

# Transcriptional repression via antilooping in the *Drosophila* embryo

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Contributed by Michael Levine, February 22, 2011 (sent for review January 31, 2010)

Transcriptional repressors are thought to inhibit gene expression by interfering with the binding or function of RNA Polymerase II, perhaps by promoting local chromatin condensation. Here, we present evidence for a distinctive mechanism of repression, whereby sequence-specific repressors prevent the looping of distal enhancers to the promoter. Particular efforts focus on the Snail repressor, which plays a conserved role in promoting epithelial-mesenchyme transitions in both invertebrates and vertebrates, including mesoderm invagination in *Drosophila*, neural crest migration in vertebrates, and tumorigenesis in mammals. Chromosome conformation capture experiments were used to examine enhancer looping at Snail target genes in wild-type and mutant embryos. These studies suggest that the Snail repressor blocks the formation of fruitful enhancer-promoter interactions when bound to a distal enhancer. This higher-order mechanism of transcriptional repression has broad implications for the control of gene activity in metazoan development.

Transcriptional repressors delineate sharp boundaries of gene expression during development (1). Repressor proteins have been proposed to work by a variety of mechanisms, including competition with activator proteins for shared DNA binding sites, inhibition or quenching of adjacent activators bound to a common enhancer, and direct repression of the RNA Polymerase II (Pol II) transcription complex (1–4). A number of sequence-specific repressors have been shown to recruit histone deacetylases, which have been implicated in chromatin condensation (5, 6). Moreover, the recent observation that many developmental control genes in *Drosophila* contain stalled Pol II raises the possibility that repressors might inhibit Pol II elongation (7–12). To investigate these different mechanisms of transcriptional repression, we used the chromosome conformation capture (3C) technique (13, 14) to examine enhancer-promoter looping at several developmental control genes in the early *Drosophila* embryo.

Different embryonic patterning mutants were used for this analysis, particularly three maternal mutants that produce abnormal Dorsal gradients controlling dorsal-ventral (DV) patterning. Each mutant transforms all cells into a single embryonic tissue: *Toll<sup>10b</sup>* (mesoderm), *Toll<sup>mm9</sup>/Toll<sup>mm10</sup>* (neuroectoderm), and *gd<sup>7</sup>/gd<sup>7</sup>* (dorsal ectoderm) (15–17). The *short gastrulation* (*sog*) and *brinker* (*brk*) genes were chosen for 3C assays because they are regulated by well-defined enhancers (18) (Fig. 1 *A* and *B*) and are differentially expressed in the three DV mutants. Both *sog* and *brk* are activated by low levels of Dorsal in wild-type and *Toll<sup>mm9</sup>/Toll<sup>mm10</sup>* embryos (16), but are silent in *gd<sup>7</sup>* mutants lacking Dorsal and actively repressed in *Toll<sup>10b</sup>* mutants containing high levels of the Snail repressor, as discussed below.

Snail is one of the best-characterized repressors in animal development and disease (4, 19, 20). It is selectively expressed in the presumptive mesoderm of gastrulating *Drosophila* embryos, where it delineates the boundary between the mesoderm and neurogenic ectoderm by repressing at least 50 target genes that are required for the patterning of the ectoderm (16, 21, 22). Snail is also essential for the formation of the ventral furrow during gastrulation by promoting epithelial-mesenchyme transitions

(EMT) through the repression of genes encoding cell-adhesion molecules, such as E-Cad (19). Snail and related repressors (e.g., Slug) have been shown to promote EMT in vertebrate developmental processes, such as the delamination of neural crest (22). Snail repressors have also been implicated in EMT in tumorigenesis; tumors that express Snail repressors display enhanced metastasis (19).

Previous studies have shown that Snail recruits two different corepressor proteins, Ebi and CtBP (5, 23, 24). Snail was classified as a short-range repressor, in that Snail binding sites must map within 100 bp of either upstream activators or the core promoter to mediate effective transcriptional repression (25). Moreover, Snail does not interfere with elongating Pol II complexes released before the onset of repression (26), but blocks the activation or release of Pol II at the promoter. Here, we explore Snail-mediated repression using 3C assays.

