Molecular Cell
Visualization of Transvection in Living Drosophila Embryos

Graphical Abstract

Highlights
- Insulators increase the stability, but not the frequency, of allele-allele pairing
- The gypsy insulator functions in an orientation-independent manner
- A shared enhancer co-activates linked PP7 and MS2 reporter genes in cis and in trans
- Linked reporter genes compete for shared resources during transvection

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In Brief
Lim et al. explore a process called transvection in living Drosophila embryos, whereby enhancers on one homolog activate transcription units on the other homolog. They show that insulators facilitate transvection by stabilizing allele-allele pairing. Surprisingly, a shared enhancer coactivates a cis-linked PP7 reporter gene along with a trans-linked MS2 reporter contained on the other homolog. This coactivation is consistent with emerging evidence for transcription “hubs” containing clusters of RNA polymerase II and associated activators.
Visualization of Transvection in Living Drosophila Embryos

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SUMMARY

How remote enhancers interact with appropriate target genes persists as a central mystery in gene regulation. Here, we exploit the properties of transvection to explore enhancer-promoter communication between homologous chromosomes in living Drosophila embryos. We successfully visualized the activation of an MS2-tagged reporter gene by a defined developmental enhancer located in trans on the other homolog. This trans-homolog activation depends on insulator DNAs, which increase the stability—but not the frequency—of homolog pairing. A pair of heterotypic insulators failed to mediate transvection, raising the possibility that insulator specificity underlies the formation of chromosomal loop domains. Moreover, we found that a shared enhancer co-activates separate PP7 and MS2 reporter genes in cis and in trans. Transvecting alleles weakly compete with one another, raising the possibility that they share a common pool of the transcription machinery. We propose that transvecting alleles form a trans-homolog “hub,” which serves as a scaffold for the accumulation of transcription complexes.

INTRODUCTION

Enhancers are short segments of genomic DNA (typically ~100 bp to ~1 kb in length) that switch genes on and off in response to a variety of intrinsic and external signals. The human genome is thought to contain on the order of ~400,000 enhancers (ENCOD Project Consortium, 2012), an average of nearly 20 enhancers per protein-coding gene. There is emerging evidence that sequence polymorphisms in enhancer DNAs represent a major source of population diversity and predilection to disease (Abraham et al., 2017; Indjeian et al., 2016). And yet, we do not understand how enhancers work over long distances to stimulate transcription at select target genes.

The phenomenon of transvection in Drosophila provides an intriguing model for analyzing enhancer-promoter interactions. Transvection was discovered by E.B. Lewis in 1954 in the course of his groundbreaking studies on the Ultrabithorax (Ubx) locus of the Bithorax complex (Lewis, 1954). He found that certain Ubx alleles are able to complement one another via transvection. Subsequent molecular studies suggested that this complementation is best explained by regulatory sequences on one homolog controlling the expression of the cognate transcription unit on the other homolog (Peifer and Bender, 1986). In this study, we sought to explore the molecular mechanisms underlying this regulatory communication between homologous chromosomes in living embryos.

Studies of transvection at Ubx and other genetic loci identified a number of properties that influence trans-homolog regulatory interactions, including chromosome pairing, promoter competition, and insulators (reviewed in Fukaya and Levine, 2017). Chromosomal inversions that disrupt pairing cause reductions in transvection at the Ubx locus (Lewis, 1954), although an unusual pairing-independent transvection process was documented at the Abdominal-B (Abd-B) locus (Hendrickson and Sakonju, 1995; Hopmann et al., 1995). Insulators positioned near transvecting alleles have been shown to augment the efficiency of trans-homolog interactions, possibly by facilitating stable chromosome pairing (Kravchenko et al., 2005). Finally, enhanced transvection was observed upon removal of nearby cis-linked promoters (Geyer et al., 1990; Martínez-Laborda et al., 1992), suggesting that enhancers on one homolog can more easily activate gene expression in trans on the other homolog when they are not impeded by proximal cis-linked promoters.

In this study, we visualized transvection in living Drosophila embryos. A well-defined developmental enhancer was placed in trans to a lacZ reporter gene containing a series of MS2 stem loops, permitting detection of nascent transcripts using an MCP-GFP fusion protein (Bertrand et al., 1998; García et al., 2013; Lucas et al., 2013). Trans-activation of the MS2-lacZ reporter is observed in early embryos only when insulators are inserted into both alleles. Evidence is presented that insulators increase the stability, but not frequency, of homolog pairing. Different classes of insulators display distinct orientation requirements, and transvection is not observed with a pair of heterotypic insulators. We also show that a shared enhancer can co-activate a cis-linked PP7 reporter gene along with a trans-linked MS2 reporter gene. We propose that transvecting alleles...
form a trans-homolog “hub,” which serves as a scaffold for the conglomeration of transcription complexes.

