SI Appendix

Transcriptional read-through is not sufficient to induce an epigenetic switch in the silencing activity of Polycomb Response Elements

Maksim Erokhin, Pavel Elizar'ev, Alexander Parshikov, Paul Schedl, Pavel Georgiev*, Darya Chetverina*

***To whom correspondence should be addressed.**

e-mail: daria.chetverina@gmail.com; georgiev_p@mail.ru

This PDF file includes

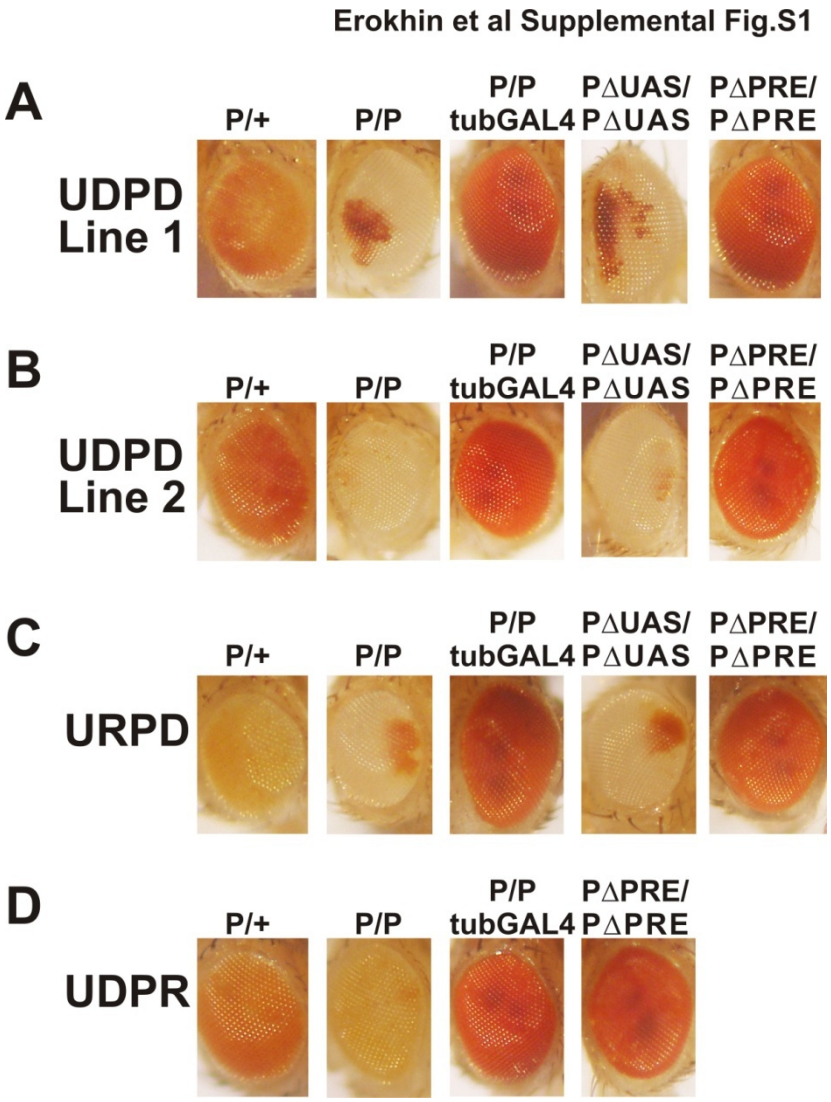
Supplemental Figures S1-S7

Supplemental Tables S1-S6

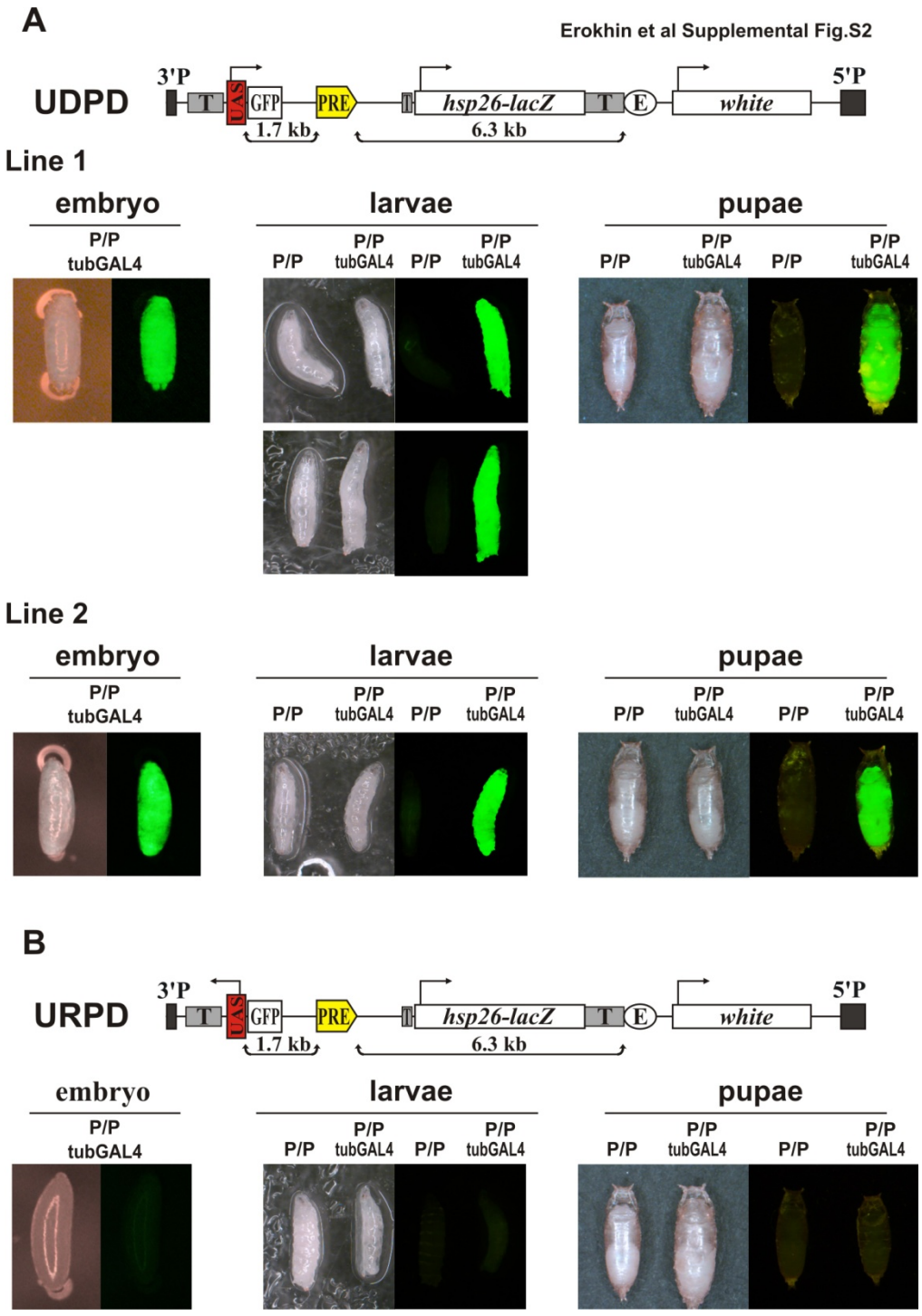
Supplemental Methods

