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Supplemental Material to:

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Highly conserved ENY2/Sus1 protein binds to Drosophila CTCF and is required for barrier activity

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Supplemental Materials

Chromatin immunoprecipitation from BG3 cells

Samples of 10^7 BG3 cells in 10 mL of Schneider medium were treated with 37% formaldehyde added to a final concentration of 1% and incubated on a rotator at room temperature for 10 min. Cross-linking was stopped by 0.125 M glycine, and the samples were washed with three portions of PBS (pH 8.0) with 0.5 mM PMSF and pelleted at 1000 rpm, 4°C, for 5 min. The pellet was resuspended in 10 mL of buffer I (25 mM HEPES, pH 7.8; 1.5 mM MgCl2, 10 mM KCl, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, Calbiochem Cocktail V) and placed on ice for 10 min. The suspension was then homogenized by 20 strokes in Dounce homogenizer with pestle B and centrifuged at 2000 rpm, 4°C, for 5 min. The pellet was resuspended in 5 mL of buffer II (50 mM HEPES, pH 7.8; 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, Calbiochem Cocktail V), sonicated in a Bioruptor sonifier (Diagenode) for 15 times, with alternating 30-s ON/OFF intervals, and centrifuged at 14 000 rpm, 4°C, for 5 min to remove cell debris. A 50-µL aliquot of the supernatant was used to test the results of sonication and measure DNA concentration: the sample was diluted with 350 µL of elution buffer (50 mM Tris, pH 8.0; 1 mM EDTA, 1% SDS, 50 mM $NaHCO_3$) and treated with RNase A (1 µL from 10 mg/mL stock) at 37°C for 1 h and then with proteinase K (1 µL from 20 mg/mL stock) at 42°C for 2 h. After subsequent incubation at 65°C for 6 h, DNA was isolated by phenol–chloroform–isoamyl alcohol extraction, concentrated by ethanol precipitation with glycogen (each tube was supplemented with 5 µL of glycogen from 20 mg/mL stock), 40 µL of sodium acetate (from 3 M stock), and 1 mL of ethanol, vortexed, and placed at –20°C for 4–5 h.

The precipitated DNA was pelleted at 14 000 rpm for 30 min, washed with 80% ethanol, and resuspended in 50 μL of MilliQ water.

Protein A Sepharose (Pierce) was washed with three portions of buffer II and incubated with 1 mg/mL BSA in the same buffer on a rotator at 4°C for 4 h. Chromatin samples containing 10–20 μg of DNA equivalent were each diluted with buffer II to a final volume of 1 mL, their 50-μL aliquots were stored as input material, and then the samples were incubated overnight, at 4°C, with appropriate antibodies—rabbit antibodies against dCTCF (1:500), ENY2 (1:200), Pc (1:1000), or with nonspecific IgG purified from rabbit preimmune sera (control)—in the presence of blocked Protein A Sepharose beads (40 μL). After incubation, the beads were washed on a rotator, at 4°C, with three 1-mL portions of buffer II and two 1-mL portions of buffer III (50 mM HEPES, pH 7.8; 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1 % SDS, 0.5 mM PMSF, Calbiochem Cocktail V), each wash for 10 min. Then 400 μL of elution buffer was added, and the beads were treated with RNase A (1 μL from 10 mg/mL stock) at 37°C for 1 h and, after adding 20 μL of NaCl (from 4 M stock), with proteinase K (1 μL from 20 mg/mL stock) at 42°C for 2 h. The samples were incubated on a thermoshaker at 65°C overnight, and DNA was isolated by phenol–chloroform–isoamyl alcohol and chloroform–isoamyl alcohol extraction in phase-lock tubes. The samples were concentrated by ethanol precipitation with glycogen (as described above), and resuspended in MilliQ water. Native chromatin and subsequent immunoprecipitations with antibodies against H3 histone (1:100) and chromatin modifications H3me3K27 (1:200) and H3acK27 (1:200) were performed as described in [73] without trypsinization steps.

The primers used for PCR in ChIP experiments for genome fragments are shown in Table S1.