RESULTS

Insulators Facilitate Transvection

Site-directed integration was used to insert the well-defined snail (sna) shadow enhancer in trans to a lacZ reporter gene at the same location on homologous chromosomes (Figure 1A; top). The ∼1.5-kb enhancer mediates stable expression in ∼800–1,000 cells comprising the presumptive mesoderm of early embryos (Figures S1A and S1B) (Dunipace et al., 2011; Perry et al., 2010). The lacZ reporter gene contains the minimal 100-bp even-skipped (eve) core promoter as well as 24 copies of the MS2 RNA stem loop sequence within the 5’ untranslated region (UTR). In situ hybridization assays were used to monitor expression of lacZ mRNAs. No significant expression was observed either in the absence of an insulator (Figure 1A), or upon insertion of a single copy of the minimal 432 bp gypsy insulator (Figure 1B). However, expression was observed when gypsy insulators were placed on both homologs, upstream of the sna shadow enhancer and MS2-lacZ reporter gene, respectively (Figure 1C). Reporter transcripts were detected in ∼3%–4% of the cells comprising the sna expression pattern within the presumptive mesoderm (Figure 1D). Importantly, trans-activation of the MS2 reporter gene was observed with different core promoters, transcription units, and genetic organization (Figures S1C and S1D). Insulators were placed adjacent to the enhancer because this configuration is commonly seen for endogenous loci that are active in the early embryo, including eve and fushi tarazu (ftz) (Fujikawa et al., 2013).

These observations are consistent with previous reports that homolog pairing is inefficient in early embryos as compared with later stages of the Drosophila life cycle (Gemkow et al., 1998; Hiraoka et al., 1993). This might be due to the slow rate of homolog pairing during the brief interphase periods that characterize the early embryo. In principle, gypsy insulators could facilitate trans-activation of MS2-lacZ by increasing the frequency or stability of homolog pairing. Previous genetic studies of transvection at the yellow locus suggest that insulators increase the efficiency of trans-homolog interactions (Kravchenko et al., 2005).

Different Classes of Insulators

To explore the contributions of insulators to transvection, we manipulated the orientation of gypsy and examined the activities of additional insulators (Figure 2). The gypsy insulator contains a highly ordered structure, with each of the 12 Suppressor of Hairy-wing (Su(Hw)) binding sites spanning the insulator positioned in the same orientation (Spana et al., 1988). Past studies have shown that insulators do not pair when placed in the same orientation in cis on the same chromosome (Kyrchanova et al., 2008). However, inverting one of the gypsy insulators did not diminish trans-activation of the MS2-lacZ reporter gene (Figures 2A and 2B), raising the possibility of distinct mechanisms for insulator interactions in cis and in trans.

We next examined the Homie insulator, which is located at the 3’ boundary of the endogenous eve locus (Fujikawa et al., 2009, 2013). Homie is bound by Su(Hw), but unlike gypsy, it is also
recognized by another insulator-binding protein CCCTC-binding factor (CTCF), which is highly conserved in vertebrates (Nègre et al., 2010). Homie insulators mediate a low rate of transvection when placed in the same orientation (Figures 2D and 2F), but not when positioned in opposite orientations (Figure 2E), similar to control embryos containing just a single Homie insulator (Figure 2C). This dependence on orientation is consistent with previous studies (Fujioka et al., 2016).

The gypsy and Homie insulators do not mediate transvection when placed in trans to one another (Figures S2A–S2C). This observation raises the possibility of separate classes of insulators: those that are bound by either Su(Hw) alone, or both Su(Hw) and CTCF. A third insulator, Fab-8, was also tested in this assay. It is bound by CTCF, but not Su(Hw) (Gerasimova et al., 2007). We examined an ~5-kb DNA fragment from the Abd-B transvection mediating region (tmr), which contains the Fab-8 insulator (Hopmann et al., 1995; Zhou et al., 1999). This tmr fragment also facilitates trans-activation of the MS2 reporter gene, although expression is slightly delayed as compared with the timing observed for the gypsy and Homie insulators (Figures S2D–S2F). These observations suggest that different classes of insulators mediate distinct kinetics of homolog interactions.

**Stabilization of Homolog Pairing**

It is possible that insulators facilitate transvection by increasing either the frequency or the stability of homolog pairing. To address this question, we analyzed sna > lacZ alleles marked with either PP7 or MS2 in living embryos (Fukaya et al., 2016; Hocine et al., 2013) (Figure 3A). Most nuclei do not exhibit pairing, whether or not the two alleles contain gypsy insulators (Figures 3B–3D and S3A–S3D; Movie S1). Approximately 20% of the transcriptionally active nuclei display pairing of sna > lacZ alleles whether there are two copies of gypsy or just one (control) (Figures 3H and 3I; Movie S2). Nuclei exhibiting pairing were divided into three classes based on the duration of association: 1-5 min, 5-10 min, and 10 min or more. Almost half of these nuclei correspond to the most stable class of pairing (10 min or more) with two copies of gypsy, but there is a 2-fold reduction with a single copy. Thus, gypsy-gypsy interactions lead to a 2-fold increase in stable pairing, and it is likely that this is important for transvection.
To determine how this frequency of stable pairing compares to endogenous loci, we examined the pairing of ftz alleles (Figures S3E and S3F). ftz is located within the Antennapedia complex, which exhibits a variety of transvection phenomena (Southworth and Kennison, 2002). Moreover, ftz contains well-defined SF1 and SF2 insulators (Belozerov et al., 2003; Li et al., 2015). MS2 and PP7 stem loops were inserted into the 3' UTR of each ftz allele using CRISPR-directed genome editing, and their activities were monitored in living embryos during nuclear cycle (nc) 14. There is an overall higher frequency of transient pairing as compared with the sna > lacZ transgenes (Figure S3E). However, stable pairing is seen in ~9% of all nuclei examined (Figure S3F), similar to the proportion of nuclei exhibiting stable pairing of the synthetic alleles. We therefore conclude that our transvection assay captures critical properties of the endogenous pairing process.