Supplemental References

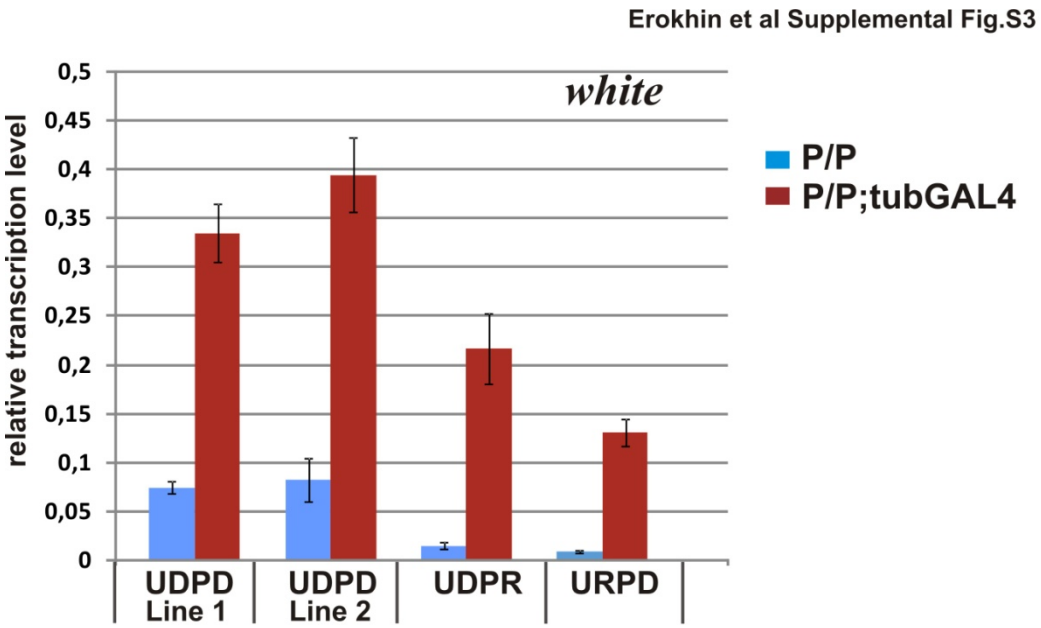
Supplemental Figures



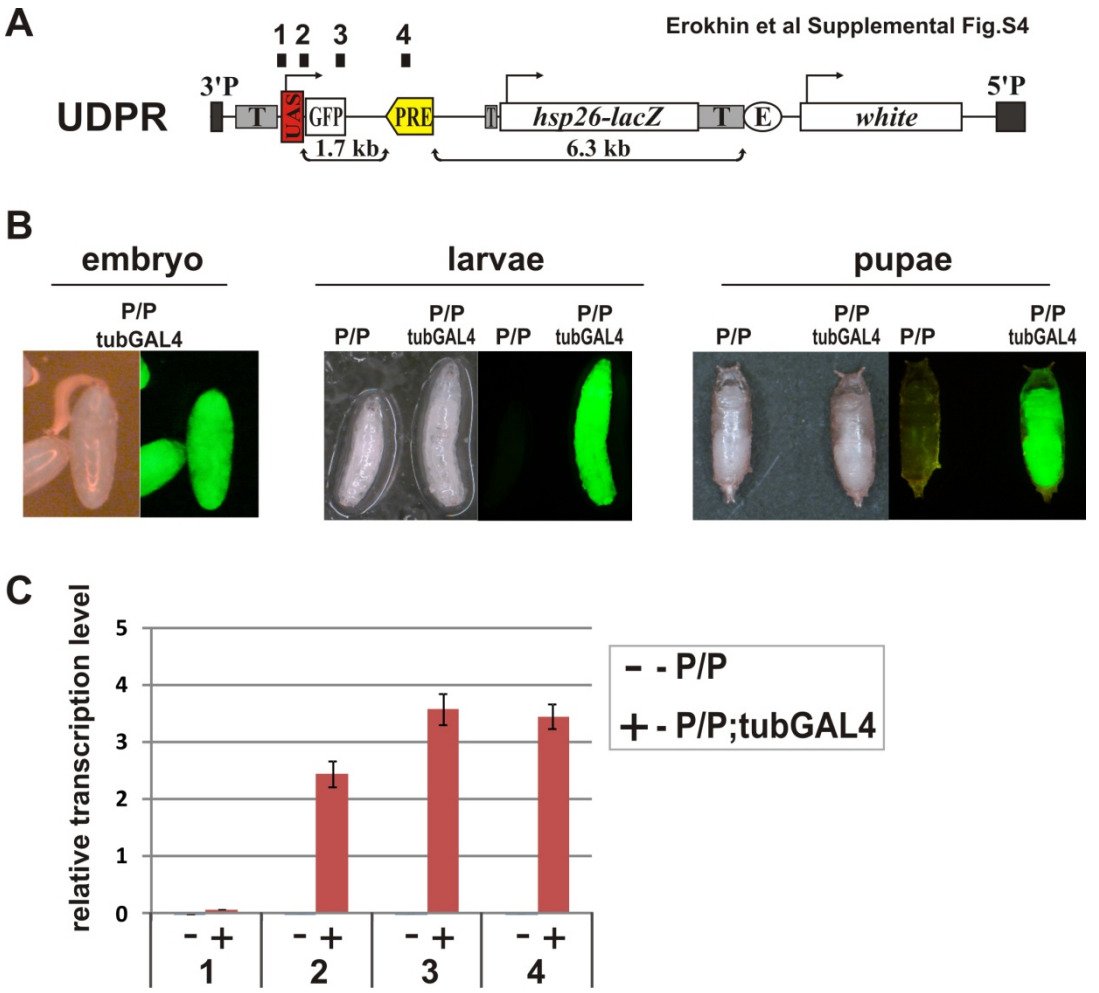
Supplemental Fig. S1. Photographs show eye pigmentation of transgenic lines and its derivates. (A) UDPD line 1; (B) UDPD line 2; (C) URPD; (D) UDPR. P/+ – lines heterozygote for the transgene; P/P – lines homozygote for the transgene; P/P; tubGAL4 – homozygote lines with GAL4 activator; PΔUAS/PΔUAS - homozygote lines with deletion of UAS promoter; PΔPRE/PΔPRE - homozygote lines with deletion of the *bxd*PRE.



