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Report

Shadow Enhancers Foster Robustness of *Drosophila* Gastrulation

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Summary

Critical developmental control genes sometimes contain "shadow" enhancers that can be located in remote positions, including the introns of neighboring genes [1]. They nonetheless produce patterns of gene expression that are the same as or similar to those produced by more proximal primary enhancers. It was suggested that shadow enhancers help foster robustness in gene expression in response to environmental or genetic perturbations [2, 3]. We critically tested this hypothesis by employing a combination of bacterial artificial chromosome (BAC) recombineering and quantitative confocal imaging methods [2, 4]. Evidence is presented that the *snail* gene is regulated by a distal shadow enhancer located within a neighboring locus. Removal of the proximal primary enhancer does not significantly perturb *snail* function, including the repression of neurogenic genes and formation of the ventral furrow during gastrulation at normal temperatures. However, at elevated temperatures, there is sporadic loss of *snail* expression and coincident disruptions in gastrulation. Similar defects are observed at normal temperatures upon reductions in the levels of Dorsal, a key activator of *snail* expression (reviewed in [5]). These results suggest that shadow enhancers represent a novel mechanism of canalization whereby complex developmental processes "bring about one definite end-result regardless of minor variations in conditions" [6].

Results and Discussion

Despite both intrinsic and environmental sources of noise, which introduce variability in complex developmental processes, the patterning of the *Drosophila* embryo unfolds with high fidelity (e.g., [7]). It has been postulated that genetic interactions in developmental regulatory networks can channel these variable inputs into faithful outcomes, as a ball bouncing inside of a funnel is channeled to the center, a process termed canalization [6]. Here we present evidence that shadow enhancers [1] are important mediators of canalization, ensuring reliable and robust expression of critical patterning genes.

snail is a key determinant of dorsal-ventral patterning [8–11]. It encodes a zinc finger repressor that establishes a sharp boundary between the presumptive mesoderm and neurogenic ectoderm and is essential for the formation of the ventral furrow and the invagination of the mesoderm. Whole-genome ChIP-chip assays identified a cluster of Dorsal and Twist (key activators of *snail* expression) binding sites in the immediate 5′ flanking region of the *snail* transcription unit that coincide with the known enhancer [11, 12]. Unexpectedly, these studies also identified a second cluster of binding sites within the neighboring Tim17b2 locus, located ~7 kb upstream of *snail*. A small genomic DNA fragment (~1 kb) encompassing this second cluster of binding sites was attached to a lacZ reporter gene and was expressed in transgenic embryos (Figure 1). The fusion gene exhibits localized expression in the presumptive mesoderm, similar to that seen for the endogenous gene (e.g., Figure 1) or obtained with the proximal enhancer (the first 2.8 kb of the 5′ flanking region; see [11]). We arbitrarily refer to the newly identified distal enhancer as the shadow enhancer and to the original, proximal enhancer as the primary enhancer [1].

A *snail* fusion gene containing only the primary enhancer rescues the gastrulation of at least some *snail* mutants in a population of mutant embryos [13]. Because *snail* is essential for the coordinated invagination of the mesoderm during early gastrulation, variability in expression could lead to occasional disruptions in morphogenesis. Perhaps the additional enhancer provides a mechanism for suppressing such variability, thereby ensuring robust expression in large populations of embryos. This hypothesis was motivated in part by previous preliminary evidence that neurogenic genes with shadow enhancers show less sensitivity to changes in activator concentration than similar genes lacking shadows [2].

An alternative view is that the proximal and shadow enhancers are primarily responsible for controlling distinct dynamic aspects of the *snail* expression pattern rather than functioning in an overlapping manner during mesoderm invagination. An expectation of the former robustness hypothesis is that transgenes containing either enhancer alone should be sufficient to induce gastrulation in *snail* mutant embryos. We tested this possibility by creating a series of recombineered bacterial artificial chromosomes (BACs) [4, 14] containing an ~25 kb genomic interval encompassing the *snail* and *Tim17b2* loci (Figure 1). Comparable BACs were prepared that either contain or lack the proximal enhancer. This enhancer was not simply deleted, but an ~1 kb segment containing critical Dorsal activator elements was replaced with a spacer DNA sequence (see Experimental Procedures) in order to retain normal spacing of the regulatory region.