Co-activation in trans
An unresolved mystery of transvection is the relative levels of expression of the cis and trans transcription units when regulated by a shared enhancer. Does the enhancer on one homolog have equal access to target genes located on both homologs? To address this question, we created a co-transvection assay, whereby a single enhancer is challenged with both a cis-linked PP7 reporter gene as well as the MS2 reporter gene in trans (Figure 4A).

Figure 3. gypsy Insulators Foster Stable Homolog Pairing
(A) Schematic representation of the lacZ reporter genes containing the 100-bp minimal eve promoter, the 1.5-kb sna shadow enhancer, the 432-bp gypsy insulator, and 24x MS2 or 24x PP7 RNA stem loop within the 5' UTR.
(B) 3D tracking of MS2-lacZ (green) and PP7-lacZ (red) transcription foci in a nucleus that exhibits allelic separation throughout nc 14. An ellipsoid with 4 μm short-axis radius and 7 μm long-axis radius was plotted as a proxy for the shape of the nucleus at mid nc 14. Location of MS2 and PP7 alleles are indicated as spheres with 0.5 μm radii to represent uncertainty in the spatial resolution in the z axis.
(C and D) Cumulative traces of MS2-lacZ (green) and PP7-lacZ (red) transcription foci during early (5 min to 29.9 min; C) and late nc 14 (30.1 min to 54.6 min; D) in the nucleus shown in (B).
(E) 3D tracking of MS2-lacZ (green) and PP7-lacZ (red) transcription foci in a nucleus that undergoes stable homolog pairing at the midpoint of nc 14. Note the association of the two alleles during later stages of nc 14.
(F and G) Cumulative traces of MS2-lacZ (green) and PP7-lacZ (red) transcription foci during early phases of nc 14 when the two alleles are unpaired (5.0 min to 29.5 min; F) and later stages when paired (29.7 min to 54.6 min; G) in the nucleus shown in (E).
(H) The pairing efficiency across nuclei (H) and the distribution of the duration of homolog pairing in those nuclei exhibiting pairing (I). The most salient impact of two copies of gypsy is the doubling in the frequency of the most stably paired alleles (from ~4% in controls to ~9% with a pair of gypsy insulators). A total of 1,135 and 936 nuclei from three independent embryos were analyzed for single gypsy and two gypsy, respectively. Error bars represent ± the SEM of three independent embryos.

See also Figure S3.
In most transvecting nuclei, the initial bursts of de novo transcription arise solely from the cis-linked PP7 reporter gene during the first 15–20 min following mitosis. Based on the kinetics of allele pairing (Figures 3E–3G; Movie S2), we believe that this delay is due to a lag in pairing, which typically occurs 10–20 min after mitosis. Alternatively, the shared enhancer may prefer the cis-linked promoter due to its proximity (see Discussion). Following this delay, there is a high incidence of co-activation of the two reporter genes (Figures 4B, 4C, S4A, and S4B; Movie S3). During transvection, the two alleles exhibit tight association, although the signals do not completely coincide with one another (Figures 4C–4E, S4B–S4D, and S5; Movies S4 and S5). It is therefore possible that trans-activation of the MS2 reporter gene depends on proximity, rather than direct physical contact with the shared enhancer (see Discussion). Importantly, trans-activation was nearly abolished when the MS2 and PP7 alleles were inserted at non-homologous locations.

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See also Figure S7.

Enhancers (blue). A total of 99 and 883 nuclei from four and three independent embryos were analyzed, respectively. Both histograms were plotted with a bin width of 0.1. These two distributions were found to be significantly different (p < 1.5 × 10⁻³).

The distribution of correlation coefficients between enhancers as shown in (C).

The most striking finding of this study is the co-activation of linked PP7 reporter gene as compared with the MS2 gene located in trans on the other homolog. Instead, once transvection commences, the shared enhancer seems to have equal access to both reporter genes. The only preference for the cis-linked PP7 is seen during the initial periods of interphase following mitosis (Figure 4). It is possible that this delay in trans-activation is due to preferential enhancer-promoter interactions with the proximal cis-linked reporter gene. A nonexclusive alternative possibility is that the delay is due to the time it takes for the two alleles to become physically associated following mitosis. We prefer the latter explanation because the analysis of sna > MS2-lacZ and sna > PP7-lacZ hetero-allelic embryos reveals a delay in association during the first 10–20 min of nc 14 (Figures 3 E–3 G; Movie S2).

Transvection is not observed during the time frame of our analysis (nc 14) without the insertion of insulators into both alleles (Figure 1). As a result, we were able to use transvection as an assay for examining insulator function. Most previous studies have centered on the role of insulators in creating chromosomal loop domains in cis, such as topologically associating domains (TADs). Insulators that are separated by large distances along a chromosome (~50 kb to 3 Mb) are thought to come into close physical proximity (Dekker and Mirny, 2016; Dixon et al., 2016). This pairing of insulators depends on their relative orientations (Guo et al., 2015; Rao et al., 2014). When in the convergent orientation in cis, they are able to form a chromosomal loop domain, but not when placed in the same orientation. We do not see an orientation dependence for the gypsy insulator in trans, although the Homie insulator does exhibit such a requirement (Figure 2).