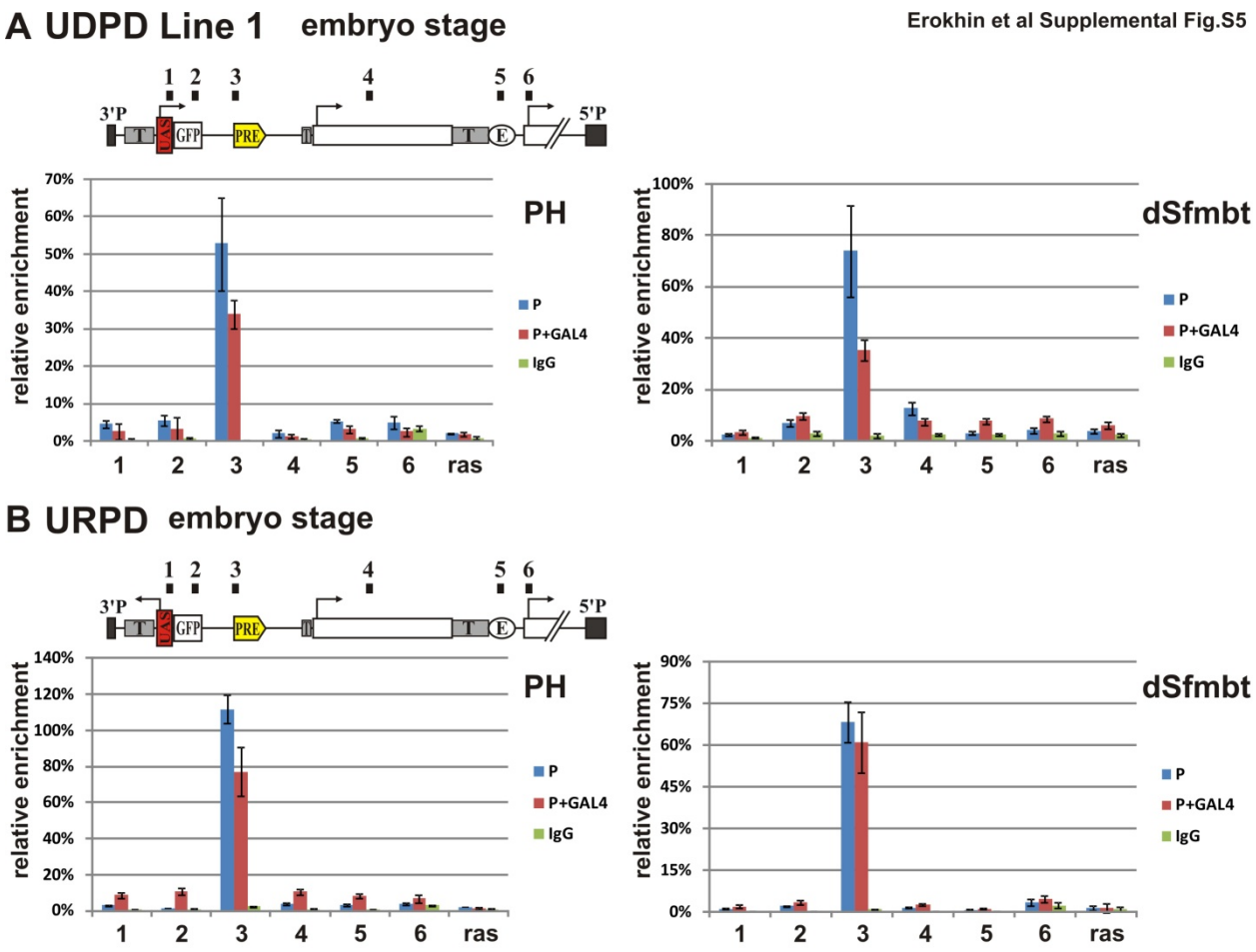
Supplemental Fig. S2. GFP is highly expressed in the UDPD but not in the URPD transgenic lines at the different stages of development. (A) UDPD line1 and 2 (B) URPD. Top: the scheme of transgenes. Bottom: visualization of the GFP expression at the embryo, larva and pupa stages of development. Transmitted light illumination (left) and illumination to visualize green fluorescence (right). P/P – lines homozygote for the transgene; P/P; tubGAL4 – homozygote lines with GAL4 activator**.**



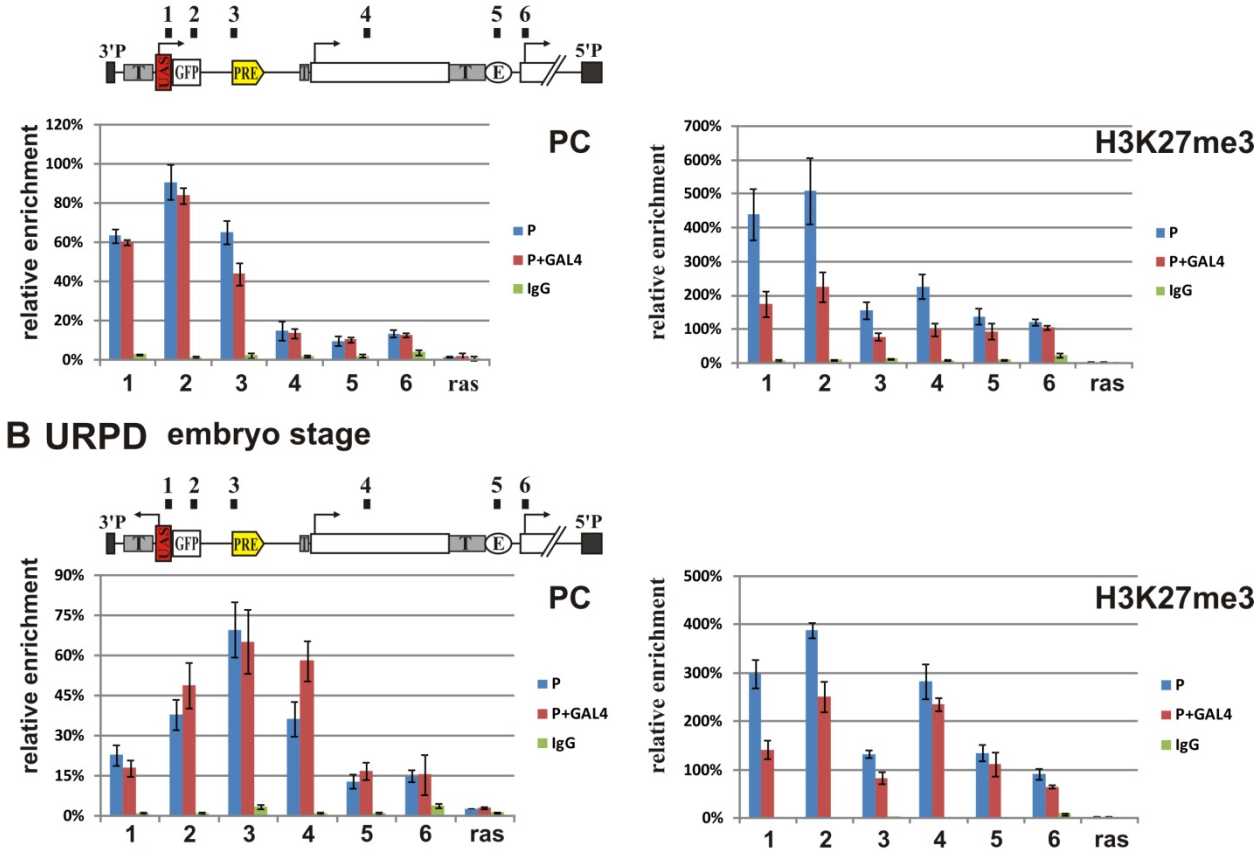
Supplemental Fig. S3. RT-qPCR analysis of *white* gene expression. RNA was isolated from adult flies. P/P - lines homozygote for transgenic construct; P/P; tubGAL4 - lines homozygote for transgenic construct expressing GAL4 activator. Individual transcript levels were normalized relative to *Ras64B* for the amount of input cDNA. Vertical lines indicate standard deviations.