Chromatin Immunoprecipitation from pupae

Chromatin was prepared from mid-late pupae. A 500-mg sample was ground in a mortar in liquid nitrogen and resuspended in 10 mL of buffer A (15 mM HEPES-KOH, pH 7.6; 60 mM KCl, 15 mM NaCl, 13 mM EDTA, 0.1 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 0.5% NP-40, 0.5 mM DTT) supplemented with 0.5 mM PMSF and Calbiochem Cocktail V. The suspension was then homogenized in a Dounce homogenizer with pestle B and filtered through Nylon Cell Strainer (BD Biosciences, United States). The homogenate was transferred to 3 mL of buffer A with 10% sucrose (AS), and the nuclei were pelleted by centrifugation at 4 000 g, 4°C, for 5 min. The pellet was resuspended in 5 mL of buffer A, homogenized again in a Dounce homogenizer, and transferred to 1.5 mL of buffer AS to collect the nuclei by centrifugation. The nuclear pellet was resuspended in wash buffer (15 mM HEPES-KOH, pH 7.6; 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, protease inhibitors) and cross-linked with 1% formaldehyde for 15 min at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 125 mM. The nuclei were washed with three 10-mL portions of wash buffer and resuspended in 1.5 mL of nuclear lysis buffer (15 mM HEPES, pH 7.6; 140 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 1% Triton X-100, 0.5 mM DTT, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitors). The suspension was sonicated in a Bioruptor sonifier (15 alternating 30-s ON and 60-s off intervals), and its 50-µL aliquot was used to test the results of sonication and measure DNA concentration. Debris was removed by centrifugation at 14 000 g, 4°C, for 10 min, and

chromatin was pre-cleared with Protein A agarose (Pierce) blocked with BSA and salmon sperm DNA, with 50 μL aliquots of such pre-cleared chromatin being stored as input material. Samples containing 10–20 μg of DNA equivalent in 1 mL of nuclear lysis buffer were incubated overnight, at 4°C, with rabbit antibodies against dCTCF (1:500), ENY2 (1:200), Pc (1:1000), or with nonspecific IgG purified from rabbit preimmune sera (control). Chromatin–antibody complexes were collected using blocked Protein A agarose at 4°C over 5 h. After several rounds of washing with lysis buffer (as such and with 500 mM NaCl), LiCl buffer (20 mM Tris-HCl, pH 8; 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, protease inhibitors), and TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA), the DNA was eluted with elution buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA, 1% SDS), the cross-links were reversed, and the precipitated DNA was extracted by the phenol–chloroform method. The enrichment of specific DNA fragments was analyzed by real-time PCR, using a StepOne Plus Thermal Cycler (Applied Biosystems). The primers used for PCR in ChIP experiments for genome fragments are shown in Table S1.

DNA constructs

To generate transgenic flies, vectors containing defective inverted P-element repeats were prepared. The 3-kb *Sal*I–*Bam*HI fragment containing the *yellow* regulatory region (yr) with the body and wing enhancers (fragment –2873 to –1266 bp relative to the transcription start site) was subcloned into the pGEM7 plasmid digested with *Bam*HI and *Xho*I. The 660-bp PRE from *Ultrabithorax* gene surrounded by frt sites (for FLP recombinase) was then inserted at –1868 from the *yellow* transcription start site (yr-PRE). The 5-kb *Bam*HI–*Bgl*II fragment containing the *yellow* coding region (yc) was subcloned into CaSpeRΔ700 (yc-C700). The CaSpeRΔ700 vector

contains the *mini-white* gene without insulator at its 3' side and defective inverted P-element repeats. The tested insulator fragments Fab-8 and Mcp (wild-type or with a mutated dCTCF binding sites) were inserted between two lox sites (for CRE recombinase). These fragments were inserted into the yr-PRE plasmid between enhancers and *yellow* promoter with *Eco*47III at position –893 from the *yellow* transcription start site. Finally, this region (including *yellow* enhancers, PRE, *yellow* promoter, and the tested fragment) was associated with yc-C700 digested with *Xba*I and *Bam*HI.

Genetic crosses

The lines with DNA fragment excisions were obtained by crossing the transposon-bearing flies with the Flp (w^{1118}*; S2CyO, hsFLP, ISA/Sco ;+*) or Cre (*yw; Cyo, P[w+,cre]/Sco; +*) recombinase-expressing lines. The Cre recombinase induces 100% excisions in the next generation. The high level of Flp recombinase was produced by heat shock treatment for 2 h during the first 3 days after hatching. All excisions were confirmed by PCR analysis. Details of the crosses and primers used for genetic analysis and the excision of functional elements are available upon request.

To determine the levels of *yellow* expression, we visually estimated the degree of pigmentation in the bristles, abdominal cuticle, and wing blades of 3- to 5-day-old males developing at 25°C. At least 50 flies from each *y* line were scored independently by two persons. A five-grade scale was used, with grade 1 corresponding to the total loss of *yellow* expression and grade 5 corresponding to wild-type pigmentation. The degree of *yellow* expression in bristles of the thorax and head was scored using a 5-point scale, where 1 denoted loss of pigmentation in all bristles on the thorax and head; ev, extreme variegation (only 1–3 bristles on the thorax and head were partially pigmented); mv, moderate variegation (about half of bristles were yellow);

wv, weak variegation (only 1–3 bristles on the thorax and head were yellow or partially pigmented); and 5, pigmentation of all bristles as in wild-type flies.