**DISCUSSION**

The most striking finding of this study is the co-activation of linked PP7 and MS2 reporter genes in cis and in trans during transvection. Promoter competition has been observed in a variety of systems including both invertebrates and vertebrates (Choi and Engel, 1988; Foley and Engel, 1992; Fukaya et al., 2016; Ohtsuki et al., 1998). Enhancers tend to prefer nearby promoters as opposed to those positioned in more remote locations. We therefore expected preferential activation of the cis-linked reporter gene with the shared enhancer as compared with the MS2 gene located in trans on the other homolog. Instead, once transvection commences, the shared enhancer seems to have equal access to both reporter genes. The only preference for the cis-linked PP7 is seen during the initial periods of interphase following mitosis (Figure 4). It is possible that this delay in trans-activation is due to preferential enhancer-promoter interactions with the proximal cis-linked reporter gene. A nonexclusive alternative possibility is that the delay is due to the time it takes for the two alleles to become physically associated following mitosis. We prefer the latter explanation because the analysis of sna > MS2-lacZ and sna > PP7-lacZ hetero-allelic embryos reveals a delay in association during the first 10–20 min of nc 14 (Figures 3 E–3 G; Movie S2).

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Moreover, we found that a pair of heterotypic insulators were unable to mediate transvection, e.g., there is no trans-activation of the MS2 reporter gene when one allele is tagged with the Su(Hw)-dependent gypsy insulator and the other with Su(Hw)/CTCF-dependent Homie insulator (Figure S2). This observation raises the possibility of insulator specificity in the creation of topological domains both in cis and in trans.

We found that insulators do not significantly increase the frequency of allelic pairing, but instead increase the stability of pairing, as suggested previously (Kravchenko et al., 2005). It is possible that somatic pairing of homologous chromosomes is mediated by DNA sequence homology, as seen for pairing during meiosis (Tsai and McKee, 2011). Support for this view stems from the analysis of trans-chromosomal interactions when the gypsy insulator, sna shadow enhancer, and PP7 reporter gene are placed in non-homologous locations in the genome (Figure S6). Although trans-activation is exceedingly rare, we have detected a few nuclei that display co-expression of MS2 and PP7, and the resulting transcriptional bursts are comparable to those seen in pairing-dependent transvection (Movie S7). One interpretation of these findings is that sequence homology increases the probability of transient associations between alleles, while insulators stabilize the association. In the case of non-homologous sites, associations between the enhancer and MS2 reporter are rare, but, when they occur, the insulators stabilize the association for trans-activation of the MS2 reporter.

There are instances of non-homologous interactions in vertebrates (Ling et al., 2006; Lomvardas et al., 2006), and we suggest that these depend on compatible insulators located near interacting loci. It is also important to note that some enhancers can act in trans independently of insulators (Bateman et al., 2012; Blick et al., 2016; Mellert and Truman, 2012), giving rise to the possibility that there might be other DNA elements that can mediate transvection.

We propose that insulator-insulator interactions create a trans-homolog topological domain, comparable to those formed in cis (Fukaya et al., 2016). This association places the shared enhancer in close proximity with both the cis-linked PP7 reporter gene and the trans-linked MS2 reporter (Figure 7). It is possible that paired insulators create a trap or “hub” for condensates of the transcriptional machinery. The formation of this transcriptional hub might be driven by the coalescence of Pol II complexes and transcription factors, accumulated at promoters and enhancers respectively (Hnisz et al., 2017). As implied by a recent live-imaging study (Gu et al., 2018), the formation of such hubs might influence the sub-diffusive mobility of enhancers. The release of Pol II from the hub could contribute to transcriptional bursting (Bothma et al., 2014), as well as co-activation of the PP7 and MS2 reporter genes in cis (Fukaya et al., 2016) and in trans.

This view of transcriptional control is quite distinct from the classical models of sequential recruitment of individual Pol II
complexes to their target promoters. It was recently proposed that transcriptional bursts are the result of periodic phase transitions of activator proteins and the Pol II machinery at active loci (Hnisz et al., 2017). These transitions are thought to arise from protein–protein interactions via intrinsically disordered domains within transcription factors, co-activators and Pol II subunits (Gemayel et al., 2015; Hnisz et al., 2017). Indeed, recent super-resolution studies suggest highly dynamic clustering of Pol II complexes and transcription factors at active foci (Cisse et al., 2013; Mir et al., 2017; Tsai et al., 2017). The proposed transcription hubs also challenge traditional models of targeted enhancer-promoter loops. The formation of extended transcription condensates consisting of multiple activators and Pol II complexes might require approximate proximity rather than direct, physical association of enhancers with their target promoters.

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and seven movies and can be found with this article online at https://doi.org/10.1016/j.molcel.2018.02.029.

