Supporting Information

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SI Materials and Methods

Chromosome Conformation Capture Assay. Chromosome conformation capture (3C) assays were performed in *Drosophila* embryos with a protocol modified from previously published study (1). Briefly, embryos were fixed with formaldehyde saturated Hexanes as described for ChIP assays (2). This process was followed by homogenization of the embryos and isolation of cross-linked chromatin. The chromatin was dissolved in respective restriction buffer in the presence of 0.1% SDS by incubation at 37 °C water bath for 25 min with intermittent gentle mixing during the incubation. Next, 0.01% Triton-X-100 was added to this dissolved chromatin and was subsequently digested with restriction enzymes (400 U) to completion. The *brk* and *sog* loci were analyzed digestion with EcoRI; saturation assays were done for *brk* using EcoRI and HindIII digestions. The *lacZ* transgenes containing Snail repressor sites were digested with Sau3AI. In all cases, digested chromatin was subjected to ligation under high-dilution conditions to facilitate intramolecular ligation. The ligation was stopped and cross-links were reversed, followed by purification of hybrid DNA. This DNA was used in subsequent PCR reactions to look for desired hybrid products using region-specific primers. The primers were designed oriented either toward or away (reverse) relative to the nearest restriction site from a specific region. Hybrid sequences were confirmed by DNA sequencing and BLAST analysis that aligned the sequences back to their different respective positions (i.e., promoter and enhancer).

Control experiments were done with noncross-linked embryos, and cross-linked embryos without ligation. The primer pairs used in the study was analyzed for their efficiency of ligation using purified genomic DNA (Fig. S1), digestion with appropriate restriction enzymes, and either with or without ligation. The 3C libraries were subjected to semiquantitative PCR reactions that were titrated from 100-ng input DNA to 10-ng input DNA for each reaction. Figs. 1 and 2 display PCR reactions using 20 ng of input DNA that displayed clear amplification of hybrid interactions compared with no cross-link control libraries. For the quantification of the *brk* locus in Fig. 2, the PCR-amplified fragments were photographed in AlphaImager (Alpha Innotech) and densitometric reading determined using the AlphaImager software. These densitometric values were used to quantify the 3C-enriched bands compared with no cross-link and no ligation libraries. Five independent libraries were made and all amplicons were quantified and plotted to get the final graph. Primer pairs used in these assays are given in Tables S1–S4.

3C-qPCR. The 3C from each background (*yw*, $Toll^{10b}$, $Toll^{rm9}/Toll^{rm10}$, and gd^{7}) was performed as described above. A similar 3C library was prepared from *brk* BAC DNA after digestion with EcoRI and HindIII. A total of three libraries were made from each background ($n = 3$). Standard curves were generated for each primer + anchor + Taqman probe combinations with a 500 ng/μL, 250 ng/μL, and 125 ng/μL diluted BAC library. The cross-linking frequency was calculated as described previously (3).

1. Lanzuolo C, Roure V, Dekker J, Bantignies F, Orlando V (2007) Polycomb response elements mediate the formation of chromosome higher-order structures in the bithorax complex. *Nat Cell Biol* 9:1167–1174.
2. Chopra VS, Hong JW, Levine M (2009) Regulation of Hox gene activity by transcriptional elongation in *Drosophila*. *Curr Biol* 19:688–693.
3. Hagège H, et al. (2007) Quantitative analysis of chromosome conformation capture assays (3C-qPCR). *Nat Protoc* 2:1722–1733.