To measure the effect that different enhancers have on transcriptional activity, we developed a reporter system for detecting nascent transcripts. The endogenous *yellow* gene is not transcribed until late in development and contains a large intron (e.g., [15, 16]), making it an ideal reporter for the detection of de novo transcripts by in situ hybridization. In contrast, the *snail* transcription unit lacks introns and is therefore not amenable to quantitative in situ hybridization methods that rely on intronic probes. Consequently, a series of BACs was created that contains *yellow* in place of *snail*. These BACs contain both enhancers or have either the primary or shadow enhancer replaced with random DNA (Figure 1). All of the

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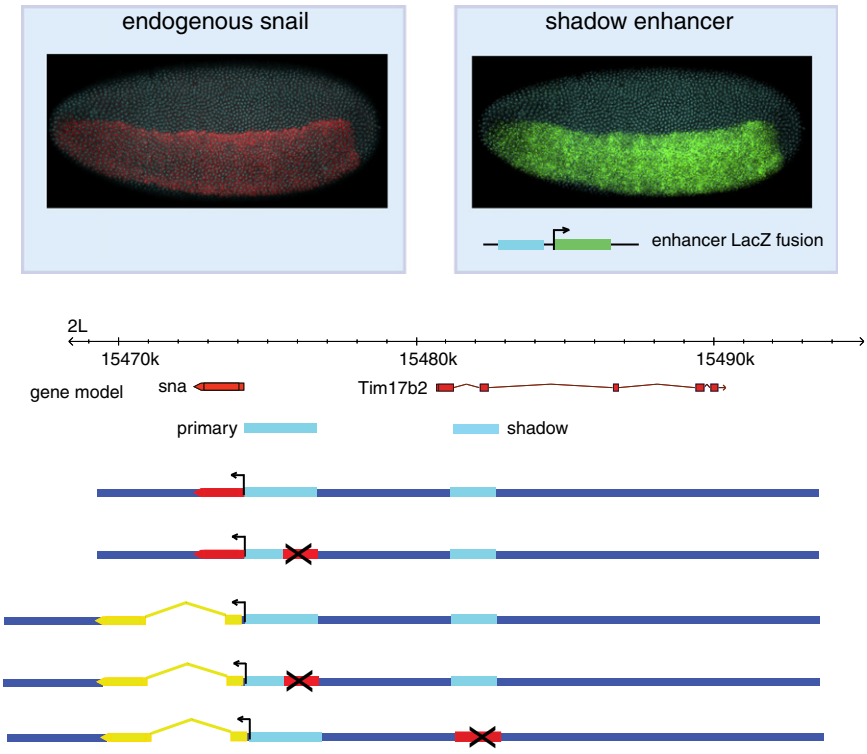

Figure 1. Identification of a *snail* Shadow Enhancer

The *snail* gene is expressed in the presumptive mesoderm (top left, red). An intronic region in neighboring Tim17b2 was shown to be bound by transcription factors that regulate *snail* [12] and is shown here to drive expression of a lacZ fusion gene in the mesoderm in a pattern qualitatively similar to the endogenous gene (top right). Bottom: schematic representations of the bacterial artificial chromosome (BAC) constructs used in subsequent experiments are aligned to the gene model. In all figures, anterior is to the left and dorsal is at the top, unless otherwise indicated.

aforementioned BACs were inserted in the same chromosomal location on 2L using phiC31 targeted integration [4, 17, 18].

