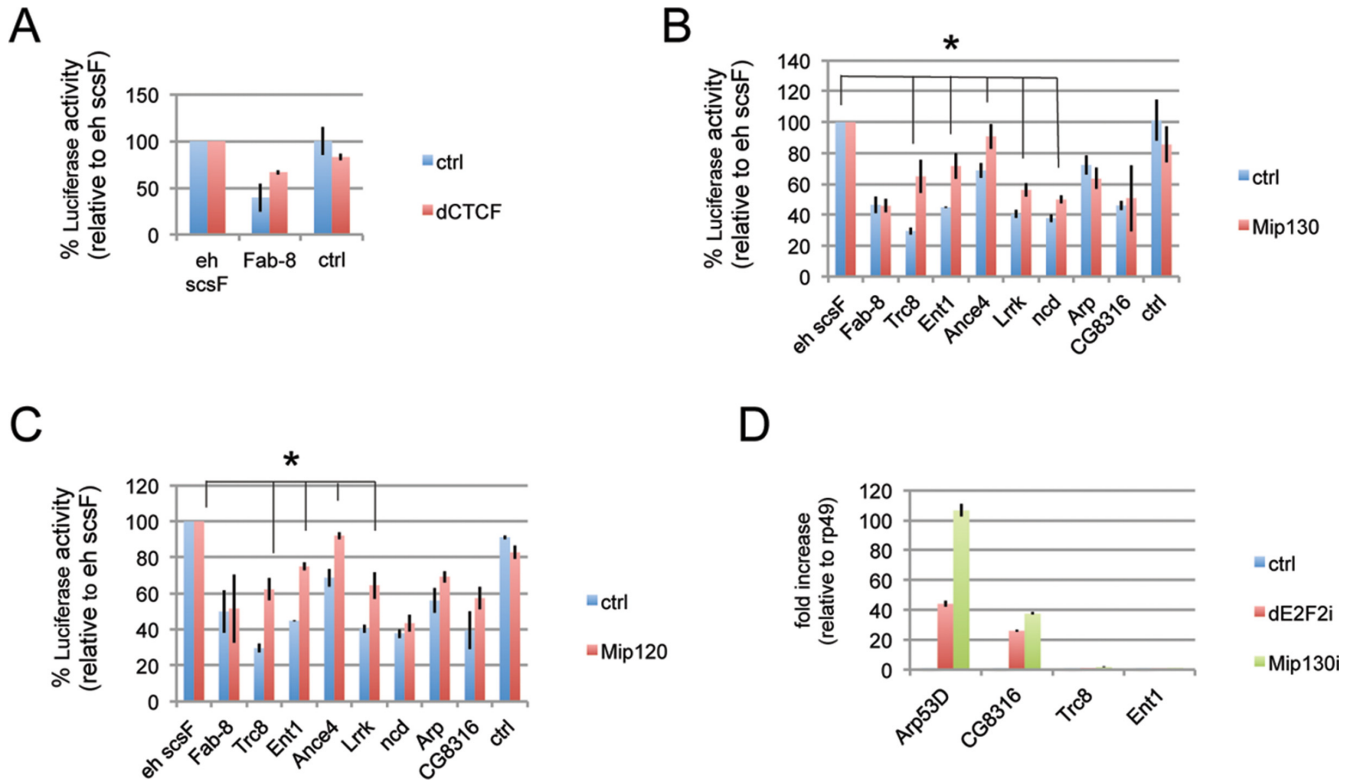


Figure 8. dREAM subunits Mip130 and Mip120 are important for enhancer-blocking activity of bound regions. (A) dCTCF is required for efficient Fab-8 enhancer blocking. Enhancer-blocking activity of Fab-8 and a control element was measured upon control or dCTCF RNAi knockdown. (B,C) Enhancer-blocking activity of control (Fab-8, positive control; ctrl, negative control), and dREAM, CP190/Beaf-32 bound regions was measured following control and Mip130 (B) or Mip120 (C) RNAi. Luciferase activity is shown relative to baseline activity. Enhancer-blocking assays were performed in triplicate. Asterisk indicates statistically significant reduction in enhancer blocking between control and specific RNAi treatments ($P < 0.05$, unpaired t Test). (D) Transcriptional effect of dREAM knockdown on endogenous genes located near enhancer-blocking fragments. RNA was isolated from RNAi-treated cells from the enhancer-blocking assay. Expression levels of the indicated genes were measured by qRT-PCR. Fold change in gene expression of specific RNAi relative to control RNAi is shown. Experiments were performed in triplicate.