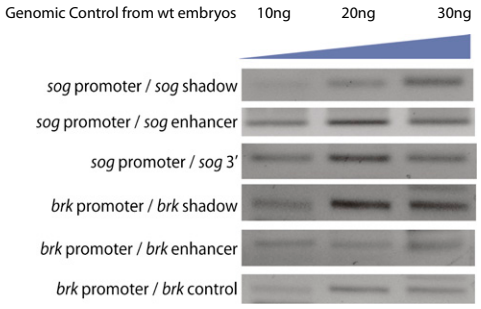

Fig. S1. Evaluation of primer pair efficiency. The primer pair efficiency was checked for all of the primer pairs on genomic DNA from wild-type embryos. The genomic DNA of wild-type embryos was digested with EcoRI and then ligated under dilute conditions followed by DNA purification for the analysis of primer pairs from the *brk* and *sog* loci. These sets serve as genomic control to check the primer pair efficiency. The PCRs were performed using increasing amounts of genomic DNA (10, 20, and 30 ng). As seen from the gels the 20-ng input DNA serves as the standard for all of the semiquantitative PCRs performed for all 3C experiments.

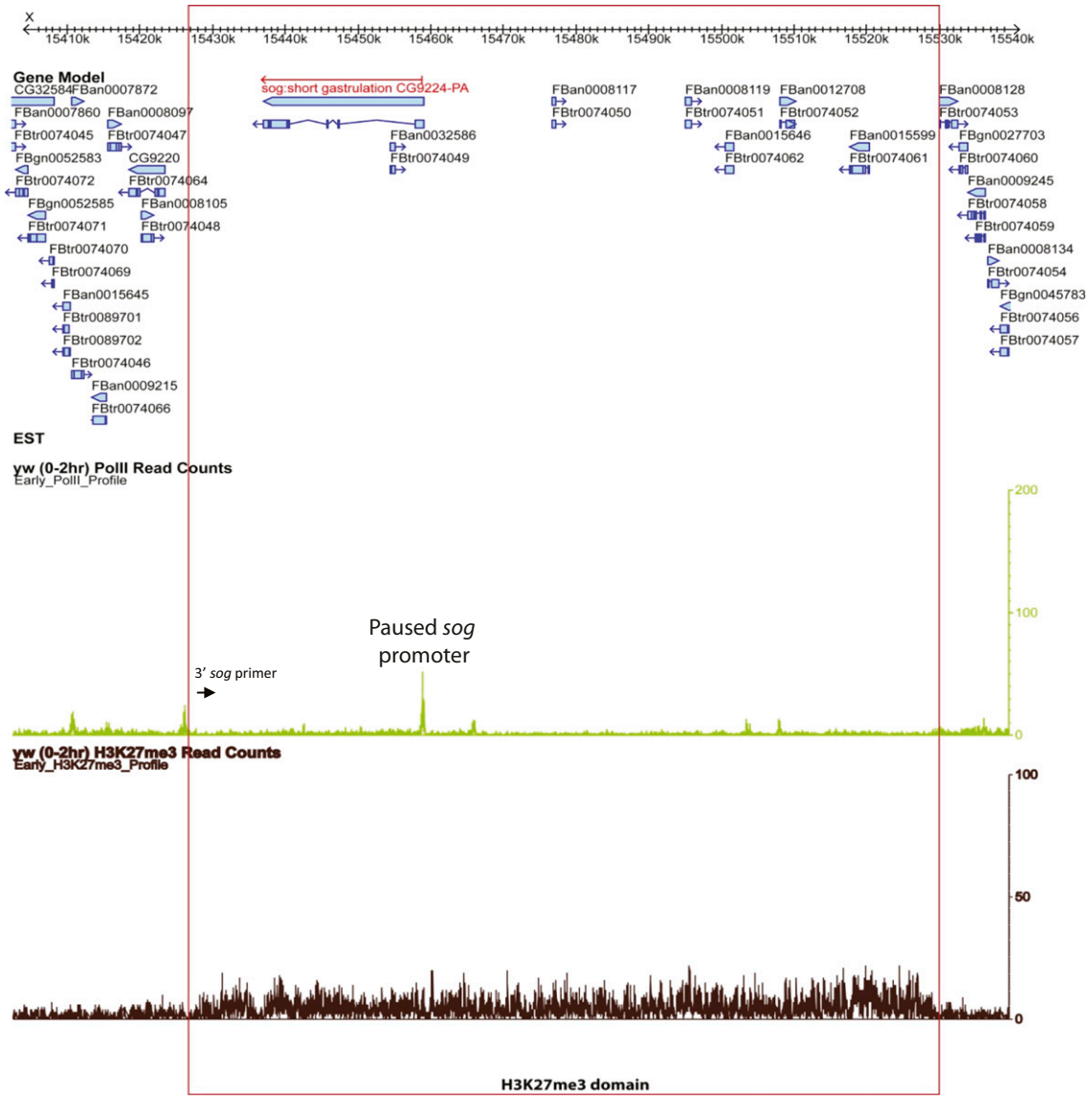

Fig. S2. Evidence for a chromatin hub within the *sog* locus. The 3C technique involves cross-linking embryos with formaldehyde, isolation of the cross-linked chromatin, digestion with a restriction enzyme, and detection of hybrid ligation products by PCR (1). These assays suggest looping of both the *sog* enhancer (primary intronic enhancer) and the 5′ (shadow) enhancer to the *sog* promoter region (Fig. 1*C*) in $Toll^{rm9}/Toll^{rm10}$ embryos (Fig. 1*C*, row 3). Importantly, these loops are lost in dorsal-ventral mutants where *sog* is inactive (Fig. 1*C*, rows 1 and 5). We observe that a 3′ *sog* primer also loops with *sog* promoter suggesting the formation of an active chromatin hub (2) (Fig. 1*C*, row 3, column 3). To confirm that the 3′ *sog* primer was uncovering a chromatin domain around the *sog* locus, we checked the whole-genome ChIP-Seq assays using antibodies against trimethylation of core histone H3 on lysine 27 (H3K27me3). The H3K27me3 ChIP-Seq assay on early embryos (0–2 h) suggests that the *sog* locus