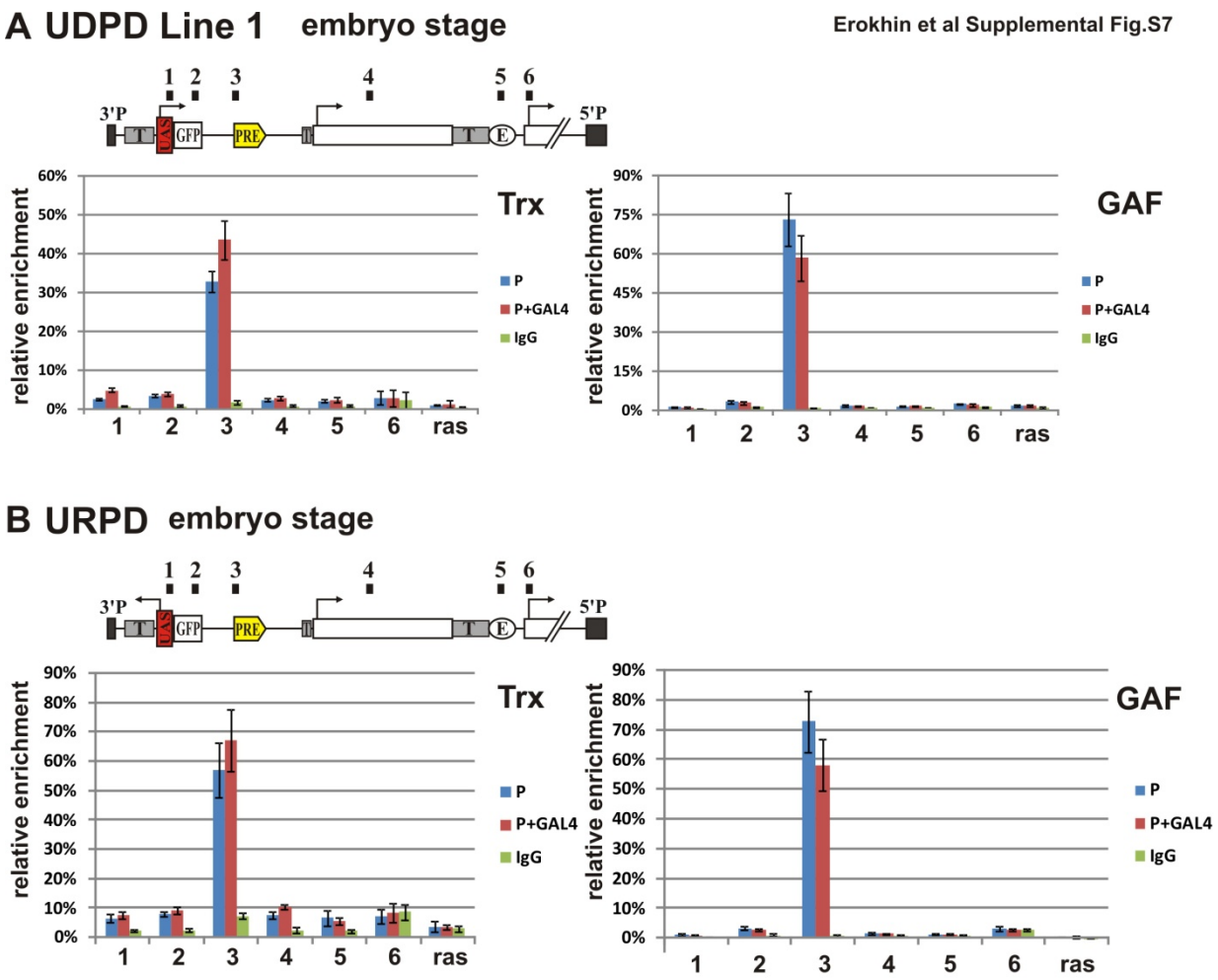
Supplemental Fig. S4. Induction of transcription from UAS promoter in UDPR lines.
(A) The scheme of UDPR transgene. The *bxd*PRE is inserted in reverse orientation. The UAS promoter drives transcription through the GFP and *bxd*PRE. "T" indicates terminators of transcription; *hsp26-lacZ* and *white* – reporter genes. E – enhancer of the *white* gene. The numbers on top indicate positions of primers used for RT-qPCR in (C). (B) GFP expression at the different stages of development (embryo, larva and pupa). Transmitted light illumination (left) and illumination to visualize green fluorescence (right). P/P – lines homozygote for the transgene; P/P; tubGAL4 – homozygote lines with GAL4 activator**.** (C) RT-qPCR analysis. Individual transcript levels were normalized relative to *Ras64B* for the amount of input cDNA. Error bars indicate standard deviations. Positions of primers are indicated in (A).



Supplemental Fig. S5. The PH (PRC1) and dSfmbt (PhoRC) proteins remain bound to PRE during transcription at the embryo stage. (A) The UDPD construct line1. (B) The URPD construct. The numbers on the top of the construct scheme indicate regions amplified by qPCR: 1) the *hsp70* minimal promoter; 2) the GFP open reading frame; 3) the transgene-specific region of *bxd*PRE; 4) the *lacZ* open reading frame; 5) the *white* enhancer and 6) the *white* promoter. The coding part of the *Ras64B (ras)* gene was used as negative control. In order standardize the amount of PH and dSfmbt associated with each region of the transgene in different transgenic samples, we calculated the enrichment relative to a positive internal control—a sequence adjacent to the *bxd*PRE in the *Bithorax* complex. The blue bars indicate relative X-ChIP signal levels in homozygote lines (P/P), red bars indicate relative X-ChIP signal levels in homozygote lines with GAL4 activator (P/P+GAL4) and green bars indicate signal levels with non-specific antibodies.