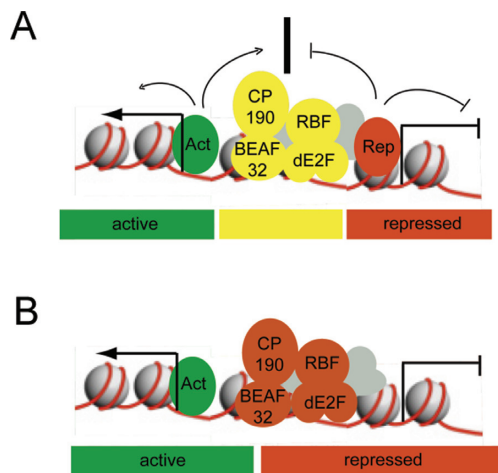


Figure 9. Model depicting potential roles for dREAM and insulator-binding proteins at DPGs. (A) dREAM/CP190-Beaf-32 as a boundary factor. dREAM, CP190 and Beaf-32 establish a boundary between independently regulated chromatin domains. Yellow, boundary element; green, active domain; red, inactive domain. Act, Activator; Rep, Repressor. (B) dREAM/CP190-Beaf-32 as a stable transcriptional repressor. Loss of the repressor (dREAM/CP190-Beaf-32) can result in spreading of the adjacent activator (Act) or vacate the binding site of a gene-specific activator.

only a few studies have addressed the potential enhancer-blocking activity of these DNA fragments (37,72). In these experiments, CP190 and dCTCF co-bound regions display strong enhancer-blocking activity compared to Su(Hw) bound sites (72). Moreover, Schwartz *et al.* show that sites associated with CP190 or CP190 and Beaf-32 exhibit strong enhancer-blocking activity, whereas sites occupied by any other combination of insulator-binding proteins show only weak or no enhancer blocking (37). In agreement with the importance of CP190 for the definition of elements with enhancer-blocking activity, dREAM-CP190 co-bound regions display robust enhancer-blocking in our cell-based assay system. The notion that dREAM functions as an enhancer-blocker may explain why a complex that is best known as a transcriptional repressor is almost exclusively found in euchromatic regions (8, Figure 2 and Supplementary Figure S2A).

The dREAM subunits Mip130 and Mip120 are important for the observed enhancer-blocking activity, but the underlying mechanism is unclear. Work in *Drosophila* suggests that the propagation of a nucleosome-free region in insulator elements is required for enhancer blocking (73). It is possible that dREAM is needed for the establishment of nucleosome-free regions through recruitment of chromatin modifying activities or their maintenance through binding

and stabilization of these regions, which in turn might be important for the recruitment of CP190.

A detailed analysis of the enhancer-blocking function of the 1A2 insulator, which harbors Su(Hw) binding sites, shows that the region adjacent to the Su(Hw) sites is important for full enhancer-blocking activity of 1A2, even though this element by itself lacks any activity (74). It is conceivable that dREAM binding sites fulfill a similar ‘facilitator’ function for CP190 and/or Beaf-32.

Although cell-based assays have been effectively used to measure the enhancer-blocking activity of characterized insulator elements (67,73,75), transfected plasmids are only partially chromatinized (76,77). In order to address the enhancer-blocking function of dREAM/CP190-bound regions in more detail and dissect the underlying mechanism it will, therefore, be interesting to test the identified elements in an *in vivo* enhancer-blocking assay.