is contained within a discrete chromosomal boundary domain. The 3C assays with a remote 3′ primer sequence interactions with the *sog* promoter (see below) does show looping because it falls within the H3K27me3 domain. This observation suggests that the *sog* locus is organized into a higher-order chromosomal structure [similar to active chromatin hubs seen at the β-globin locus (2)].

1. Dekker J, Rippe K, Dekker M, Kleckner N (2002) Capturing chromosome conformation. *Science* 295:1306–1311.
2. Patrinos GP, et al. (2004) Multiple interactions between regulatory regions are required to stabilize an active chromatin hub. *Genes Dev* 18:1495–1509.

Table S1. Primers used for 3C assays in wt and mutant backgrounds (EcoRI site)

Primer name	Sequence	Region amplified
Brksha3CL	TTC AGG TGG CAA GTA TGT ATG TAT G	*brk* shadow enhancer
Brk3CL	GGT GGA TGT GTG TAT AGG TTT CTT C	*brk* promoter
Brkenh3CL	TAT CCC CCA AAA ACT CAC ATA AAA T	*brk* primary enhancer
Sog3CL	TTC AGG ATT GTA TTG TTT GTT GAG A	*sog* promoter
Sogsha3CL	ATA AAA GAA CAT TGT AAC CCC TGG A	*sog* shadow enhancer
Sogenh3CL	CAA AGG GTT TAC AAT GCA CAT CTA T	*sog* primary enhancer
Brk control 3C	ATT TTG GCT TCA ATA GTG TGT ACT G	*brk* control region
Sog control 3C	TGC ATA AAT AAC AAT ACG TTT GAA CA	*sog* control region
Brk3CR	AAA AGG CGA GAG AAA TAC ATT TTG	*brk* promoter (Reverse)
Brksha3CR	TAG TGC CTG GCC TTA AAT ATA CAA A	*brk* shadow (Reverse)
Sogcontrol3CR	AGG CTA ATG GAA AAC AAT TCC AG	*sog* control (Reverse)
Sogenh3CR	ATT AAA TTC AAT GGT TCA ACA AAA A	*sog* primary (Reverse)
Sog3CR	TAT TAG TTG TGG AAA TTG CAG CTT A	*sog* promoter (Reverse)
Sogsha3CR	GTA GTT GAA GAC GAG GTG GAC TTT	*sog* shadow (Reverse)
Brkenh3CR	CAG TCA TTA AAA ATT GGT TCG AAA A	*brk* enhancer (Reverse)