BACs containing the *snail* gene were crossed into a mutant background with a deletion spanning the entire *snail* transcription unit (*Df (2L)osp*^{29}), along with a marked balancer to identify homozygous *snail* null mutants. As noted earlier, the reciprocal situation, proximal enhancer without shadow, can sometimes rescue gastrulation [13]. Mutant embryos homozygous for the *snail* deficiency chromosome (*osp*^{29}) are easily recognized by the absence of *snail* expression and ectopic *single-minded* (*sim*) expression, a key regulator of midline formation within the central nervous system that is normally excluded from the mesoderm by the Snail repressor [19, 20] (Figures 2E and 2F).

There is neither a ventral furrow nor subsequent ingression of the mesoderm in these mutants (e.g., [8, 9]). BAC transgenes containing both enhancers (Figure 2A) or just the shadow enhancer alone (Figure 2B) rescue gastrulation of mutant embryos (Figures 2C and 2D; compare with Figures 2E and 2F). In both cases, a complete ventral furrow is formed, followed by invagination of the mesoderm indistinguishable from that seen in wild-type embryos. Both BACs restore *snail* expression in the presumptive mesoderm, and *sim* transcripts are restricted to lateral regions that form the ventral midline of the central nervous system after gastrulation. These observations, along with previous studies (e.g., [13]), indicate that neither the primary nor shadow enhancer is necessary for the gastrulation of embryos raised at optimal, permissive conditions.

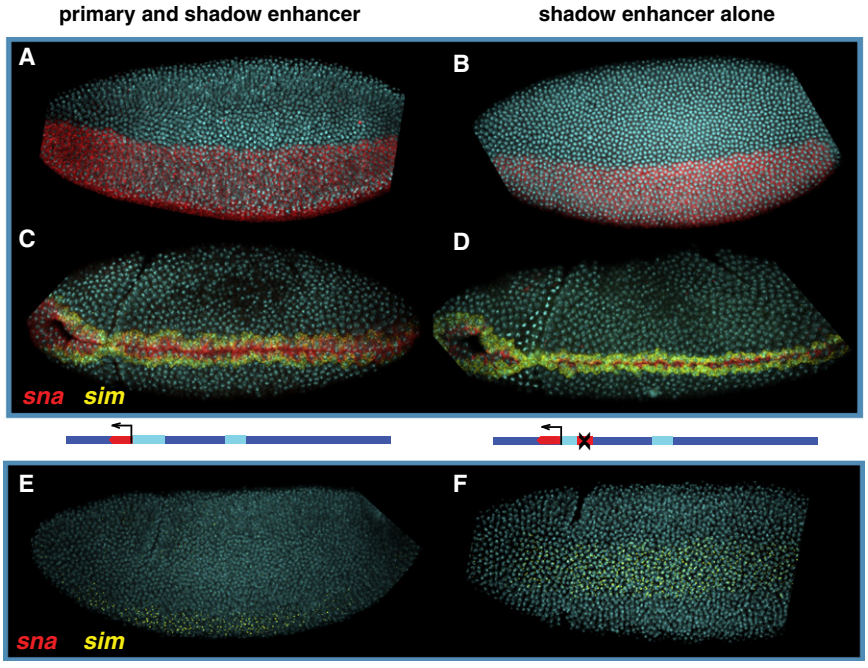

Figure 2. The *snail* Shadow Enhancer Rescues Gastrulation

(A) The rescue BAC construct in a *sna* mutant background drives *sna* expression (red) uniformly throughout the mesoderm in cycle 14 embryos.
(B) The pattern driven by the BAC with the primary enhancer deleted is qualitatively similar.
(C) During gastrulation, all *sna*-expressing cells migrate into the interior of the embryo. A single row of cells flanking the *sna* domain express *sim*, shown in yellow.
(D) *sna* driven without the primary enhancer is sufficient to induce normal gastrulation and normal *sim* expression when these embryos are raised at 22°C.
(E) In embryos lacking the *snail* BAC rescue construct, no *sna* is expressed. Instead, *sim* is expressed throughout the ventral region. Without *sna*, there is no mesodermal invagination. Lateral view.
(F) Embryo as in (E), ventral view.