dREAM facilitates CP190 chromatin binding

The underlying mechanism(s) by which dREAM cooperates with CP190 is likely to be connected with the ability of dREAM to help establish or maintain CP190 chromatin association. Interestingly, CP190, but not Beaf-32 chromatin binding was reduced in *de2f2* mutant animals (Figure 5D). Beaf-32 can bind to DNA in a sequence-specific manner through recognition of the CGATA motif (78). In contrast, CP190 does not possess known DNA-binding activity and is thought to get recruited indirectly by DNA-binding factors. This view is based on physical and functional interactions with sequence-specific insulator-binding proteins as well as the high degree of co-localization of these factors in genomic binding studies (30,32,34–36,44). Despite the high degree of overlap in their genomic binding profiles, the significance of Beaf-32 for CP190 chromatin association is unclear. Recent studies found contrary results regarding the significance of Beaf-32 for CP190 recruitment (37,44). CP190 chromatin association is reduced, however, in *dctcf* mutant animals and upon dCTCF knockdown in tissue culture cells (35,37,42). A recent study in *Drosophila* cells has shown that CP190 associates with the majority of its binding sites in distinct combinatorial patterns with other insulator-binding proteins. At a subset of binding sites, however, it does not co-localize with any known DNA-binding protein, suggesting that CP190 either has intrinsic DNA-binding activity or depends on a not yet identified factor for recruitment (37). Given the physical interaction between dREAM complex subunits and CP190, we speculate that dREAM complexes may be directly involved in the recruitment of CP190 to these sites.

Interestingly, the strong enrichment of DPGs among dREAM-bound genes is conserved in human cells (Supplementary Figure S7), but, to date, CTCF is the only known mammalian ortholog of fly insulator-binding proteins. Based on the extensive co-localization among insulator-binding proteins in the fly genome (34,37,45) and the presence of CP190 as a common insulator co-factor, which suggests a shared mechanism, it has been proposed that the functions of the *Drosophila* proteins were integrated in CTCF (79). CP190 is a chromatin architectural protein (43,44), and it is conceivable that another protein with sim-

ilar properties has adopted its role. Interestingly, the function of mammalian CTCF, including its role in chromatin looping, has been intimately linked to the Cohesin complex (80). Remarkably, inactivation of pRB in mammalian cells results in reduced chromatin association of Cohesin and the functionally related Condensin II-subunit Cap-D3 (81,82). Furthermore, pRB physically interacts with Condensin II-subunits in fly and human cells and RBF1 co-localizes extensively with Cap-D3 on polytene chromosomes (26), raising the fascinating possibility that these specialized architectural protein complexes have taken over a CP190-related role in higher organisms.

Over the past years, several studies have shown that a variety of different proteins co-localize with insulator-binding proteins and/or contribute to insulator function. These factors range from different combinations of insulator proteins (34,36,37,45) to factors like L(3)MBT (83), Topoisomerase II (48), the ubiquitin ligase dTopors (46), Ago2 (42), the Rm62 helicase (47) and exosome components (84). Further studies are clearly needed to determine how dREAM function is integrated with the array of factors acting in concert with insulator-binding proteins and to delineate a potential role of dREAM complexes in the organization of chromosome architecture.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Pierre Spierer, Harald Saumweber, Craig Hart, Elissa Lei, Jordan Raff and Mike Botchan for reagents used in this paper, and all members of the Dyson, Motamedi, Näär, Rocco, Whetstine and Zou labs for helpful comments and discussions.

M.K. was supported by a Tosteson Postdoctoral Fellowship Award and a DFG Forschungsstipendium. N.J.D is the James and Shirley Curvey MGH Research Scholar.

FUNDING

National Institutes of Health [R01CA64402 and GM053203 to N.J.D.].

Conflict of interest statement. None declared.

REFERENCES

- Cooper, K. (2006) Rb, whi it's not just for metazoans anymore. *Oncogene*, **25**, 5228–5232.
- Gutzat, R., Borghi, L. and Gruijsem, W. (2012) Emerging roles of RETINOBLASTOMA-RELATED proteins in evolution and plant development. *Trends Plant Sci.*, **17**, 139–148.
- van den Heuvel, S. and Dyson, N.J. (2008) Conserved functions of the pRB and E2F families. *Nat. Rev. Mol. Cell Biol.*, **9**, 713–724.
- Du, W. and Dyson, N. (1999) The role of RBF in the introduction of G1 regulation during *Drosophila* embryogenesis. *EMBO J.*, **18**, 916–925.
- Datar, S.A., Jacobs, H.W., de la Cruz, A.F., Lehner, C.F. and Edgar, B.A. (2000) The *Drosophila* cyclin D-Cdk4 complex promotes cellular growth. *EMBO J.*, **19**, 4543–4554.
- Dimova, D.K., Stevaux, O., Frolov, M.V. and Dyson, N.J. (2003) Cell cycle-dependent and cell cycle-independent control of transcription by the *Drosophila* E2F/RB pathway. *Genes Dev.*, **17**, 2308–2320.