Supplemental References

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2. Samara NL, Datta AB, Berndsen CE, Zhang X, Yao T, Cohen RE, Wolberger C. Structural insights into the assembly and function of the SAGA deubiquitinating module. Science 2010; 328:1025-9.

3. Schwartz YB, Linder-Basso D, Kharchenko PV, Tolstorukov MY, Kim M, Li HB, Gorchakov AA, Minoda A, Shanower G, Alekseyenko AA, et al. Nature and function of insulator protein binding sites in the Drosophila genome. Genome research 2012; 22:2188-98.

Supplemental Figure Legends

Figure S1. Molecular docking of two Sus1 molecules and Sus1/dCTCF complex. (A) X-ray structure of Sus1, yeast homolog of ENY2, in complex with ySgf11 [1, 2]. (B) Molecular docking of two Sus1 molecules. (C) Molecular docking of complex of Sus1 and hCTCF zinc fingers.

Figure S2. Genetic interactions between mutations in the genes encoding ENY2, dCTCF, and components of SAGA. (A) Morphological defects in males hemizygous for *e(y)2*^{u1} or homozygous for *dCTCF* mutation (*GE24185/GE24185*) at 25^{o}C. Arrows indicate observed morphological defects. (B) Expression of the *e(y)2* gene in *wt* (y1w1) and *e(y)2*^{u1} (e(y)2 u1, y1w1) males aged 2–3 days at 18^{o}C or 25^{o}C. RNAs were extracted and individual transcript levels were quantified by RT-qPCR using appropriate pairs of primers normalized relative to *rpl32* and *tub*.

Figure S3. Chromosomal landscape at the dCTCF-dependent boundaries flanking H3me3K27 domains [3]. Relative amounts of H3me3K27 were determined by ChIP with the pairs of primers inside (ins), at the boundary (cts), and outside (out) of the H3me3K27 domains. Their relative locations on the cytological map are indicated. The ENY2-independent boundary is marked "R".

Figure S4. Efficiency of dCTCF and ENY2 knockdown in BG3 cells. (A) Depletion levels of dCTCF and ENY2 quantified by RT-qPCR according to quantitative real-time PCR with cDNAs synthesized on RNAs extracted from BG3 cells after treatment with corresponding dsRNAs (dCTCF_Ri or ENY2_Ri). “C” is the mock RNAi control obtained with BG3 cells treated with

GFP dsRNA. Individual transcript levels were determined by quantitative PCR with corresponding primers were normalized relative to rpl32 for the amount of input cDNA (C). Error bars show standard deviations of triplicate PCR measurements. (B) Western blotting for dCTCF and ENY2 of extracts from control GFP dsRNA-treated (C) and specific RNAi treated (dCTCF_Ri or ENY2_Ri) BG3 cells. Lamin was used as internal control. The antibodies used for detection are indicated on the right of each blot.

Figure S5. Comparing relative amounts of Pc and H3K27ac at the selected regions in BG3 cells before and after treatment with specific dsRNA from *dCTCF* or *e(y)2* coding regions. Histograms show the results of ChIP for the relative amounts of Pc, H3K27ac and H3 in previously defined dCTCF-binding regions (see Figure S3). Designations are as in Figure 3.

Figure S6. The mutant Mcp insulator lacking dCTCF-binding site does not block the spreading of Pc in transgenic line. (A) Binding of dCTCF, ENY2, and Pc to the constructs from the homozygous lines. (B) Binding of the same proteins to the construct from the derivative homozygous line obtained by deletion of PRE. Protein binding to the constructs from the homozygous transgenic lines was analyzed by ChIP followed by real-time PCR quantification. Other designations are as in Figure 4.

Table S1. The list of the used primers.

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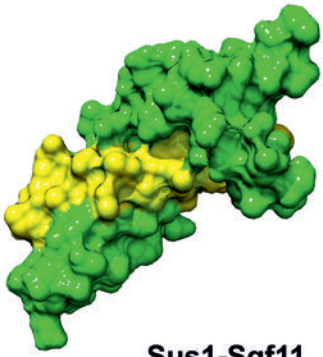

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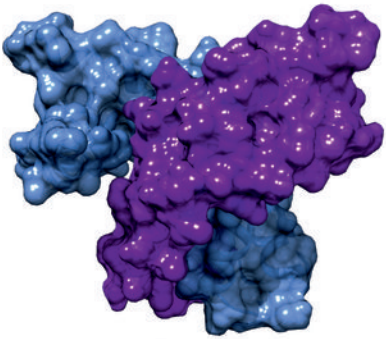