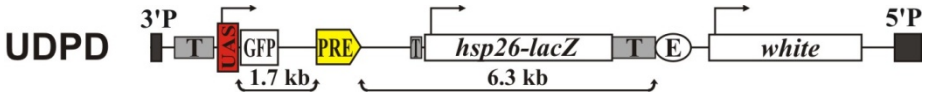
Supplemental Fig. S6. The PC (PRC1) and H3K27me3 histone hallmark remain bound to PRE during transcription at the embryo stage. (A) The UDPD construct line1. (B) The URPD construct. The numbers on the top of the construct scheme indicate regions amplified by qPCR: 1) the *hsp70* minimal promoter; 2) the GFP open reading frame; 3) the transgene-specific region of *bxd*PRE; 4) the *lacZ* open reading frame; 5) the *white* enhancer and 6) the *white* promoter. The coding part of the *Ras64B* gene was used as negative control. In order standardize the amount of PC and H3K27me3 associated with each region of the transgene in different transgenic samples, we calculated the enrichment relative to a positive internal control—a sequence adjacent to the *bxd*PRE in the *Bithorax* complex. The blue bars indicate relative X-ChIP signal levels in homozygote lines (P/P), red bars indicate relative X-ChIP signal levels in homozygote lines with GAL4 activator (P/P+GAL4) and green bars indicate signal levels with non-specific antibodies.



Supplemental Fig. S7. The Trx and GAF proteins are bound to PRE during transcription at the embryo stage. (A) The UDPD construct line1. (B) The URPD construct. The numbers on the top of the construct scheme indicate regions amplified by qPCR: 1) the *hsp70* minimal promoter; 2) the GFP open reading frame; 3) the transgene-specific region of *bxd*PRE; 4) the *lacZ* open reading frame; 5) the *white* enhancer and 6) the *white* promoter. The coding part of the *Ras64B* gene was used as negative control. In order standardize the amount of Trx and GAF associated with each region of the transgene in different transgenic samples, we calculated the enrichment relative to a positive internal control—a sequence adjacent to the *bxd*PRE in the *Bithorax* complex. The blue bars indicate relative X-ChIP signal levels in homozygote lines (P/P), red bars indicate relative X-ChIP signal levels in homozygote lines with GAL4 activator (P/P+GAL4) and green bars indicate signal levels with non-specific antibodies.

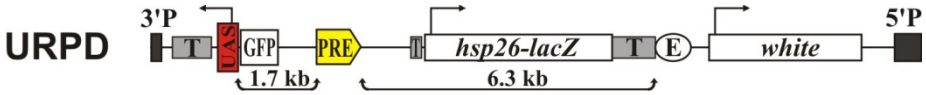
Supplemental Tables

Supplemental Table S1



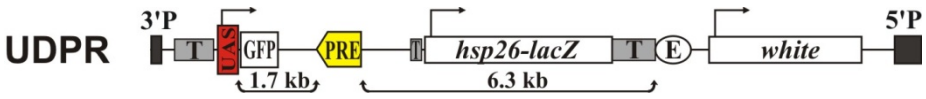
	P/+	P/P	P/+; tubGAL4	PΔPRE/+	PΔUAS/+
Lines with PRE in repressing state					
Line 1	Mosaic: Yellow(80%) and Brown(20%) dots	Mosaic: White(85%) and Brown (15%) dots	Red	Red	Mosaic: Yellow(80%) and Brown(20%) dots
Line 2	Mosaic: Yellow (50%) and dark-Orange(50%) dots	Mosaic: White(95%) and Yellow(5%) dots	Red	Red	Mosaic: Yellow (50%) and dark-Orange(50%) dots
Line 3	Mosaic: Orange(50%) and Red(50%) dots	Mosaic: Yellow(70%) and Orange(30%) dots	Red	Red	Mosaic: Orange(50%) and Red(50%) dots
Line 4	Red	Mosaic: pale-Yellow(80%) and Yellow(20%) dots	Red	Red	Red
Line 5	dark-Brown	Mosaic: White(80%) and dark-Brown (20%) dots	Red	Red	dark-Brown
Line 6	Mosaic: Yellow(50%) and dark-Orange(50%) dots	Mosaic: White(80%) and pale-Yellow(20%) dots	Red	Red	Mosaic: Yellow(50%) and dark-Orange(50%) dots
Line 7	Mosaic: Orange(50%) and Red(50%) dots	Mosaic: pale-Yellow(50%) and dark-Yellow(50%) dots	Red	Red	Mosaic: Orange(50%) and Red(50%) dots

Supplemental Table S2



	P/+	P/P	P/+; tubGAL4	PΔPRE/+	PΔUAS/+
Lines with PRE in repressing state					
Line 1	Mosaic: pale-Yellow and Yellow (50%) dots	Mosaic: White (85%) and Orange (15%) dots	Red	Red	Mosaic: pale-Yellow and Yellow (50%) dots
Line 2	dark-Yellow	pale-Yellow	Red	Red	dark-Yellow
Line 3	Yellow	Mosaic: White (50%) and pale-Yellow(50%) dots	Red	Red	Yellow
Line 4	Mosaic: Yellow (50%) and dark-Orange (50%) dots	Mosaic: White (50%) and pale-Yellow (50%) dots	Red	Red	Mosaic: Yellow (50%) and dark-Orange (50%) dots
Line 5	Mosaic: White (90%) and Red(10%) dots	Mosaic: White (90%) and Red(10%) dots	Red	Red	Mosaic: White (90%) and Red(10%) dots
Line 6	Red	Mosaic: dark-Yellow (50%) and Brown(50%) dots	Red	Red	Red
Line 7	Mosaic: pale-Yellow (50%) and dark-Yellow(50%) dots	pale-Yellow	Red	Red	Mosaic: pale-Yellow (50%) and dark-Yellow(50%) dots
Line 8	Yellow	pale-Yellow	Red	Red	Yellow
Line 9	Yellow	pale-Yellow	Red	Red	Yellow
Line 10	Mosaic: White (40%) and dark-Yellow (60%) dots	Mosaic: White (40%) and dark-Yellow (60%) dots	Red	Red	Mosaic: White (40%) and dark-Yellow (60%) dots
Line 11	Mosaic: Yellow (50%) and Orange (50%) dots	Mosaic: White (98%) and Orange (2%) dots	Red	Red	Mosaic: Yellow (50%) and Orange (50%) dots