7. Moon, N.S., Di Stefano, L. and Dyson, N. (2006) A gradient of epidermal growth factor receptor signaling determines the sensitivity of *rbf1* mutant cells to E2F-dependent apoptosis. *Mol. Cell Biol.*, **26**, 7601–7615.
8. Korenjak, M., Taylor-Harding, B., Binne, U.K., Satterlee, J.S., Stevaux, O., Aasland, R., White-Cooper, H., Dyson, N. and Brehm, A. (2004) Native E2F/RBF complexes contain Myb-interacting proteins and repress transcription of developmentally controlled E2F target genes. *Cell*, **119**, 181–193.
9. Lewis, P.W., Beall, E.L., Fleischer, T.C., Georgette, D., Link, A.J. and Botchan, M.R. (2004) Identification of a *Drosophila* Myb-E2F2/RBF transcriptional repressor complex. *Genes Dev.*, **18**, 2929–2940.
10. Sadasivam, S. and DeCaprio, J.A. (2013) The DREAM complex: master coordinator of cell cycle-dependent gene expression. *Nat. Rev. Cancer*, **13**, 585–595.
11. Harrison, M.M., Ceol, C.J., Lu, X. and Horvitz, H.R. (2006) Some *C. elegans* class B synthetic multivulva proteins encode a conserved LIN-35 Rb-containing complex distinct from a NuRD-like complex. *Proc. Natl Acad. Sci. U.S.A.*, **103**, 16782–16787.
12. Litovchick, L., Sadasivam, S., Florens, L., Zhu, X., Swanson, S.K., Velmurugan, S., Chen, R., Washburn, M.P., Liu, X.S. and DeCaprio, J.A. (2007) Evolutionarily conserved multisubunit RBL2/p130 and E2F4 protein complex represses human cell cycle-dependent genes in quiescence. *Mol. Cell*, **26**, 539–551.
13. Schmit, F., Korenjak, M., Mannefeld, M., Schmitt, K., Franke, C., von Eyss, B., Gagraca, S., Hanel, F., Brehm, A. and Gaubatz, S. (2007) LINC, a human complex that is related to pRB-containing complexes in invertebrates regulates the expression of G2/M genes. *Cell Cycle*, **6**, 1903–1913.
14. Georgette, D., Ahn, S., MacAlpine, D.M., Cheung, E., Lewis, P.W., Beall, E.L., Bell, S.P., Speed, T., Manak, J.R. and Botchan, M.R. (2007) Genomic profiling and expression studies reveal both positive and negative activities for the *Drosophila* Myb MuvB/dREAM complex in proliferating cells. *Genes Dev.*, **21**, 2880–2896.
15. Sim, C.K., Perry, S., Tharadra, S.K., Lipsick, J.S. and Ray, A. (2012) Epigenetic regulation of olfactory receptor gene expression by the Myb-MuvB/dREAM complex. *Genes Dev.*, **26**, 2483–2498.
16. Sadasivam, S., Duan, S. and DeCaprio, J.A. (2012) The MuvB complex sequentially recruits B-Myb and FoxM1 to promote mitotic gene expression. *Genes Dev.*, **26**, 474–489.
17. Muller, G.A., Quaa, M., Schumann, M., Krause, E., Padi, M., Fischer, M., Litovchick, L., DeCaprio, J.A. and Engeland, K. (2012) The CHR promoter element controls cell cycle-dependent gene transcription and binds the DREAM and MMB complexes. *Nucleic Acids Res.*, **40**, 1561–1578.
18. Litovchick, L., Florens, L.A., Swanson, S.K., Washburn, M.P. and DeCaprio, J.A. (2011) DYRK1A protein kinase promotes quiescence and senescence through DREAM complex assembly. *Genes Dev.*, **25**, 801–813.