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**AUTHOR CONTRIBUTIONS**

T.F. and M.L. designed the experiments. T.F. and T.H. performed the experiments. B.L. and T.H. performed the image analysis. All the authors discussed the results and wrote the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


### STAR METHODS

#### KEY RESOURCES TABLE

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<td>BDSC # 9748</td>
</tr>
<tr>
<td>D. melanogaster gypsy-snaPr-MS2-yellow</td>
<td>This study</td>
<td>N/A</td>
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<td><strong>Oligonucleotides</strong></td>
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<td>Primers are listed in STAR Methods.</td>
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<td>MATLAB 2016b</td>
<td>MathWorks</td>
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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Michael Levine (msl2@princeton.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

In all experiments, we studied *Drosophila melanogaster* embryos at nuclear cycle 14. The following fly lines were used in this study: *nos > MCP-GFP, His2Av-mRFP* (this study), *nos > MCP-GFP, nos > mCherry-PCP, His2Av-eBFP2* (this study), *sna shadow enhancer* (this study), *gypsy-sna shadow enhancer* (this study), *sna shadow enhancer-gypsy* (this study), *sna shadow enhancer-evePr-MS2-lacZ* (this study), *gypsy-sna shadow enhancer-evePr-MS2-lacZ* (this study), *gypsy-evePr-MS2-lacZ* (this study), *gypsy (inverted)-evePr-MS2-lacZ* (this study), *Homie-evePr-MS2-lacZ* (this study), *Homie (inverted)-evePr-MS2-lacZ* (this study), *Homie-sna shadow enhancer* (this study), *tmr-sna shadow enhancer* (this study), *tmr-evePr-MS2-lacZ* (this study), *gypsy-sna shadow enhancer-evePr-PP7-lacZ* (this study), *gypsy-snaPr-MS2-yellow* (this study), *fushi tarazu-MS2* (this study), *fushi tarazu-PP7* (this study), *y¹ w¹¹¹⁸, PBac[+attP-3B] VK00033* (Bloomington Drosophila Stock Center #9750), *y¹ w¹¹¹⁸, PBac[y¹+attP-3B] VK00031* (Bloomington Drosophila Stock Center #9748).

*fushi tarazu-MS2* and *fushi tarazu-PP7* were generated using CRISPR/Cas9 based insertion of 24x MS2 RNA stem loop or 24x PP7 RNA stem loop into the 3’ UTR of endogenous *fushi tarazu*. In brief, ~1-kb DNA fragment of 5’ and 3’ homology arm sequences were PCR amplified from the genomic DNA, and inserted into the pBS-MS2-loxP-dsRed-loxP and pBS-PP7-loxP-dsRed-loxP donor plasmids (see below). These plasmids were co-injected with the pCFD3 gRNA expression plasmid to *nos-Cas9/CyO* embryos (Ren et al., 2013).

METHOD DETAILS

Site-specific transgenesis by phiC31 system

All reporter plasmids were integrated into a unique landing site on the third chromosome using strain 9750 (Bloomington Drosophila Stock Center). In Figure S6, PP7-lacZ reporter plasmid was integrated into a unique landing site on the third chromosome using strain 9748 (Bloomington Drosophila Stock Center). Microinjection was performed as described (Ringrose, 2009). Zero to 1-hour embryos were collected and dechorionated with bleach. Aligned embryos were dried with silica gel for 12 min and covered with Halocarbon oil 27 (Sigma). Subsequently, microinjection was performed using Picospritzer III (Parker) and Narishige M-152 Micromanipulator (Narishige). Injection mixture contains 500 ng/µl plasmid DNA, 5 mM KCl, 0.1 mM phosphate buffer, pH 6.8. mini-White marker was used for subsequent screening.

Genome editing by CRISPR/Cas9

pCFD3 gRNA expression plasmid and pBS-dsRed donor plasmid were co-injected to *nos-Cas9/CyO* embryos (Ren et al., 2013). Microinjection was performed as described (Ringrose, 2009). Injection mixture contains 500 ng/µl pCFD3 gRNA expression plasmid, 500 ng/µl pBS-dsRed donor plasmid, 5 mM KCl, 0.1 mM phosphate buffer, pH 6.8. 3xP3-dsRed marker was used for subsequent screening.

Fly strains

**MCP-GFP, mCherry-PCP, His2Av-eBFP2**

The *nos > SV40NLS-mCherry-PCP, His2Av-eBFP2* expression plasmid (Fukaya et al., 2017) was integrated into a unique landing site on the third chromosome using strain 9750 (Bloomington Drosophila Stock Center) to obtain maternal expression of the mCherry-PCP and His2Av-eBFP2 fusion proteins. Maternal expression of the MCP-GFP fusion protein was obtained using a transgenic strain carrying a *nos > MCP-GFP* transgene that was integrated into the third chromosome by P-element mediated transformation (Garca et al., 2013). These were mated to create the fly line *mCherry-PCP, MCP-GFP, His2Av-eBFP2* in order to obtain co-expression of MCP-GFP, mCherry-PCP and His2Av-eBFP2 fusion proteins.

**MCP-GFP, His2Av-mRFP**

The *nos > MCP-GFP, His2Av-mRFP* expression plasmid was integrated into a unique landing site on the third chromosome using strain 9750 (Bloomington Drosophila Stock Center) to obtain maternal expression of the MCP-GFP and His2Av-mRFP fusion proteins.