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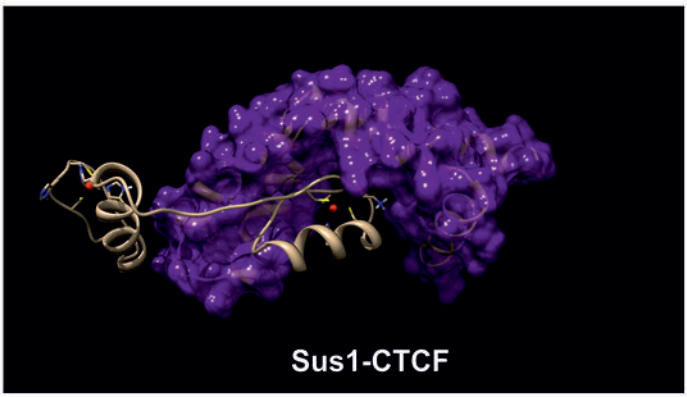

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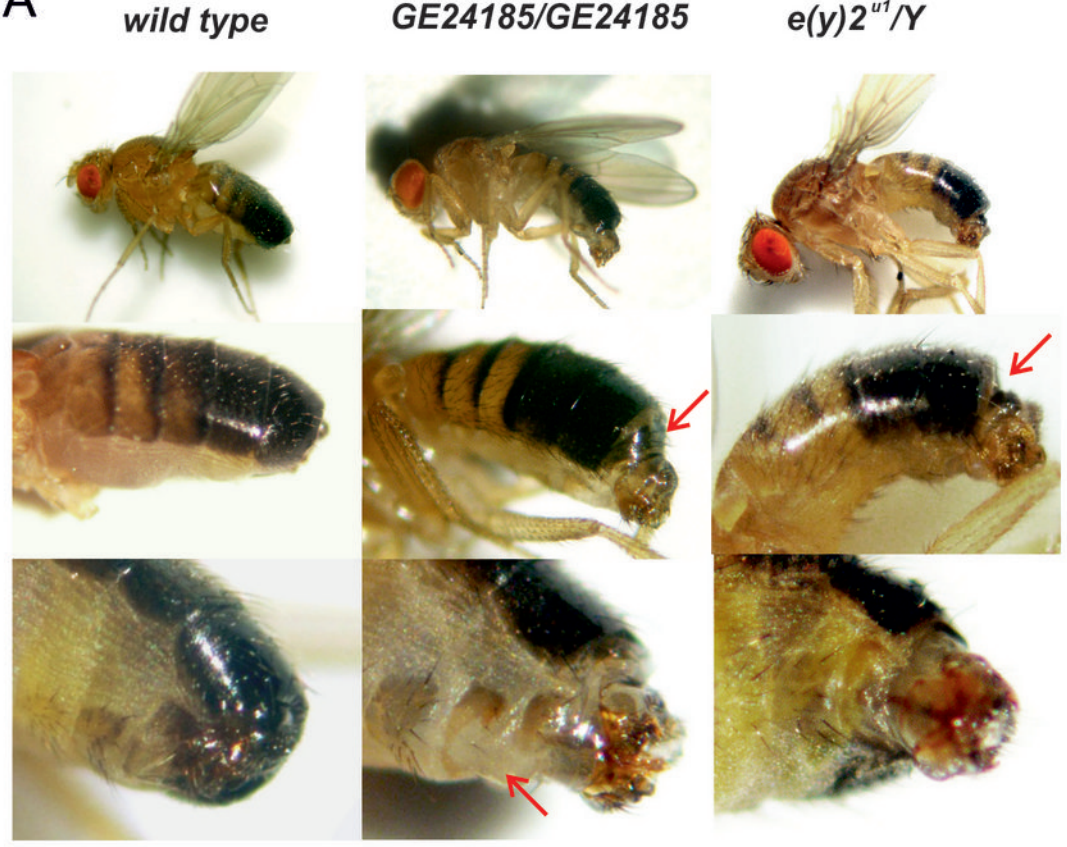