19. Tschop, K., Conery, A.R., Litovchick, L., Decaprio, J.A., Settlemann, J., Harlow, E. and Dyson, N. (2011) A kinase shRNA screen links LATS2 and the pRB tumor suppressor. *Genes Dev.*, **25**, 814–830.
20. Wen, H., Andrejka, L., Ashton, J., Karess, R. and Lipsick, J.S. (2008) Epigenetic regulation of gene expression by *Drosophila* Myb and E2F2-RBF via the Myb-MuvB/dREAM complex. *Genes Dev.*, **22**, 601–614.
21. Kudron, M., Niu, W., Lu, Z., Wang, G., Gerstein, M., Snyder, M. and Reinke, V. (2013) Tissue-specific direct targets of *Caenorhabditis elegans* Rb/E2F dictate distinct somatic and germline programs. *Genome Biol.*, **14**, R5.
22. Gonzalo, S., Garcia-Cao, M., Fraga, M.F., Schotta, G., Peters, A.H., Cotter, S.E., Eguia, R., Dean, D.C., Esteller, M., Jenuwein, T. et al. (2005) Role of the Rb1 family in stabilizing histone methylation at constitutive heterochromatin. *Nat. Cell Biol.*, **7**, 420–428.
23. Isaac, C.E., Francis, S.M., Martens, A.L., Julian, L.M., Seifried, L.A., Erdmann, N., Binne, U.K., Harrington, L., Sicinski, P., Berube, N.G. et al. (2006) The retinoblastoma protein regulates pericentric heterochromatin. *Mol. Cell Biol.*, **26**, 3659–3671.
24. Siddiqui, H., Fox, S.R., Gunawardena, R.W. and Knudsen, E.S. (2007) Loss of RB compromises specific heterochromatin modifications and modulates HP1 α dynamics. *J. Cell. Physiol.*, **211**, 131–137.
25. Montoya-Durango, D.E., Liu, Y., Teneng, I., Kalbfleisch, T., Lacy, M.E., Steffen, M.C. and Ramos, K.S. (2009) Epigenetic control of mammalian LINE-1 retrotransposon by retinoblastoma proteins. *Mutat. Res.*, **665**, 20–28.
26. Longworth, M.S., Herr, A., Ji, J.Y. and Dyson, N.J. (2008) RBF1 promotes chromatin condensation through a conserved interaction with the Condensin II protein dCAP-D3. *Genes Dev.*, **22**, 1011–1024.
27. Longworth, M.S., Walker, J.A., Anderssen, E., Moon, N.S., Gladden, A., Heck, M.M., Ramaswamy, S. and Dyson, N.J. (2012) A shared role for RBF1 and dCAP-D3 in the regulation of transcription with consequences for innate immunity. *PLoS Genet.*, **8**, e1002618.
28. Van Bortle, K. and Corces, V.G. (2012) Nuclear organization and genome function. *Annu. Rev. Cell. Dev. Biol.*, **28**, 163–187.
29. Gerasimova, T.I., Gdula, D.A., Gerasimov, D.V., Simonova, O. and Corces, V.G. (1995) A *Drosophila* protein that imparts directionality on a chromatin insulator is an enhancer of position-effect variegation. *Cell*, **82**, 587–597.
30. Pai, C.Y., Lei, E.P., Ghosh, D. and Corces, V.G. (2004) The centrosomal protein CP190 is a component of the gypsy chromatin insulator. *Mol. Cell*, **16**, 737–748.
31. Gerasimova, T.I., Lei, E.P., Bushey, A.M. and Corces, V.G. (2007) Coordinated control of dCTCF and gypsy chromatin insulators in *Drosophila*. *Mol. Cell*, **28**, 761–772.
32. Mohan, M., Bartkuhn, M., Herold, M., Philippen, A., Heinel, N., Bardenhagen, I., Leers, J., White, R.A., Renkawitz-Pohl, R., Saumweber, H. et al. (2007) The *Drosophila* insulator proteins CTCF and CP190 link enhancer blocking to body patterning. *EMBO J.*, **26**, 4203–4214.