Supplemental Table S3



	P/+	P/P	P/+; tubGAL4	PΔPRE/+	PΔUAS/+
Lines with PRE in repressing state					
Line 1	Mosaic: dark-Yellow (50%) and Orange(50%) dots	Mosaic: pale-Yellow (95%) and Orange(5%) dots	Red	Red	Mosaic: dark-Yellow (50%) and Orange(50%) dots
Line 2	Mosaic: pale-Yellow (70%) and Orange (30%) dots	Mosaic: White(95%) and pale-Yellow(5%) dots	Red	Red	Mosaic: pale-Yellow (70%) and Orange (30%) dots
Line 3	Mosaic: White(90%) and Red(10%) dots	Mosaic: White(90%) and Red(10%) dots	Red	Red	Mosaic: White(90%) and Red(10%) dots
Line 4	Mosaic: pale-Yellow (90%) and Orange (10%) dots	Mosaic: White(95%) and pale-Yellow(5%) dots	Red	Red	Mosaic: pale-Yellow (90%) and Orange (10%) dots
Line 5	Mosaic: Orange (30%) and Red (70%) dots	Mosaic: White (70%) and Red (30%) dots	Red	Red	Mosaic: Orange (30%) and Red (70%) dots
Line 6	Mosaic: pale-Yellow (90%) and Orange (10%) dots	Mosaic: White(95%) and pale-Yellow(5%) dots	Red	Red	Mosaic: pale-Yellow (90%) and Orange (10%) dots
Line 7	Mosaic: pale-Yellow (70%) and Orange (30%) dots	Mosaic: White(95%) and Red(5%) dots	Red	Red	Mosaic: pale-Yellow (70%) and Orange (30%) dots
Line 8	pale-Yellow	Mosaic: White(95%) and pale-Yellow(5%) dots	Red	Red	pale-Yellow
Line 9	Mosaic: Red(95%) and Orange (5%) dots	Mosaic: White(70%) and Red(30%) dots	Red	Red	Mosaic: Red(95%) and Orange (5%) dots

Supplemental Table S4 Primers used for RT-qPCR analysis

Primer name	**Sequence**
UDPD and UDPR constructs	
1-RT-forward	5'-AGCGGAGACTCTAGCGAGC-3'
1-RT-reverse	5'-TAGCGACGTGTTCACTTTGC-3'
2-RT-forward	5'-GAGAACTCTGAATAGGGAATTGG-3'
2-RT-reverse	5'-AGCTCCTCGCCCTTGCTCACCAT-3'
3-RT-forward	5'-CCGACCACTACCAGCAGAAC-3'
3-RT-reverse	5'-GTCCATGCCGAGAGTGATCC-3'
4-RT-forward	5'-CCTTAAGTGATTTTTAGTGGCCTTG-3'
4-RT-reverse	5'-TTAAAACGAGACTTTCATGAGCC-3'
URPD construct	
1-RT-forward	5'-GATAATTCCCAATTCCCTATTCAG-3'
1-RT-reverse	5'-TAACCAGCAACCAAGTAAATCAAC-3'
2-RT-forward	5'-TAGCGACGTGTTCACTTTGC-3'
2-RT-reverse	5'-AGCGGAGACTCTAGCGAGC-3'
3-RT-forward	5'-CCGACCACTACCAGCAGAAC-3'
3-RT-reverse	5'-GTCCATGCCGAGAGTGATCC-3'
4-RT-forward	5'-AAATTGCTCCGGCAACAGAAGATTA-3'
4-RT-reverse	5'-GCTTGATGATCCAACCCAAGATAAA-3'
Analysis of *white* gene expression	
white RT-forward	5'-GCAAATGTCAGCACACGATCAT-3'
white RT-reverse	5'-GTGGGCTCATCGCAGATCA-3'
Control gene	
Ras64B-forward	5'-GAGGGATTCCTGCTCGTCTTCG-3'
Ras64B-reverse	5'-GTCGCACTTGTTACCCACCATC-3'

Supplemental Table S5. Primers used for PCR in X-ChIP experiments

Primer name	**Sequence**
Primer pairs UDPD and UDPR constructs	
1-ChIP-forward (downstream UAS, construct specific)	5'-GAGAACTCTGAATAGGGAATTGG-3'
1- ChIP-reverse (downstream UAS, construct specific)	5'-AGCTCCTCGCCCTTGCTCACCAT-3'
Primer pairs URPD construct	
1-ChIP-forward (downstream UAS, construct specific)	5'-GATAATTCCCAATTCCCTATTCAG-3'
1- ChIP-reverse (downstream UAS, construct specific)	5'-TAACCAGCAACCAAGTAAATCAAC-3'
Identical for all constructs	
2-ChIP-forward (GFP)	5'-CCGACCACTACCAGCAGAAC-3'
2- ChIP-reverse (GFP)	5'-GTCCATGCCGAGAGTGATCC-3'
3-ChIP-forward (PRE construct specific)	5'-TCCTCGACGGTATCGATAAGCTTG-3'
3- ChIP-forward (PRE construct specific)	5'-CCATAATGGCTGCGCCGTAAAG-3'
4-ChIP-forward (*LacZ* coding region)	5'-GGTGAAATTATCGATGAGCGTGG-3'
4- ChIP-reverse (*LacZ* coding region)	5'-CAGTTCAACCACCGCACGATAGA-3'
5-ChIP-forward (Eye enhancer)	5'-AAAACTTTCTACGCCTCAGTTC-3'
5- ChIP-reverse (Eye enhancer)	5'-GCTTATTAGCCCTGCAATTGA-3'
6-ChIP-forward (*white* promoter)	5'- GCACTGGATATCATTGAACTTATCTG -3'
6- ChIP-reverse (*white* promoter)	5'- TGGACAGAGAAGGAGGCAAACA -3'
Ras64B-forward (negative control)	5'-GAGGGATTCCTGCTCGTCTTCG-3'
Ras64B-reverse (negative control)	5'-GTCGCACTTGTTACCCACCATC-3'
bxd PRE adjacent-forward (genome specific, positive control)	5'-AAGAGCAAGGCGAAAGAGAGC-3'

bxd PRE adjacent- reverse (genome specific, positive control)	5'-CGTTTTAAGTGCGACTGAGATGG-3'

Supplementary Table S6. Antibodies.

Antibody	**Reference**	**Source**	**Antigene**
PH (Polyhomeotic)	Erokhin et al. Epigenetics Chromatin. 2013; 6: 31. doi: 10.1186/1756-8935-6-31	Rabbit polyclonal	86-520 aa, ph-p-PA; FlyBase ID FBpp0070416
dSfmbt (SCM-related protein containing four MBT domains)	Erokhin et al. Epigenetics Chromatin. 2013; 6: 31. doi: 10.1186/1756-8935-6-31	Rabbit polyclonal	1-348 aa, Sfmbt-PB ; FlyBase ID FBpp0080070
PC (Polycomb)	this paper	Rabbit polyclonal	191-354 aa, Pc-PA; FlyBase ID FBpp0078059
H3K27me3	Abcam (ab6002) - ChIP Grade	Mouse monoclonal	Synthetic peptide within Human Histone H3 aa 1-100 (tri methyl K27) conjugated to KLH (Sulfo-SMCC).
TRX-N (Trithorax)	this paper	Rabbit polyclonal	8-351 aa, trx-PA; FlyBase ID FBpp0082406
GAF (GAGA Factor)	this paper	Rabbit polyclonal	1-519 aa, Trl-PB FlyBase ID FBpp0089419

Supplemental Methods

Drosophila strains, germline transformation, and genetic crosses

All flies were maintained at 25°C on the standard yeast medium. The construct, together with a P element containing defective inverted repeats (P25.7wc) that was used as a transposase source (1), was injected into *yacw*^{1118} preblastoderm embryos as described (2, 3). The resulting flies were crossed with *yacw*^{1118} flies, and the transgenic progeny were identified by their eye pigmentation. The transformed lines were tested for transposon integrity and copy number by Southern blot hybridization. Only single-copy transformants were included in the study.