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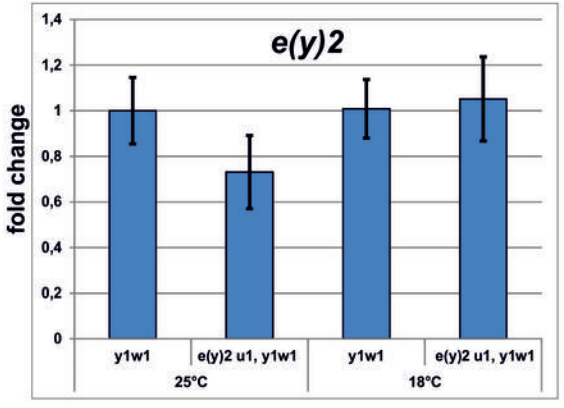
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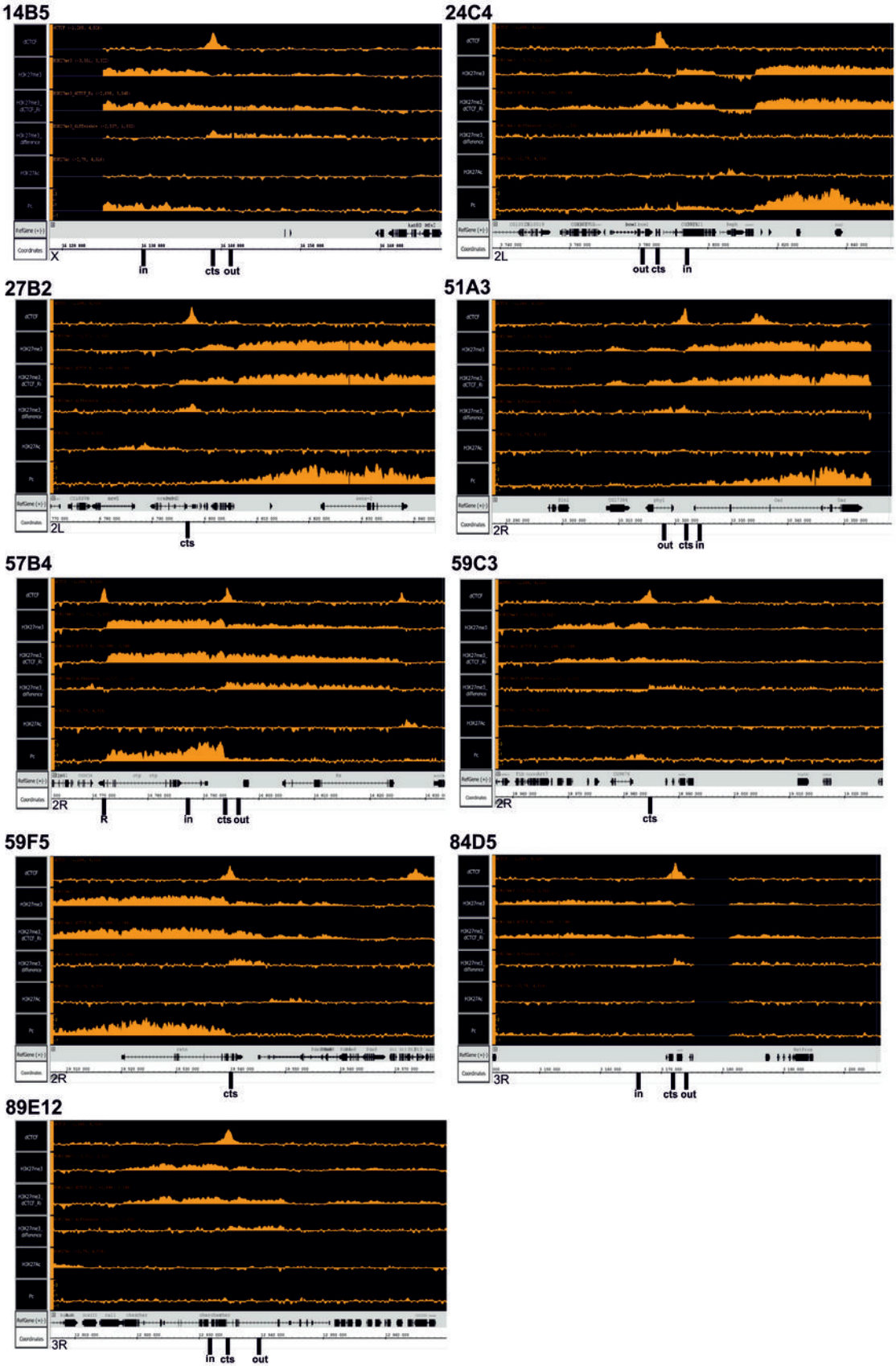