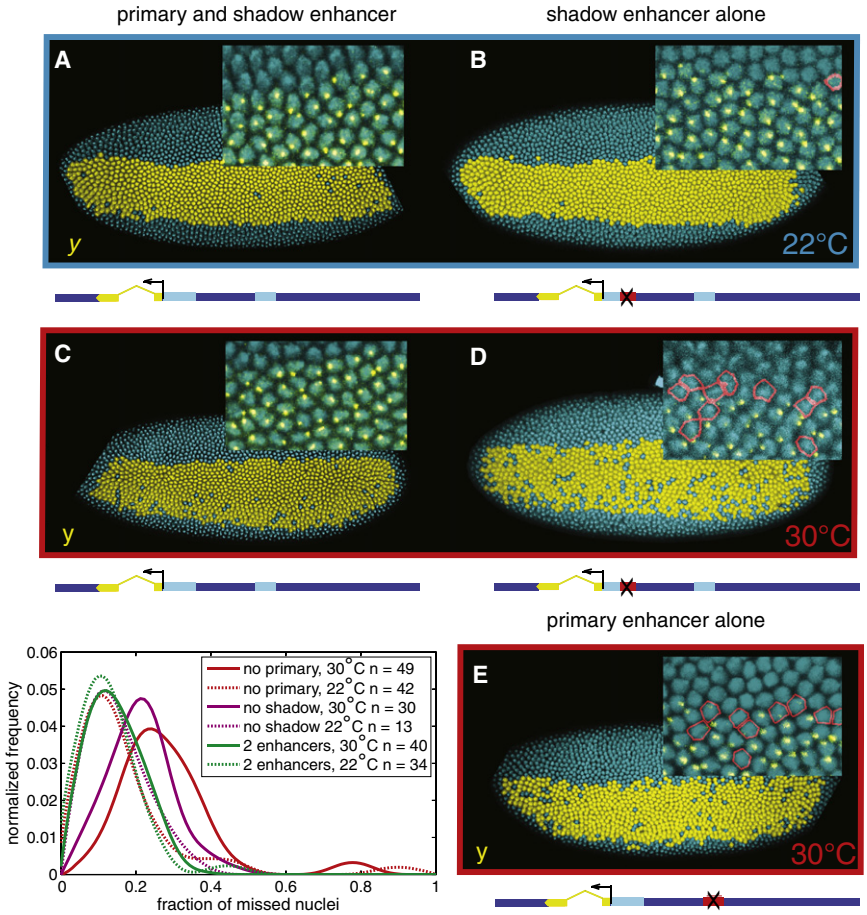

Figure 3. Multiple Enhancers Ensure Robust Gene Expression under Different Thermal Conditions

(A) Visualization of expression of the *yellow* reporter gene from the BAC containing the *sna* locus, stained for the *yellow* intron. Cells actively transcribing the reporter are shown in yellow. Intronic probes show a single bright point of transcription inside actively transcribing nuclei (inset); all embryos are heterozygous and have one copy of the reporter. Nuclei that express the endogenous gene but not the reporter are outlined in red. A schematic representation of the BAC is shown below the embryo.

(B) At 22°C, a similar degree of uniform expression is exhibited by embryos carrying a *yellow* BAC lacking the primary enhancer.

(C) At 30°C, embryos with both enhancers still show straight boundaries and a small percent of inactive nuclei.

(D) Embryos lacking the primary enhancer at 30°C show substantially more ragged boundaries of expression and a greater percent of inactive cells in the mesoderm.

(E) Embryos lacking the shadow enhancer are similar to those lacking the primary at both temperatures.

Bottom left: Frequency distributions of the fraction of cells in the *sna*-expressing region that lack *yellow* nascent expression are plotted for each of the six different embryo populations. n indicates the number of embryos in each population sample.