33. Perez-Lluch, S., Cuartero, S., Azorin, F. and Espinas, M.L. (2008) Characterization of new regulatory elements within the *Drosophila* bithorax complex. *Nucleic Acids Res.*, **36**, 6926–6933.
34. Bushey, A.M., Ramos, E. and Corces, V.G. (2009) Three subclasses of a *Drosophila* insulator show distinct and cell type-specific genomic distributions. *Genes Dev.*, **23**, 1338–1350.
35. Bartkuhn, M., Straub, T., Herold, M., Herrmann, M., Rathke, C., Saumweber, H., Gilfillan, G.D., Becker, P.B. and Renkawitz, R. (2009) Active promoters and insulators are marked by the centrosomal protein 190. *EMBO J.*, **28**, 877–888.
36. Negre, N., Brown, C.D., Shah, P.K., Kheradpour, P., Morrison, C.A., Henikoff, J.G., Feng, X., Ahmad, K., Russell, S., White, R.A. et al. (2010) A comprehensive map of insulator elements for the *Drosophila* genome. *PLoS Genet.*, **6**, e1000814.
37. Schwartz, Y.B., Linder-Basso, D., Kharchenko, P.V., Tolstorukov, M.Y., Kim, M., Li, H.B., Gorchakov, A.A., Minoda, A., Shanower, G., Alekseyenko, A.A. et al. (2012) Nature and function of insulator protein binding sites in the *Drosophila* genome. *Genome Res.*, **22**, 2188–2198.
38. Matzat, L.H., Dale, R.K., Moshkovich, N. and Lei, E.P. (2012) Tissue-specific regulation of chromatin insulator function. *PLoS Genet.*, **8**, e1003069.
39. Sexton, T., Yaffe, E., Kenigsberg, E., Bantignies, F., Leblanc, B., Hoichman, M., Parrinello, H., Tanay, A. and Cavalli, G. (2012) Three-dimensional folding and functional organization principles of the *Drosophila* genome. *Cell*, **148**, 458–472.
40. Hou, C., Li, L., Qin, Z.S. and Corces, V.G. (2012) Gene density, transcription, and insulators contribute to the partitioning of the *Drosophila* genome into physical domains. *Mol. Cell*, **48**, 471–484.
41. Li, H.B., Muller, M., Bahechar, I.A., Kyrchanova, O., Ohno, K., Georgiev, P. and Pirrotta, V. (2011) Insulators, not Polycomb response elements, are required for long-range interactions between Polycomb targets in *Drosophila melanogaster*. *Mol. Cell Biol.*, **31**, 616–625.
42. Moshkovich, N., Nisha, P., Boyle, P.J., Thompson, B.A., Dale, R.K. and Lei, E.P. (2011) RNAi-independent role for Argonaute2 in CTCF/CP190 chromatin insulator function. *Genes Dev.*, **25**, 1686–1701.
43. Wood, A.M., Van Bortle, K., Ramos, E., Takenaka, N., Rohrbach, M., Jones, B.C., Jones, K.C. and Corces, V.G. (2011) Regulation of chromatin organization and inducible gene expression by a *Drosophila* insulator. *Mol. Cell*, **44**, 29–38.
44. Liang, J., Lacroix, L., Gamot, A., Cuddapah, S., Queille, S., Lhouaud, P., Lepetit, P., Martin, P.G., Vogelmann, J., Court, F. et al. (2014) Chromatin Immunoprecipitation Indirect Peaks Highlight Long-Range Interactions of Insulator Proteins and Pol II Pausing. *Mol. Cell*, **53**, 672–681.
45. Van Bortle, K., Ramos, E., Takenaka, N., Yang, J., Wahi, J.E. and Corces, V.G. (2012) *Drosophila* CTCF tandemly aligns with other