18°C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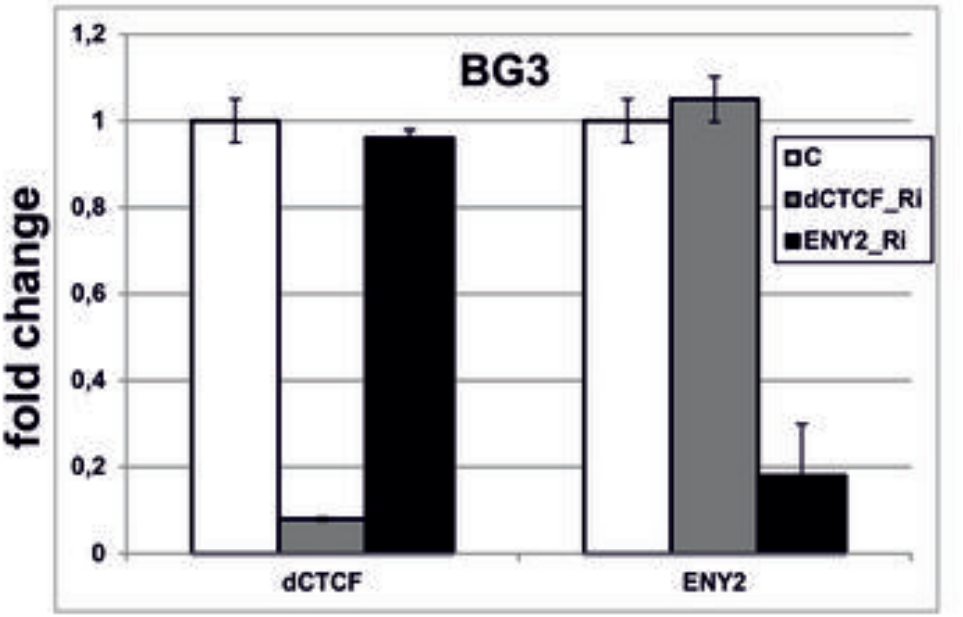
A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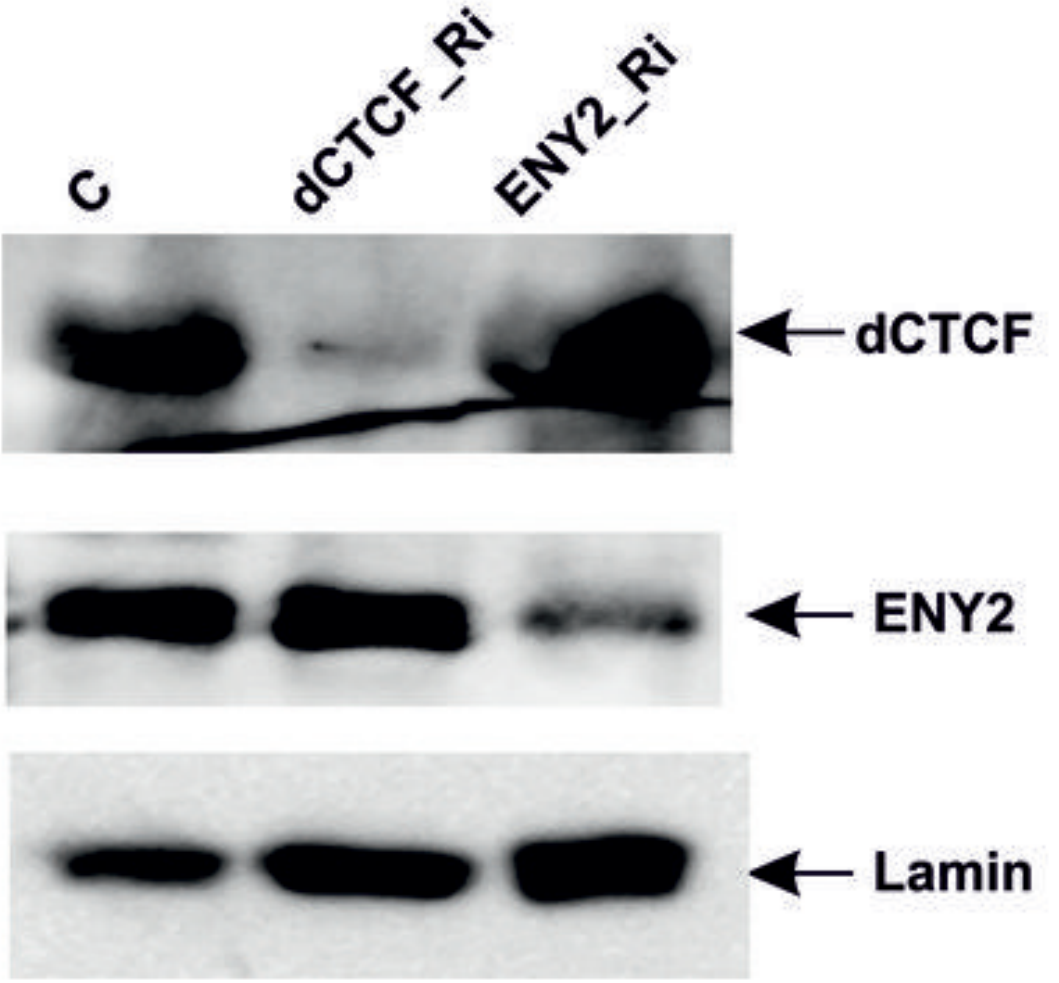
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