Although the shadow enhancer is sufficient for generating a qualitatively normal pattern of *snail* expression, additional assays were done to determine whether there might be subtle changes in expression. Quantitative confocal imaging methods were used to investigate this possibility (see [2]). As mentioned earlier, BAC transgenes were prepared that contain the *yellow* reporter gene in place of the *snail* transcription unit. In situ hybridization assays with intronic probes permit direct detection of *yellow* de novo transcripts, and, hence, precise measurements of *snail* transcription with single cell (nucleus) resolution. At normal culturing temperatures (22°C), there is no discernible difference in the initial de novo transcription patterns of BAC transgenes containing both enhancers (Figure 3A) or containing just a single enhancer, either the primary enhancer or shadow enhancer (Figure 3B). In the majority of cases, more than 90% of the nuclei in the presumptive mesoderm express *yellow* nascent transcripts.

Less-reliable expression is observed for BAC transgenes containing a single enhancer at elevated temperatures (30°C; Figures 3C and 3D). More than 20% of the nuclei in the presumptive mesoderm lack *yellow* nascent transcripts in over half of the embryos expressing the BAC transgene without the shadow enhancer (Figure 3E). This effect is even more pronounced upon removal of the primary enhancer. The same cutoff value, absence of *yellow* nascent transcripts in at least 20% of all mesodermal nuclei, occurs in over three-fourths of these embryos (Figure 3D). In contrast, the BAC transgene containing both the primary and shadow enhancers continues to display nearly complete patterns of de novo transcription at the elevated temperature (Figure 3C).

Similar results were obtained in response to genetic perturbations (Figures 4A and 4B). For example, the *yellow* transgene BAC containing both enhancers exhibits a normal pattern of expression in embryos derived from *dl*/+ mothers containing half the normal dose of the Dorsal gradient (Figure 4A). The distribution of nuclei failing to maintain active expression is similar to that seen for wild-type embryos (Figure 4C). However, the comparable BAC transgene containing only the shadow enhancer exhibits erratic patterns of activation in these embryos, particularly in lateral regions (Figure 4B, quantification in C). These results, along with the preceding analysis of embryos grown at elevated temperatures, suggest that the *snail* shadow enhancer helps ensure accurate and reproducible patterns of gene expression in large populations of embryos subject to genetic and environmental perturbations.

The preceding results document quantitative changes in the variability and reliability of *snail* expression upon removal of the primary or shadow enhancer. We next asked whether such variation causes changes in cellular morphogenesis, particularly the formation of the ventral furrow and subsequent invagination of the mesoderm (Figures 4D and 4E). *snail* mutant embryos carrying BACs with both enhancers (Figure 4D) or just the shadow enhancer (Figure 4E) were grown at elevated temperatures (30°C). Embryos carrying the transgene with both enhancers exhibit normal patterns of gastrulation (Figure 4D). In contrast, comparable embryos lacking the primary enhancer display erratic patterns of gastrulation, including the formation of incomplete ventral furrows that do not extend along the entire germband (Figure 4E) and