In situ hybridization

Embryos were dechorionated and fixed in fixation buffer (0.5x PBS, 25 mM EGTA, 4% formaldehyde and 50% Heptane) for 20 min at room temperature. Antisense RNA probes labeled with digoxigenin (DIG RNA Labeling Mix 10 × conc, Roche) and biotin (Biotin RNA Labeling Mix 10 × conc, Roche) were used to detect *lacZ* and *snail* RNAs, respectively. Hybridization was performed at 55 °C overnight in hybridization buffer (50% formamide, 5x SSC, 50 µg/ml Heparin, 100 µg/ml salmon sperm DNA, 0.1% Tween-20). Subsequently, embryos were washed with hybridization buffer at 55 °C and incubated with Western Blocking Buffer (Roche) at
room temperature for one hour. Then, embryos were incubated with sheep anti-digoxigenin (Roche) and mouse anti-biotin primary
antibodies (Invitrogen) at 4°C for overnight, followed by incubation with Alexa Fluor 488 donkey anti-sheep (Invitrogen) and Alexa
Flour 555 goat anti-mouse (Invitrogen) fluorescent secondary antibodies at room temperature for two hours. DNA was stained with
Hoechst 33342 (Thermo Fisher Scientific), and embryos were mounted in ProLong Gold Antifade Mountant (Thermo Fisher
Scientific). Imaging was performed on a Zeiss LSM 880 confocal microscope. Plan-Apochromat 20x/0.8 NA. objective was
used. Images were captured in 16 bit. Z-stack images were visualized with the Maximum intensity projection method.

**Dual-color MS2/PP7 live imaging**

MCP-GFP, mCherry-PCP, His2Av-eBFP2 virgins were mated with homozygous males carrying PP7-lacZ reporter genes. Resulting
trans-heterozygote virgins were collected and mated with homozygous males carrying MS2 reporter genes. The resulting embryos
were dechorinated and mounted between a semipermeable membrane (In Viro Systems & Services) and a coverslip (18 mm x
18 mm), and embedded in Halocarbon oil 27 (Sigma). Embryos were imaged using a Zeiss LSM 880 at room temperature. Plan-Apochromat 40x/1.3 NA. oil immersion objective was used. At each time point, a stack of 21 images separated by 0.5 μm was acquired and the final time resolution is 13 s. Images were captured in 16 bit. For each cross, more than three biological replicates were taken using the same setting of the microscope. The same laser power and microscope setting were consistently used in the same experiments.

**Single-color MS2 live imaging**

MCP-GFP, His2Av-mRFP virgins were mated with homozygous males carrying the MS2 reporter gene. Resulting trans-heterozygote
virgins were collected and mated with homozygous males carrying snail shadow enhancer. In Figure S1A and B, heterozygote MCP-
GFP, His2Av-mRFP virgins were mated with homozygous males carrying snail shadow enhancer-evePr-MS2-lacZ reporter gene. The resulting embryos were dechorinated and mounted between a semipermeable membrane (In Viro Systems & Services) and a coverslip (18 mm x 18 mm), and embedded in Halocarbon oil 27 (Sigma). Embryos were imaged using a Zeiss LSM 880 at room temperature. Plan-Apochromat 40x/1.3 NA. oil immersion objective was used. At each time point, a stack of 21 images separated by 0.5 μm was acquired and the final time resolution is 13 s. Images were captured in 16 bit. For each cross, three biological replicates were taken using the same setting of the microscope. The same laser power and microscope setting were used throughout this study.

**Plasmids**

**pbphi-snail shadow enhancer**

A DNA fragment containing snail shadow enhancer was amplified from the genomic DNA using primers (5′-GGG GGA AGC TTG CAT
TGA GTT TTG TTG T-3′) and (5′-CCC CCG GTA CTA AAA TTC CGA TTT TTG TTG T-3′), and digested with HindIII and NheI. The resulting fragment was inserted between the HindIII and NheI sites in pbphi-multi cloning site. Subsequently, a DNA fragment containing Burkholderia cenocepacia ParSc3 sequence (Saad et al., 2014) was inserted into the unique NdeI site of the plasmid. ParSc3 was not used in this study.

**pbphi-gypsy-snail shadow enhancer**

A DNA fragment containing gypsy insulator was amplified from the genomic DNA using primers (5′-GGG GGG CGG CCT GCC
CAC GTA ATG GTG CG-3′) and (5′-CCC CCT CTA GAA AGC TTG AGC TTC GCA TGG TTT TGC C-3′), and digested with NOTI and
BamHI. The resulting fragment was inserted between the NOTI and BglII sites in pbphi-snail shadow enhancer.

**pbphi-snail shadow enhancer-gypsy**

A DNA fragment containing gypsy insulator was amplified from the genomic DNA using primers (5′-GGG GGG CTA GCC TGG CCA
CGT AAT AAG TGT GCG-3′) and (5′-CCC CCT CTA GAA AGC TTG AGC TTC GCA TGG TTT TGC C-3′), and digested with NheI and
XbaI. The resulting fragment was inserted between the NheI and XbaI sites in pbphi-snail shadow enhancer. ParSc3 sequence is depleted from this plasmid.

**pbphi-lacZ-α-Tub 3′ UTR**

A DNA fragment containing α-Tubulin at 84B 3′ UTR was amplified from the genomic DNA using primers (5′-GGG GGC TCG AGG GT
CAC GCC ACT TCA ACG C-3′) and (5′-CCC CCT CTA GAA AGC TTG AGC TTC GCA TGG TTT TGC C-3′), and digested with XhoI and
XbaI. The resulting fragment was inserted between the XhoI and XbaI sites in pbphi-multi cloning site. Subsequently, a DNA fragment
containing lacZ was amplified using primers (5′-GGG GAT CTA TGC AGA ACT GGG AGA CGA C-3′) and (5′-CCC CCT CTC AGT TAT TTT TGA CAC CAG ACC A-3′), and digested with BglII and XhoI. The resulting fragment was inserted between the BglII and XhoI sites in the plasmid.