## Results

**Snail Blocks Enhancer-Promoter Interactions.** The 3C assays were used to gain additional insights into the mechanisms by which Snail works as a repressor. The 3C technique involves cross-linking embryos with formaldehyde, isolation of cross-linked chromatin, digestion with a restriction enzyme, and detection of hybrid ligation products by PCR (13, 27). Noncross-linked chromatin and nonligated chromatin serve as controls and primer pair efficiency was checked by a genomic DNA control (Fig. S1). PCR amplicons were cloned, sequenced, and mapped to their respective positions in the genome to verify the presence of hybrid DNA products and thereby confirming long-range interactions. In contrast, PCR reactions performed on noncross-linked controls failed to produce amplicons (e.g., Fig. 1C, rows 2, 4, and 6).

These assays suggest looping of both the *sog* enhancer (primary intronic enhancer) and the 5' (shadow) enhancer to the *sog* promoter region (Fig. 1C) in *Toll<sup>mm9</sup>/Toll<sup>mm10</sup>* embryos, which exhibit constitutive *sog* expression (Fig. 1C, row 3). Importantly, these loops are lost in DV mutants where *sog* is inactive (Fig. 1C, rows 1 and 5). The absence of loops in *gd<sup>7</sup>* mutants (Fig. 1C, row 5) presumably results from a failure to recruit appropriate coactivators because these embryos lack nuclear Dorsal. In contrast, the loss of looping in *Toll<sup>10b</sup>* mutants (Fig. 1C, row 1) results from active repression by Snail, which is ubiquitously expressed in these mutants. Snail inhibits the expression of neurogenic genes such as *sog* and *brk* in the mesoderm (1).

Previous ChIP-chip assays suggest that both Dorsal and Snail co-occupy the *sog* and *brk* enhancers in *Toll<sup>10b</sup>* embryos (17) (Fig. 1 *A* and *B*). Thus, we propose that Snail, along with its corepressors CtBP and Ebi (5, 23, 24), somehow block enhancer-promoter looping (summarized in Fig. 4). The extent of looping was investigated in detail for the *brk* locus (Fig. 2), as discussed below.

Author contributions: V.S.C. and M.L. designed research; V.S.C. and N.K. performed research; V.S.C. and M.L. analyzed data; and V.S.C. and M.L. wrote the paper.

The authors declare no conflict of interest.

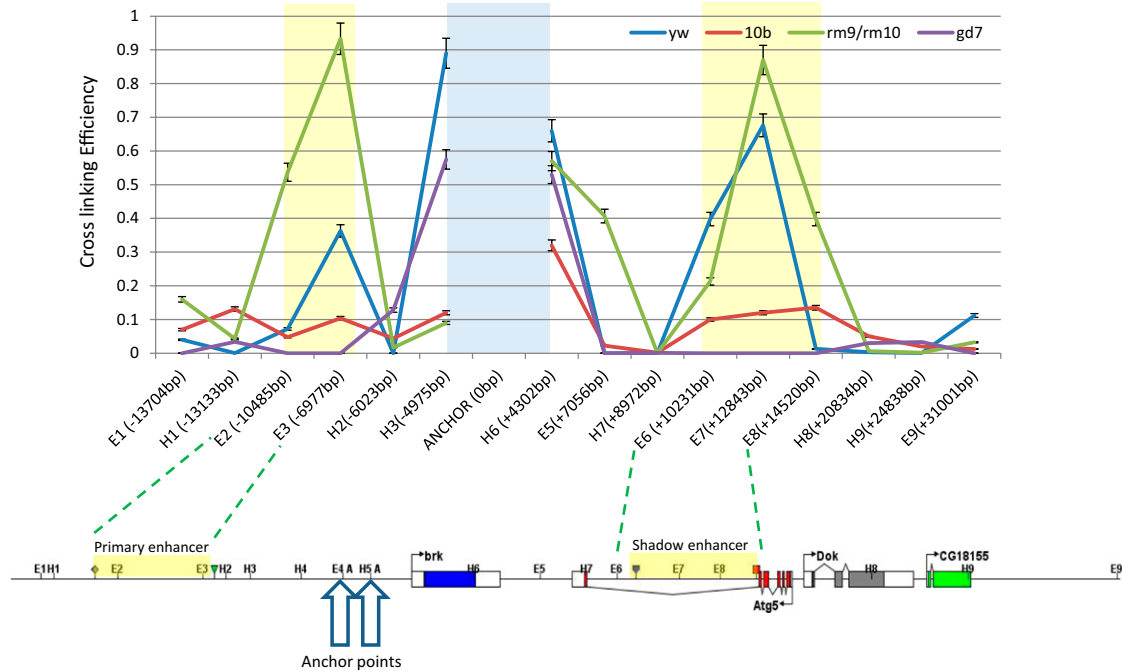
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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1102625108/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1102625108/-DCSupplemental).