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 (*y*^{1}*w*^{1}*; Cyo, P[w+,cre]/Sco;* +) recombinase-expressing lines (4, 5). All excisions were confirmed by PCR analysis.

To induce GAL4 expression under control of ubiquitous tubulin promoter, we used the modified *yw*^{1118}; *P[w*$^{-}$*, tubGAL4]117/TM3,Sb* line (Bloomington Stock Center #5138), in which the marker *mini-white* gene was deleted as described (6).

To induce GAL4 expression under control of heat shock 70 promoter, we used *hsp70-GAL4*$^{7-1}$ line (kindly provided by Renato Paro). To induce expression flies were heat shocked at 37^{o}C for 2 hours.

For phenotype analysis of *white* expression, we visually determined the degree of pigmentation in the eyes (*white*) of 3- to 5-day-old males developing at 25°C, with reference to standard color scales. Pigmentation of all flies was analyzed in hetero- (P/+) or homozygote (P/P). The pigmentation scores were independently determined by two investigators.

The levels of GFP expression were analyzed on the Leica MZ16 F stereomicroscope.

The details of crosses used for genetic analysis are available upon request.

Plasmid construction

The constructs were made on the basis of the CaSpeR vector (7). The sequence corresponding to the eye enhancer (Ee) of the *white* gene (regulatory sequences from position –1180 to –1849 bp relative to the transcription start site) was cut out of the Ee- pBluescript SK+ plasmid (8) without flanking sequences and cloned in direct orientation into the CaSpeR4 vector cleaved by *Not*I [En-*white*]. The 4324bp *Sma*I–*Sal*I fragment of the CaSpeR-hs43-lacZ gene transformation vector (GenBank: X81643.1, containing the *lacZ* gene with *adh gene leader* and the SV40 terminator at the 5′ end) was cloned into the pBluescript SK+ cleaved with *Sma*I and *Sal*I [LacZ-SV40-pSK]. The 472bp sequence corresponding to the hsp26 promoter was PCR-amplified with the 5′-ctagaaacttcggctctctca-3′ and 5′-gttgaatgaacttgtttgacttgt-3′ primers and cloned into the pBluescript SK+ plasmid cleaved by *Eco*RV[hsp26-pSK]. The *Hind*III–*Pst*I fragment of the hsp26-pSK plasmid was inserted into the LacZ-SV40-pSK plasmid cleaved by *Sma*I [hsp26-LacZ-SV40-pSK]. The *Not*I–*Sal*I fragment of the hsp26-LacZ-SV40-pSK plasmid was inserted into the En-*white* vector cleaved by *Bam*HI [hsp26-LacZ-SV40-En-*white*].

UDPD: The *Hind*III–*Eco*RI fragment containing the minimal hsp70 promoter with five GAL4 binding sites upstream of it was excised from the pUAST vector (9) and cloned into the pBluescript SK+-lox2 cleaved by *Eco*RV [lox(UAS)]. The 717bp sequence corresponding to the eGFP coding sequences was PCR-amplified with the 5′-atggtgagcaagggcgaggagct-3′ and 5′-cttgtacagctcgtccatgccga-3′ primers and cloned into the pBluescript SK+ plasmid cleaved by *Eco*RV[eGFP-pSK].

The *Hind*III–*Pst*I fragment of the eGFP-pSK was inserted in direct orientation into the lox(UAS) plasmid cleaved by *Apa*I [(UAS)dir-eGFP].

The *Xba*I–*Bam*HI fragment of the pUAST vector containing the 702-bp SV40 terminator was inserted into the (UAS)dir-eGFP plasmid cleaved by *Bam*HI [SV40-(UAS)dir-eGFP].

The 1828bp *Hinc*II–*Hinc*II fragment of the Lac*Z*-SV40-pSK plasmid was inserted into the pBluescript SK+ plasmid cleaved by *Eco*RV [*linker*1828bp-pSK].

The *Pst*I-*Pst*I fragment sequence corresponding to the 656bp PRE (3R:16764122..16764777) was cut out of frt(PRE) plasmid (10) and cloned into the pBluescript SK+-frt2 plasmid cleaved by *Eco*RV [frt(*PRE656*)].

The *Hinc*II–*Sma*I fragment of the frt(*PRE656*) plasmid was inserted in direct orientation into the *linker*1828bp-pSK plasmid cleaved by *Aor*I [*linker785*(PREdir)*linker1043*-pSK].

The *Xba*I–*Bam*HI fragment of the pGL3basic vector containing the 222-bp SV40 terminator was inserted into the *linker785*(PREdir)*linker1043*-pSK plasmid cleaved by *Sma*I [*linker785*(PREdir)*linker1043*-SV40s-pSK].

The *Xba*I–*Xba*I fragment of the SV40-(UAS)dir-eGFP plasmid was inserted into the *linker785*(PREdir)*linker1043*-SV40s-pSK plasmid cleaved by *Eco*RV [SV40-(UAS)dir-eGFP-l-(PREdir)-l-SV40s].

To produce **UDPD** the *Apa*I–*Not*I fragment of the SV40-(UAS)dir-eGFP-l-(PREdir)-l-SV40s plasmid was inserted into the hsp26-Lac*Z*-SV40-En-*white* vector cleaved by *Bam*HI.

URPD: The *Hind*III–*Pst*I fragment of the eGFP-pSK plasmid was inserted in head-to-head orientation into the lox(UAS) plasmid cleaved by *Bam*HI [(UAS)rev-eGFP]. The *Xba*I-*Bam*HI fragment of the pUAST vector containing the 702-bp SV40 terminator was inserted into the (UAS)rev-eGFP plasmid cleaved by *Apa*I [SV40-(UAS)rev-eGFP].

The *Xba*I–*Xba*I fragment of the SV40-(UAS)rev-eGFP plasmid was inserted into the *linker785*(PREdir)*linker1043*-SV40s-pSK plasmid cleaved by *Eco*RV [SV40-(UAS)rev-eGFP-l-(PREdir)-l-SV40s].