**pbphi-evePr-MS2-lacZ-α-Tub 3′ UTR**

A DNA fragment containing eve core promoter was amplified from the genomic DNA using primers (5′-GGG GGG CCG CCT GGC
CAC GTA ATG GTG CG-3′) and (5′-CCC CCG CCG CGT TGT TTG TGG TGA CAC CAC-3′), and digested with NOTI. The

**pbphi-gypsy-evePr-MS2-lacZ-α-Tub 3′ UTR**

A DNA fragment containing gypsy insulator was amplified from the genomic DNA using primers (5′-GGG GGG CCG CCT GGC
CAC GTA ATG GTG CG-3′) and (5′-CCC CCG CCG CGT TGT TTG TGG TGA CAC CAC-3′), and digested with NOTI. The

**pbphi-PP7-α-Tub 3′ UTR**

A DNA fragment containing α-Tubulin at 84B 3′ UTR was amplified from the genomic DNA using primers (5′-GGG GGC TCG AGG GT
CAC GCC ACT TCA ACG C-3′) and (5′-CCC CCT CTA GAA AGC TTG AGC TTC GCA TGG TTT TGC C-3′), and digested with XhoI and
XbaI. The resulting fragment was inserted between the XhoI and XbaI sites in pbphi-multi cloning site. Subsequently, a DNA fragment
containing lacZ was amplified using primers (5′-GGG GAT CTA TGC AGA ACT GGG AGA CGA C-3′) and (5′-CCC CCT CTC AGT TAT TTT TGA CAC CAG ACC A-3′), and digested with BglII and XhoI. The resulting fragment was inserted between the BglII and XhoI sites in the plasmid.

**pbphi-evePr-MS2-lacZ-α-Tub 3′ UTR**

A DNA fragment containing eve core promoter was amplified from the genomic DNA using primers (5′-GGG GGG CCG CCT GGC
CAC GTA ATG GTG CG-3′) and (5′-CCC CCG CCG CGT TGT TTG TGG TGA CAC CAC-3′), and digested with NOTI. The

**pbphi-PP7-α-Tub 3′ UTR**

A DNA fragment containing α-Tubulin at 84B 3′ UTR was amplified from the genomic DNA using primers (5′-GGG GGC TCG AGG GT
CAC GCC ACT TCA ACG C-3′) and (5′-CCC CCT CTA GAA AGC TTG AGC TTC GCA TGG TTT TGC C-3′), and digested with XhoI and
XbaI. The resulting fragment was inserted between the XhoI and XbaI sites in pbphi-multi cloning site. Subsequently, a DNA fragment
containing lacZ was amplified using primers (5′-GGG GAT CTA TGC AGA ACT GGG AGA CGA C-3′) and (5′-CCC CCT CTC AGT TAT TTT TGA CAC CAG ACC A-3′), and digested with BglII and XhoI. The resulting fragment was inserted between the BglII and XhoI sites in the plasmid.
resulting fragment was inserted into the unique NOTI site in the pbphi-evePr-MS2-lacZ-αTub 3′UTR. By sequencing resulting plasmids, orientation of gypsy insulator was determined.

**pbphi-gypsy-snaill shadow enhancer-evePr-MS2-lacZ-αTub 3′UTR**

A DNA fragment containing gypsy insulator and snail shadow enhancer was purified from pbphi-gypsy-snail shadow enhancer by digesting with NOTI and NheI. The resulting fragment was inserted between the NOTI and Spel sites in the pbphi-evePr-MS2-lacZ-αTub 3′UTR.

**pbphi-snail shadow enhancer-evePr-MS2-lacZ-αTub 3′UTR**

A DNA fragment containing snail shadow enhancer was purified from pbphi-snail shadow enhancer by digesting with NOTI and NheI. The resulting fragment was inserted between the NOTI and Spel sites in the pbphi-evePr-MS2-lacZ-αTub 3′UTR.

**pbphi-evePr-PP7-lacZ-αTub 3′UTR**

A DNA fragment containing eve core promoter was amplified from the genomic DNA using primers (5′-GGG GCC GCG CCG CGC TAC TTA AAA GTT TTT TCT CTA ACT TTA CTC ACC TGC TTC TGC AAC-3′) and (5′-CCC CCA TTA GAT ACG TCG TGT GGT TTG TTA CAC GCA CCA C-3′), and digested with NheI and BglII. The resulting fragment was inserted into the unique BglII site in the plasmid.

**pbphi-gypsy-snaill shadow enhancer-evePr-PP7-lacZ-αTub 3′UTR**

A DNA fragment containing gypsy insulator and snail shadow enhancer was purified from pbphi-gypsy-snail shadow enhancer by digesting with NOTI and NheI. The resulting fragment was inserted between the NOTI and NheI sites in the pbphi-evePr-PP7-lacZ-αTub 3′UTR.

**pbphi-Homie-snaill shadow enhancer**

A DNA fragment containing Homie insulator was amplified from the genomic DNA using primers (5′-GGG GCC GCG CCG CAA TAC TAA AAA GTT TTT TCT CTA ACT TTA CTC ACC TGC TTC TGC AAC-3′) and (5′-CCC CCA TTA GAT ACG TCG TGT GGT TTG TTA CAC GCA CCA C-3′), and digested with NOTI and BglII. The resulting fragment was inserted between the NOTI and BglII sites in the plasmid.