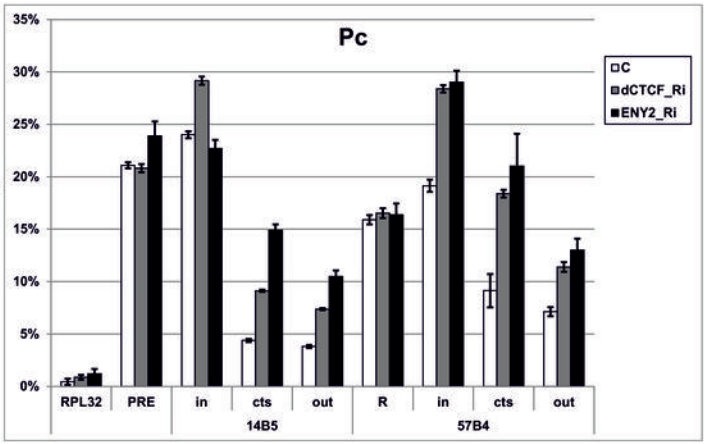
Lamin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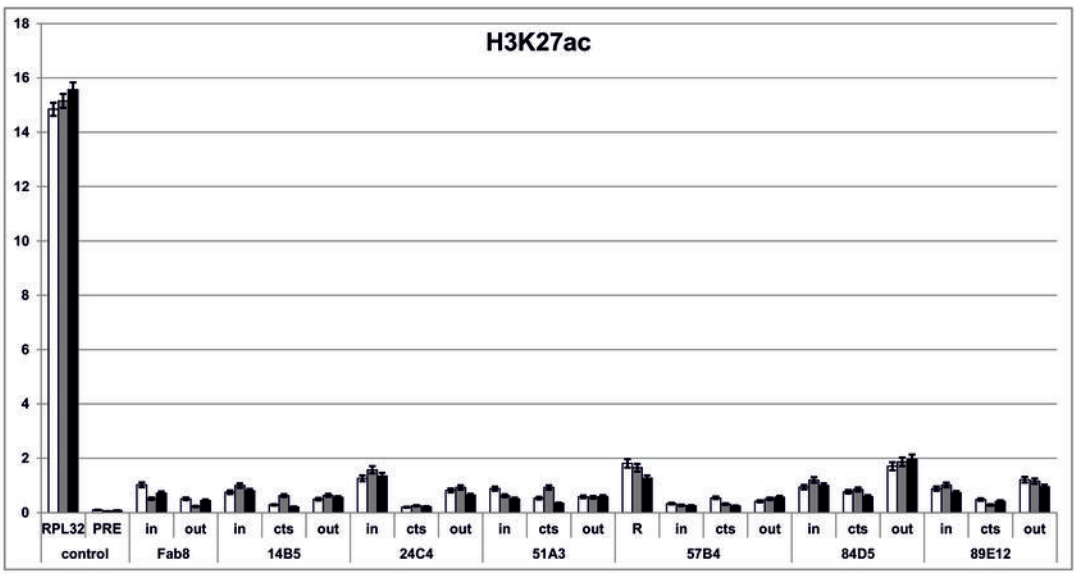
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