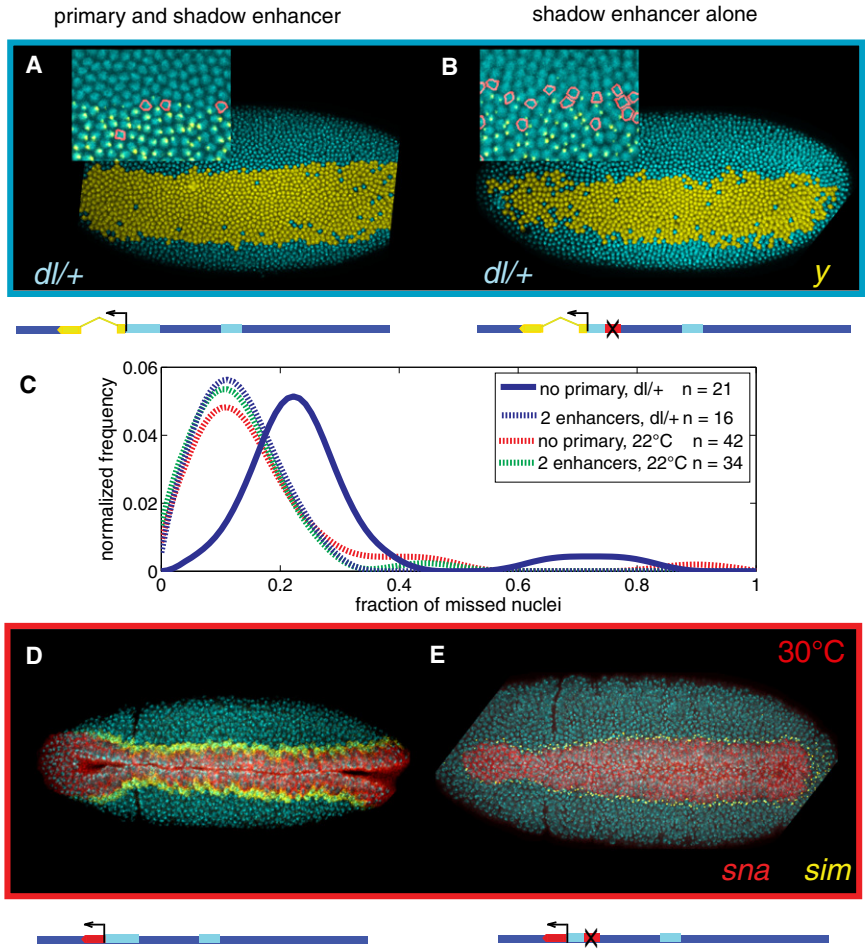

Figure 4. The Effect of Intrinsic and Extrinsic Variability

(A) Embryos from dorsal heterozygote mothers raised at 25°C show uniform *yellow* expression when driven with both enhancers. Only a few cells are lacking active expression (inset).

(B) Embryos with a single enhancer in this background show substantially greater loss of expression and ragged boundaries.

(C) The distribution nuclei that fail to maintain active transcription shifts to the right in the dorsal heterozygote background only for embryos lacking one of the enhancers.

(D) All observed embryos raised at 30°C (n = 28) from a population heterozygous for the BAC constructs containing both enhancers gastrulate normally, forming a straight ventral furrow; note stage of development by presence of cephalic furrow. Exonic *sim* shown in yellow, exonic *sna* shown in red.

(E) Some embryos from a similar population, but with only the single enhancer and raised at 30°C, show various defects in gastrulation (n = 10 of 14). Note embryo stage by presence of cephalic furrow yet lack of significant mesodermal invagination. The number of *snail*-expressing cells anterior to the cephalic furrow is also reduced. Figure S2 shows a range of defects observed in these embryos; a narrower pattern of anterior expression may result in delays in involution of anterior regions, and some exhibit a more erratic midline. Intronic *sim* shown in yellow.

disruptions in the symmetry of the involuted mesodermal tube (see Figure S2 available online). As shown earlier, such defects are not observed at normal temperatures (22°C; Figure 2).

We have presented evidence that the *snail* shadow enhancer located within the *Tim17b2* locus helps ensure reliable and reproducible patterns of *snail* expression in the presumptive mesoderm during gastrulation. BAC transgenes lacking either the primary enhancer or the shadow enhancer display erratic patterns of de novo transcription at elevated temperatures. We propose that shadow enhancers come to be fixed in populations by ensuring robustness in the activities of key patterning genes such as *snail*. Increases in temperature should cause less-stable occupancy of critical binding sites, but an additional enhancer could suppress this noise by increasing the probability of gene activation. This increased time of active transcription per cell might augment the overall levels of expression, which could be an important function of shadow enhancers.

Other critical dorsal-ventral determinants also contain shadow enhancers, including *brinker*, *vnd*, and *sog* [1]. The recent analysis of *shavenbaby* suggests that shadow enhancers are essential for the reliable morphogenesis of embryonic bristles in older embryos [21]. There is also evidence that shadow enhancers might be a common feature of vertebrate systems such as zebrafish [22].