### 3C-qPCR analysis using Taqman probes for brk locus



**Fig. 3.** 3C-qPCR was performed using cross-linked embryos from wild-type (*yw*, blue line), *Toll<sup>10b</sup>* (red line), *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* (green line), and *gd<sup>7</sup>* (purple line) 2- to 4-h embryos. The locus was saturated with a total of 18 primers [9 each for HindIII (H1–9) and EcoRI (E1–9)] spanning ~43 kb encompassing the *brk* locus. A BAC DNA library containing the *brk* locus was used to run standard controls for each primer and anchor + Taqman probe mixture and absolute levels of hybrid target was quantified from 3C libraries in each genetic background. The wild-type primary and shadow enhancers loop to the *brk* promoter (blue shaded area) and strong looping is also observed in *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* (green line) background when the gene is active in neuroectodermal tissues. When *brk* is actively repressed by Snail as in *Toll<sup>10b</sup>* (red line), there is no looping of the primary enhancer but residual looping of the shadow enhancer (possibly because of progressive repression of *brk* expression during development). In the total absence of Snail or activator proteins like Dorsal as in *gd<sup>7</sup>* (purple line), there is no enhancer–promoter looping.

this higher-order mechanism of transcriptional repression will prove to be generally used in a variety of developmental processes. For example, gap repressors controlling segmentation, including Kruppel and Knirps, recruit the same corepressor

CtBP that is used by Snail (24). It is possible that CtBP (or Ebi) somehow interferes with the recruitment or function of coactivators [e.g., CBP (28)] or other factors required for the formation of enhancer–promoter loops. Antilooping is a flexible form of repression, in that it need not interfere with the binding or function of Pol II at the core promoter. Such a mechanism might be particularly useful for repressing “poised” genes (containing paused or stalled Pol II) that are rapidly activated during development.

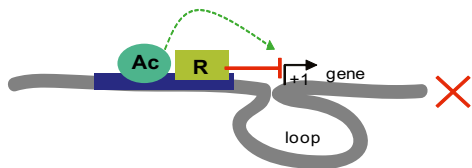
#### Materials and Methods

**Fly Strains.** The different alleles of the maternal gene *Toll* used were *Toll<sup>10b</sup>* and *Toll<sup>rm9</sup>/Toll<sup>rm10</sup>* as described previously (16). The *gastrulation defective* (*gd<sup>7</sup>*) mutant flies were used as described previously (15, 29). All of the mutant backgrounds were 2- to 4-h staged embryos.

**3C Assay.** The 3C assay was performed in *Drosophila* wild-type (*yw*) and DV mutant embryos, as described previously (30) (see *SI Materials and Methods*). The 3C-qPCR assays were performed as described in Hagège et al. (14). Briefly, Taqman-MGB probes were designed using Primer Express 3 software and qPCRs performed in ABI Vii7 real-time machine using Perfecta FastMix II Low Rox (VWR) master mix. The BAC clone for the *brk* locus was ordered from <http://bacpac.chori.org/>. The *brk* BAC DNA was digested with either EcoRI or HindIII restriction enzymes followed by ligation. This BAC template library was used as control quantification template for the standard curves for each primer pair in 3C-qPCR assays. The details of all primers and probes used are illustrated in Tables S1–S4.

**ACKNOWLEDGMENTS.** We thank D. Papatsenko, M. Perry, C. Tsui, and P. Paliwal for providing critical feedback and helpful advice. This work was supported by a National Institutes of Health Grant GM46638.

#### A Direct repression



#### B Repression via “anti-looping”



**Fig. 4.** Mechanisms of transcriptional repression. (A) Direct interference of transcription occurs when repressors (R) bind to enhancers (blue rectangle) and prevent the interaction of activator proteins (Ac) with the transcriptional machinery. According to this model, the requirement for close linkage of the repressor sites is the need for the repressor to loop to the promoter to inhibit Pol II or other general transcription factors. (B) Repression via antilooping. Repressors like Snail bind to enhancer regions and prevent the formation of enhancer–promoter loops, thus disrupting target-gene activation.

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