Table S2. *brk* locus saturation primers

Name	Sequence	Restriction site
Brk 1L	TTC TAG CAG GGG TCA CAC TGT T	HindIII
Brk 2L	TAT CCC CCA AAA ACT CAC ATA AAA T	EcoRI
Brk 3L	ACC CAA AAA TAC TTA ACC TGT TTT CTT	HindIII
Brk 4L	CTG GGT ATC GAA AAC AAA CAG TAG A	HindIII
Brk 5L	AAA CAC TTA CTA TTT GGT GCC AAT G	EcoRI
Brk 6L	AAA AGC CTA GGG AGA CCT AAA ACT A	EcoRI
Brk 7L	AAG ACT GGC GAC ATT CTG TCT G	EcoRI
Brk 8L	CGT TGT TGT GTT GTT GTT AAC GTG	HindIII
Brk 9L	CAG ACG ACG CCA AGA AAA TAT AAC	HindIII
Brk 10L	AGG AGA CTT TAA AAA GGA GAC CAA A	HindIII
Brk 11L	GGT GGA TGT GTG TAT AGG TTT CTT C	EcoRI (promoter)
Brk 12L	CAG GAG AAC AGA AGA AGC AGA GTA A	HindIII (promoter)
Brk 13L	CCT TTT AGT TCC CTA AGC CAT ATT T	HindIII
Brk 14L	AGA GAG AAA ATG TGA AAA ATG CAA C	HindIII
Brk 15L	GCC AAG TTT TAC AAA GTA AAT AGG G	EcoRI
Brk 16L	TCA CTT TCC GTA ATT AAA AGC TTG A	HindIII
Brk 17L	TTC AGG TGG CAA GTA TGT ATG TAT G	EcoRI
Brk 18L	AGT AGC TCA GCC AGT TTT AGC TTG T	EcoRI
Brk 19L	CCC TCA CAA TAT AAT TCG TAA ATC C	EcoRI
Brk 20L	CAA TGA ATA CGA ATT GAT CGC TAG T	HindIII
Brk 21L	GTT GAC ATG TTA ATT TGG ATT TTC C	HindIII
Brk 22L	AAG ACC CTC GTT TCC AAG TAG ATA	HindIII
Brk 23L	ATT TTG GCT TCA ATA GTG TGT ACT G	EcoRI

Table S3. *brk* locus 3C-qPCR primers

Restriction site	Sequence
Eco 1F	GATTTATCCCCCAAAAACTCACAT
Hind 1F	GTGTGCCTGGGTATCGAAAAC
Eco 2F	GCGATGGCCCGTTGAC
Eco 3F	GTGGTGGTGCAGCAATCG
Hind 2F	CACTGTGCGGCTCTCTTTCTC
Hind 3F	GCGCTGTTTTTGTAGTTGTTGCT
Hind 4F	GTCATTCGAAGGAAAAGCATAAGAG
Eco 4F	TGTGTATAGGTTTCTTCGATTTTTACGT (Anchor)
Hind 5f	GGCAGGAGAACAGAAGAAGCA (Anchor)
Hind 6F	TGATTCTCGTGGCGGATACTG
Eco 5F	CGTTGCCCCGTGTAAACTTT
Hind 7F	TGGGTCTCCTCGTGTAGATCAA
Eco 6F	TGGCGCTACCCCAAAAATAA
Eco 7F	CATTCGAGAGCACCCTACACAA
Eco 8F	GAATGGAAAGCAGAAACTCTCTATAGG
Hind 8F	AATTTGTACGCCAGCGATCAG
Hind 9F	GGATGCGATCAAGACATACTGCTA
Eco 9F	CTCTTCATCACACAAAACCACACA

Table S4. Taqman MGB probes

Probe	Sequence
HindIII brk prom anchor	CAC ATA TTG TTG CTG CAC TT
EcoRI brk prom anchor	TCA ATT TTT ATC TCT TTG CCT TC