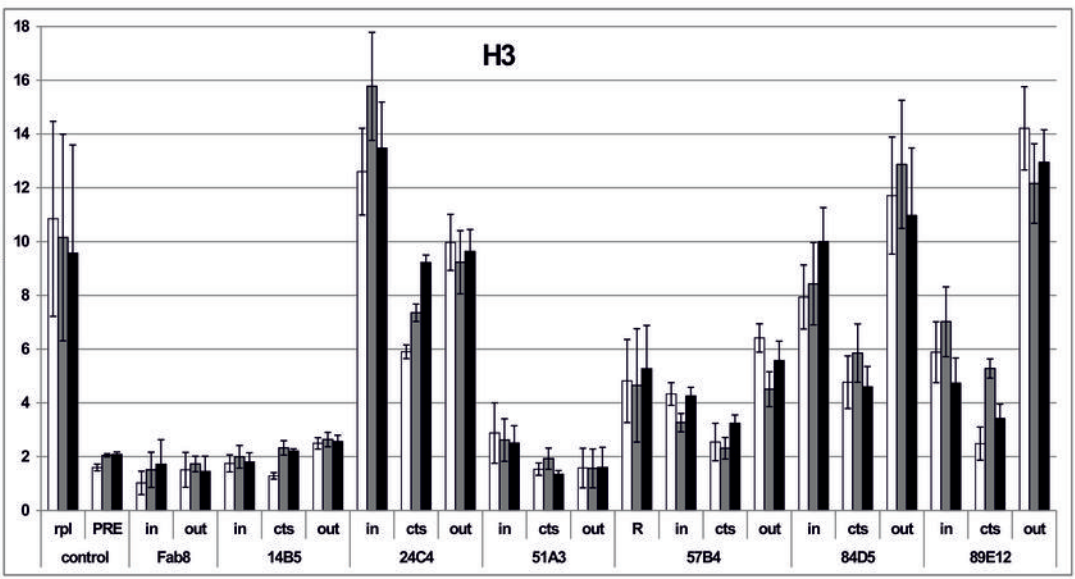
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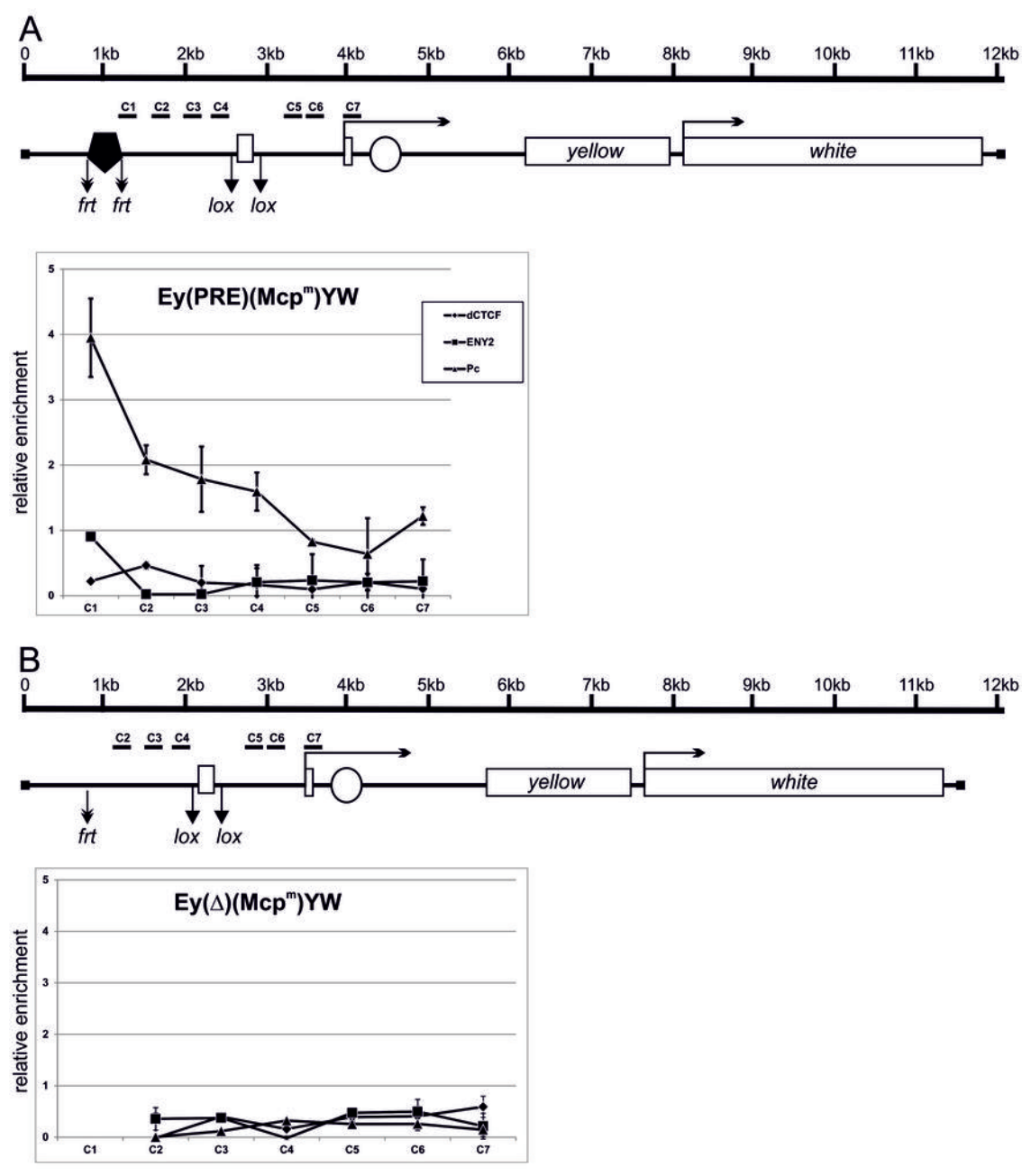
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