Shadow enhancers appear to represent a novel mechanism of canalization [6], whereby complex developmental processes lead to a fixed outcome despite genetic and environmental perturbations. Other mechanisms of canalization have been suggested, including recursive wiring of gene regulatory networks and "capacitors" such as hsp90 that suppress both altered folding of mutant proteins and transpositioning of mobile elements [23–26].

It is conceivable that primary and shadow enhancers mediate overlapping patterns of activity only during early embryogenesis. They might come to possess distinctive regulatory activities at later stages of development. Nonetheless, during the time when their activities coincide during gastrulation, they maintain reliable patterns of *snail* expression in response to environmental and genetic variability. Although either enhancer might be sufficient, both enhancers are required for accurate and reliable patterns of expression in response to variability. This precise patterning enables rapid development, without delays arising from corrective feedback mechanisms. It is easy to imagine that delays in embryogenesis would result in selective disadvantages to the resulting larvae, which must compete for limiting sources of food. Regardless of the specific mechanisms that select for shadow enhancers, the occurrence of such enhancers provides an opportunity for the evolution of novel patterns of gene expression. As long as the two enhancers maintain overlapping activities during developmental hot spots such as gastrulation, they can drift or be selected to produce novel patterns of gene expression.

Experimental Procedures

Fly Genetics

Positive BAC line males (labeled with w+) were crossed to yw; wg^{Sp}/CyO; Pr,Dr/TM3,Sb,Ser virgins. Homozygous BAC lines were created by selfing

the red-eyed, Sb,Ser flies from the F1 generation. Males from the BAC lines carrying the yellow reporter constructs were crossed to dl^6/CyO virgins.

To test for rescue of the BAC constructs, we generated a white-eyed, double balancer strain carrying a CyO-linked hunchback-LacZ reporter by crossing and backcrossing wnt4/CyO, hb-lacZ (BSC 6650) to yw; wg^{Sp}/CyO; Pr,Dr/TM3,Sb,Ser virgins. Positive BAC males were crossed into this line to create w; +/CyO, hb-lacZ; BAC[snail,w+]/TM3,Sb,Ser virgins. Simultaneously, w; *Df (2L)osp*^{29}/CyO, (BSC 3078) flies carrying a deletion spanning the *snail* gene were crossed to yw; wg^{Sp}/CyO; Pr,Dr/TM3,Sb,Ser virgins. The *Df (2L)osp*^{29}/wg^{Sp}; +/TM3,Ser males were crossed to the virgins containing the labeled balancer and the BAC. The progeny were selfed to create homozygous stable lines for the BAC carrying the snail deletion over the hb-lacZ-marked CyO balancer. Populations still containing the Ser balancer or a wild-type chromosome III were also analyzed to test the effect of single-copy rescue. The labeled balancer allowed for the reliable identification of embryos lacking a functional copy of endogenous *sna*.

Recombineering and Transgenesis

Recombineering was performed as described previously [4, 14, 27–29] with modifications described in the Supplemental Experimental Procedures. These supplemental sections also describe construction of the *yellow* intronic reporter, the use of plasmids with a conditional origin of replication to reduce recombineering colony background [30], and preparation of modified BAC constructs for microinjection. Table S1 lists primers used to make BAC modifications; the *sna* primary enhancer sequence was replaced using an ampicillin resistance cassette as a nonregulatory spacer. BAC CH321-18I14 [14] was used as the basis for all other modifications.

Fluorescence In Situ Hybridization and Quantitative Imaging Methods

Fluorescence in situ hybridization was performed as described in [31]. Embryos were imaged on a Leica scanning confocal SL microscope as a 14–20 section Z stack through the nuclear layer at 1/2 micron intervals, with scanning resolution of approximately 250 nm/pixel. Images were maximum projected and computationally segmented to localize and count nuclei, mRNA expression domains, and nascent transcripts. More details on the automation of image analysis are included in the Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at doi:10.1016/j.cub.2010.07.043.

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