To produce **URPD** the *Apa*I–*Not*I fragment of the SV40-(UAS)rev-eGFP-l-(PREdir)-l-SV40s plasmid was inserted into the hsp26-Lac*Z*-SV40-En-*white* vector cleaved by *Bam*HI.

UDPR: The *Hinc*II–*Sma*I fragment of the frt(*PRE656*) plasmid was inserted in reverse orientation into the *linker*1828bp-pSK plasmid cleaved by *Aor*I [*linker785*(PRErev)*linker1043*-pSK]. The *Xba*I–*Bam*HI fragment of the pGL3basic vector containing the 222-bp SV40 terminator was inserted into the *linker785*(PRErev)*linker1043*-pSK plasmid cleaved by *Sma*I [*linker785*(PRErev)*linker1043*-SV40s-pSK].

The *Xba*I–*Xba*I fragment of the SV40-(UAS)dir-eGFP plasmid was inserted into the *linker785*(PRErev)*linker1043*-SV40s-pSK plasmid cleaved by *Eco*RV [SV40-(UAS)dir-eGFP-l-(PRErev)-l-SV40s].

To produce **UDPR** the *Apa*I–*Not*I fragment of the SV40-(UAS)dir-eGFP-l-(PRErev)-l-SV40s plasmid was inserted into the hsp26-LacZ-SV40-En-*white* vector cleaved by *Bam*HI.

RT–PCR

RNA was isolated from approximately 20 adult flies homozygote for the construct with TRI reagent (Ambion) according to the manufacturer's instructions. Purified RNA pools were digested by RNase-free DNase I (BioLabs) and re-purified by using RNeasy Mini kit (Quagen). For reverse transcription, 3 μg of the generated RNA was incubated with ArrayScript Reverse Transcriptase (Ambion) in the presence of dNTPs, Oligo(dT) (Fermentas), and RNase Inhibitor (Ambion) in the supplied reaction buffer at 42°C for 1.5 h, according to the manufacturer's instructions. The reverse transcriptase was inactivated by heating at 95°C for 5 min. To control DNA digestion by DNase I, additional negative control experiments were performed without Reverse Transcriptase in the reaction mixture. The generated cDNA pools were used as templates in real-time qPCR, using a C1000™ Thermal Cycler with CFX96 real-time PCR detection module (Bio-Rad) or a StepOne Plus Thermal Cycler (Applied Biosystems, United States). Each PCR was performed in triplicate, cDNA pools were obtained in technical duplicate. Relative levels of mRNA expression were calculated in the linear amplification range by calibration to a DNA fragment standard curve (for genomic DNA) to account for differences in

primer efficiency. The results of RT–PCR detection of *Ras64B* were used to standardize the overall amount of cDNA used in PCR assays. Primers are listed in Supplemental Table S4.

X-ChIP

For each experiment, 150–200 mg of the initial material (0- to 16-hour embryos or from 2-to 5-day-old adult flies homozygote for the construct) was collected. The material was homogenized in 5 ml of buffer A1 (15 mM HEPES, pH 7.6; 60 mM KCl, 15 mM NaCl, 4 mM $MgCl_2$, 0.5% Triton X-100, 0.5 mM DTT) supplemented with the EDTA-free protease inhibitor cocktail (Roche, Switzerland) and formaldehyde as a crosslinking agent (final concentration 1.8%). The reaction was stopped by adding glycine (final concentration 225 mM). The homogenate was cleared by passing through 100-μm nylon cell strainer (BD Falcon) and pelleted by centrifugation at 4000 *g,* 4°C for 5 min. After washing in three 3-ml portions of buffer A1 at 4°C (5 min each) and 3 ml of lysis buffer without SDS, the pellet was treated with 0.5 ml of complete lysis buffer (15 mM HEPES, pH 7.6; 140 mM NaCl, 1mM EDTA, 0.5 mM EGTA, 1%Triton X-100, 0.5 mM DTT, 0.1% sodium deoxycholate, 0.1% SDS, 0.5 % N-lauroylsarcosine, EDTA-free protease inhibitor cocktail) and sonicated to break chromatin into fragments with an average length of 700 bp. The material was pelleted by centrifugation at 18 000 *g* for 5 min, and the supernatant fluid was transferred to a new tube. The pellet was treated with the second 0.5-ml portion of lysis buffer, and the preparation was centrifuged at 18 000 *g* for 5 min. The two portions of the supernatant fluid were pooled, cleared by centrifuging twice at 18 000 *g* for 10 min, and the resultant chromatin extract (1 ml) was used in four ChIP experiments after preincubation with A-Sepharose or G-Sepharose (see below). One aliquot (1/10 volume) of chromatin extract after preincubation with Sepharose was kept as a control sample (Input).

ChIP experiments involved incubation with specific rabbit/mouse antibody or with corresponding nonimmune IgG that were used as nonspecific antibody controls. Antibody–chromatin complexes were collected with either protein A-Sepharose (for antibodies raised in

rabbits) or G-Sepharose (for mouse antibody) beads (Thermo Scientific). The enrichment of specific DNA fragments was analyzed by real-time qPCR, using a C1000™ Thermal Cycler with CFX96 real-time PCR detection module (Bio-Rad) or a StepOne Plus Thermal Cycler (Applied Biosystems, United States).

Primers used in ChIP/real-time PCR analyses are listed in Supplemental Table S5.

Supplemental References

1. Karess RE & Rubin GM (1984) Analysis of P transposable element functions in Drosophila. *Cell* 38(1):135-146.
2. Rubin GM & Spradling AC (1982) Genetic transformation of Drosophila with transposable element vectors. *Science* 218(4570):348-353.
3. Spradling AC & Rubin GM (1982) Transposition of cloned P elements into Drosophila germ line chromosomes. *Science* 218(4570):341-347.
4. Golic KG & Lindquist S (1989) The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. *Cell* 59(3):499-509.
5. Siegal ML & Hartl DL (2000) Application of Cre/loxP in Drosophila. Site-specific recombination and transgene coplacement. *Methods in molecular biology* 136:487-495.
6. Kyrchanova O, Toshchakov S, Parshikov A, & Georgiev P (2007) Study of the functional interaction between Mcp insulators from the Drosophila bithorax complex: effects of insulator pairing on enhancer-promoter communication. *Molecular and cellular biology* 27(8):3035-3043.
7. Pirrotta V (1988) Vectors for P-mediated transformation in Drosophila. *Biotechnology* 10:437-456.
8. Erokhin M*, et al.* (2013) Transcription through enhancers suppresses their activity in Drosophila. *Epigenetics & chromatin* 6(1):31.
9. Brand AH & Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118(2):401-415.
10. Erokhin M, Parshikov A, Georgiev P, & Chetverina D (2010) E(y)2/Sus1 is required for blocking PRE silencing by the Wari insulator in Drosophila melanogaster. *Chromosoma* 119(3):243-253.