- insulator proteins at the borders of H3K27me3 domains. *Genome Res.*, **22**, 2176–2187.
46. Capelson, M. and Corces, V.G. (2005) The ubiquitin ligase dTopors directs the nuclear organization of a chromatin insulator. *Mol. Cell*, **20**, 105–116.
 47. Lei, E.P. and Corces, V.G. (2006) RNA interference machinery influences the nuclear organization of a chromatin insulator. *Nat. Genet.*, **38**, 936–941.
 48. Ramos, E., Torre, E.A., Bushey, A.M., Gurudatta, B.V. and Corces, V.G. (2011) DNA topoisomerase II modulates insulator function in *Drosophila*. *PLoS One*, **6**, e16562.
 49. Stevaux, O., Dimova, D., Frolov, M.V., Taylor-Harding, B., Morris, E. and Dyson, N. (2002) Distinct mechanisms of E2F regulation by *Drosophila* RBF1 and RBF2. *EMBO J.*, **21**, 4927–4937.
 50. Frasch, M., Glover, D.M. and Saumweber, H. (1986) Nuclear antigens follow different pathways into daughter nuclei during mitosis in early *Drosophila* embryos. *J. Cell Sci.*, **82**, 155–172.
 51. Whitfield, W.G., Chaplin, M.A., Oegema, K., Parry, H. and Glover, D.M. (1995) The 190 kDa centrosome-associated protein of *Drosophila melanogaster* contains four zinc finger motifs and binds to specific sites on polytene chromosomes. *J. Cell Sci.*, **108**(Pt 11), 3377–3387.
 52. Jiang, N., Emberly, E., Cuvier, O. and Hart, C.M. (2009) Genome-wide mapping of boundary element-associated factor (BEAF) binding sites in *Drosophila melanogaster* links BEAF to transcription. *Mol. Cell Biol.*, **29**, 3556–3568.
 53. Frolov, M.V., Huen, D.S., Stevaux, O., Dimova, D., Balczarek-Strang, K., Elsdon, M. and Dyson, N.J. (2001) Functional antagonism between E2F family members. *Genes Dev.*, **15**, 2146–2160.
 54. Beall, E.L., Bell, M., Georgette, D. and Botchan, M.R. (2004) Dm-myb mutant lethality in *Drosophila* is dependent upon mip130: positive and negative regulation of DNA replication. *Genes Dev.*, **18**, 1667–1680.
 55. Butcher, R.D., Chodagam, S., Basto, R., Wakefield, J.G., Henderson, D.S., Raff, J.W. and Whitfield, W.G. (2004) The *Drosophila* centrosome-associated protein CP190 is essential for viability but not for cell division. *J. Cell Sci.*, **117**, 1191–1199.
 56. Royzman, I., Austin, R.J., Bosco, G., Bell, S.P. and Orr-Weaver, T.L. (1999) ORC localization in *Drosophila* follicle cells and the effects of mutations in dE2F and dDP. *Genes Dev.*, **13**, 827–840.
 57. Frolov, M.V., Moon, N.S. and Dyson, N.J. (2005) dDP is needed for normal cell proliferation. *Mol. Cell Biol.*, **25**, 3027–3039.
 58. Roy, S., Gilbert, M.K. and Hart, C.M. (2007) Characterization of BEAF mutations isolated by homologous recombination in *Drosophila*. *Genetics*, **176**, 801–813.
 59. Negre, N., Lavrov, S., Hennetin, J., Bellis, M. and Cavalli, G. (2006) Mapping the distribution of chromatin proteins by ChIP on chip. *Methods Enzymol.*, **410**, 316–341.
 60. Toedling, J., Skylar, O., Krueger, T., Fischer, J.J., Sperling, S. and Huber, W. (2007) Ringo—an R/Bioconductor package for analyzing ChIP-chip readouts. *BMC Bioinform.*, **8**, 221.
 61. Filion, G.J., van Bommel, J.G., Braunschweig, U., Talhout, W., Kind, J., Ward, L.D., Brugman, W., de Castro, I.J., Kerkhoven, R.M., Bussemaker, H.J. *et al.* (2010) Systematic protein location mapping reveals five principal chromatin types in *Drosophila* cells. *Cell*, **143**, 212–224.
 62. Korenjak, M., Anderssen, E., Ramaswamy, S., Whetstone, J.R. and Dyson, N.J. (2012) RBF binding to both canonical E2F targets and noncanonical targets depends on functional dE2F/dDP complexes. *Mol. Cell Biol.*, **32**, 4375–4387.
 63. Taylor-Harding, B., Binne, U.K., Korenjak, M., Brehm, A. and Dyson, N.J. (2004) p55, the *Drosophila* ortholog of RbAp46/RbAp48, is required for the repression of dE2F2/RBF-regulated genes. *Mol. Cell Biol.*, **24**, 9124–9136.
 64. Yang, L. and Yu, J. (2009) A comparative analysis of divergently-paired genes (DPGs) among *Drosophila* and vertebrate genomes. *BMC Evol. Biol.*, **9**, 55.
 65. Yang, J., Ramos, E. and Corces, V.G. (2012) The BEAF-32 insulator coordinates genome organization and function during the evolution of *Drosophila* species. *Genome Res.*, **22**, 2199–2207.
 66. Raab, J.R. and Kamakaka, R.T. (2010) Insulators and promoters: closer than we think. *Nat. Rev. Genet.*, **11**, 439–446.
 67. Tehurikov, N.A., Kretova, O.V., Moiseeva, E.D. and Sosin, D.V. (2009) Evidence for RNA synthesis in the intergenic region between enhancer and promoter and its inhibition by insulators in *Drosophila melanogaster*. *Nucleic Acids Res.*, **37**, 111–122.
 68. Kyrchanova, O., Ivlieva, T., Toshchakov, S., Parshikov, A., Maksimenko, O. and Georgiev, P. (2011) Selective interactions of boundaries with upstream region of Abd-B promoter in *Drosophila* bithorax complex and role of dCTCF in this process. *Nucleic Acids Res.*, **39**, 3042–3052.
 69. Blanton, J., Gaszner, M. and Schedl, P. (2003) Protein:protein interactions and the pairing of boundary elements in vivo. *Genes Dev.*, **17**, 664–675.
 70. Hart, C.M., Cuvier, O. and Laemmli, U.K. (1999) Evidence for an antagonistic relationship between the boundary element-associated factor BEAF and the transcription factor DREF. *Chromosoma*, **108**, 375–383.
 71. Emberly, E., Blattes, R., Schuettengruber, B., Hennion, M., Jiang, N., Hart, C.M., Kas, E. and Cuvier, O. (2008) BEAF regulates cell-cycle genes through the controlled deposition of H3K9 methylation marks into its conserved dual-core binding sites. *PLoS Biol.*, **6**, 2896–2910.
 72. Negre, N., Brown, C.D., Ma, L., Bristow, C.A., Miller, S.W., Wagner, U., Kheradpour, P., Eaton, M.L., Loriaux, P., Sealfon, R. *et al.* (2011) A cis-regulatory map of the *Drosophila* genome. *Nature*, **471**, 527–531.
 73. Li, M., Belozero, V.E. and Cai, H.N. (2010) Modulation of chromatin boundary activities by nucleosome-remodeling activities in *Drosophila melanogaster*. *Mol. Cell Biol.*, **30**, 1067–1076.
 74. Soshnev, A.A., Li, X., Wehling, M.D. and Geyer, P.K. (2008) Context differences reveal insulator and activator functions of a Su(Hw) binding region. *PLoS Genet.*, **4**, e1000159.
 75. Li, M., Belozero, V.E. and Cai, H.N. (2008) Analysis of chromatin boundary activity in *Drosophila* cells. *BMC Mol. Biol.*, **9**, 109–119.
 76. Wolffe, A.P. (1999) *Chromatin: Structure and Function*, 3rd edn. Academic press, London, UK.
 77. Hebbar, P.B. and Archer, T.K. (2008) Altered histone H1 stoichiometry and an absence of nucleosome positioning on transfected DNA. *J. Biol. Chem.*, **283**, 4595–4601.
 78. Zhao, K., Hart, C.M. and Laemmli, U.K. (1995) Visualization of chromosomal domains with boundary element-associated factor BEAF-32. *Cell*, **81**, 879–889.
 79. Gurudatta, B.V. and Corces, V.G. (2009) Chromatin insulators: lessons from the fly. *Brief. Funct. Genomics Proteomics*, **8**, 276–282.
 80. Merckenschlager, M. and Odom, D.T. (2013) CTCF and cohesin: linking gene regulatory elements with their targets. *Cell*, **152**, 1285–1297.
 81. Manning, A.L., Longworth, M.S. and Dyson, N.J. (2010) Loss of pRB causes dysfunction and chromosomal instability. *Genes Dev.*, **24**, 1364–1376.
 82. Coschi, C.H., Ishak, C.A., Gallo, D., Marshall, A., Talluri, S., Wang, J., Cecchini, M.J., Martens, A.L., Percy, V., Welch, I. *et al.* (2014) Haploinsufficiency of an RB-E2F1-Condensin II complex leads to aberrant replication and aneuploidy. *Cancer Disc.*, April 16 [Epub ahead of print]
 83. Richter, C., Oktaba, K., Steinmann, J., Muller, J. and Knoblich, J.A. (2011) The tumour suppressor L(3)mbt inhibits neuroepithelial proliferation and acts on insulator elements. *Nat. Cell Biol.*, **13**, 1029–1039.
 84. Lim, S.J., Boyle, P.J., Chinen, M., Dale, R.K. and Lei, E.P. (2013) Genome-wide localization of exosome components to active promoters and chromatin insulators in *Drosophila*. *Nucleic Acids Res.*, **41**, 2963–2980.
 85. Minervini, C.F., Ruggieri, S., Traversa, M., D’Aiuto, L., Marsano, R.M., Leronni, D., Centomani, I., De Giovanni, C. and Viggiano, L. (2010) Evidences for insulator activity of the 5’UTR of the *Drosophila melanogaster* LTR-retrotransposon ZAM. *Mol. Genet. Genomics*, **283**, 503–509.