**pbphi-Homie-evePr-MS2-lacZ-αTub 3′UTR**

A DNA fragment containing eve core promoter was amplified from the genomic DNA using primers (5′-GGG GCC GCG CCG CAA TAC TAA AAA GTT TTT TCT CTA ACT TTA CTC ACC TGC TTC TGC AAC-3′) and (5′-CCC CCA TTA GAT ACG TCG TGT GGT TTG TTA CAC GCA CCA C-3′), and digested with NheI and BglII. The resulting fragment was inserted into the unique BglII site in the plasmid.

**pbphi-tmr-snaill shadow enhancer**

A DNA fragment containing Abd-B transvection mediating region was amplified from the genomic DNA using primers (5′-GGG GCC GCG CCG CCG GTA ATT CGT CTG GCT ACC TGC CC-3′) and (5′-CCC CCA GAT CTC GTG TGG AAT TCT GTT CTG TCA CAA-3′), and digested with NotI and XhoI. The resulting fragment was inserted between the NotI and XhoI sites of the plasmid.

**pbphi-tmr-evePr-MS2-lacZ-αTub 3′UTR**

A DNA fragment containing Abd-B transvection mediating region was amplified from the genomic DNA using primers (5′-GGG GCC GCG CCG CCG GTA ATT CGT CTG GCT ACC TGC CC-3′) and (5′-CCC CCA GAT CTC GTG TGG AAT TCT GTT CTG TCA CAA-3′), and digested with NOTI and BglII. The resulting fragment was inserted between the NOTI and BglII sites in the plasmid.

**pbphi-nanos > MCP-GFP**

A DNA fragment containing MCP was amplified from pUC18-Phos-MCP-GFP-αTub 3′UTR plasmid (Garcia et al., 2013) using primers (5′-GGG GCC AGA TGA TGG CTT GTA ACT TTA CTC A-3′) and (5′-CCC CCA AGC TTG GCG GAT CAG TGG TGT GCT G-3′), digested with XhoI and HindIII. The resulting fragment was inserted into the unique XhoI and HindIII sites in pbphi-nanos promoter-αTubulin 3′UTR expression vector (Fukaya et al., 2016). Subsequently, a DNA fragment containing GFP was amplified using primers (5′-GGG GCC GTA AGC TGA TGG CTT GTA ACT TTA CTC A-3′) and (5′-CCC CCA AGC TTG GCG GAT CAG TGG TGT GCT G-3′), digested with NheI and BamHI. The resulting fragment was inserted between the NheI and BamHI sites in the plasmid.

**pbphi-His2Av-mRFP**

A DNA fragment containing mRFP was amplified using primers (5′-GGG GCC AGA TGA TGG CTT GTA ACT TTA CTC A-3′) and (5′-CCC CCA AGC TTG GCG GAT CAG TGG TGT GCT G-3′), digested with HindIII and NheI. The resulting fragment was inserted between the HindIII and NheI sites in the pbphi-His2Av (Fukaya et al., 2017).
**pbphi-nanos > MCP-GFP, His2Av-mRFP**

A DNA fragment containing His2Av-mRFP was purified from pbphi-His2Av-mRFP by digesting with Xbal. The resulting fragment was inserted into the unique XbaI site in the pbphi-nanos > MCP-GFP.

**pCFD3-dU6-ftz gRNA**

Two DNA oligos (5'-GTC GAG TTA CTC TCT TCC CCA GAG-3') and (5'-AAA CCT CTG GGG AAG AGA GTA ACT-3') were annealed and inserted into the pCFD3-dU6:3gRNA vector (addgene # 49410) using BbsI sites.

**pBS-loxP**

Two DNA oligos (5'-CCA AGG ATC CAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT ATC ATA TGC GAC TAG TGG GAT-3') and (5'-GGC CAC TAG TCG CAT AGT ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TGG ATC CTT GGG-3') were annealed and inserted into the pBlueScript vector using BamHI and SpeI restriction sites.

**pBS-MS2-loxP-dsRed-loxP**

A DNA fragment containing 24x MS2 stem loops was purified from pbBlueScript-24x MS2SL-stable (Bertrand et al., 1998) by digesting with BamHI and BglII. The resulting fragment was inserted into the unique BamHI site in pBS-loxP plasmid. Subsequently, a DNA fragment containing dsRed and loxP site was amplified from pHD-DsRed-attP (addgene #51019) using primers (5'-GGG GGC ATA TGG GAT CTA ATT CAA TTA GAG A-3') and (5'-TCT TTA CTA GTA CCG GTT AAG ATA CAT TGA TGA G-3'), and digested with NdeI and Spel. The resulting fragment was inserted between the NdeI and Spel sites of the plasmid.

**pBS-PP7-loxP-dsRed-loxP**

A DNA fragment containing 24x PP7 stem loops was purified from pbBlueScript-24x PP7 (Fukaya et al., 2016) by digesting with BamHI and BglII. The resulting fragment was inserted into the unique BamHI site in pBS-loxP plasmid. Subsequently, a DNA fragment containing dsRed and loxP site was amplified from pHD-DsRed-attP (addgene #51019) using primers (5'-GGG GGC ATA TGG GAT CTA ATT CAA TTA GAG A-3') and (5'-TCT TTA CTA GTA CCG GTT AAG ATA CAT TGA TGA G-3'), and digested with NdeI and Spel. The resulting fragment was inserted between the NdeI and Spel sites of the plasmid.

**pBS-ftz5'-MS2-loxP-dsRed-loxP-ftz3'**

A DNA fragment containing 24x MS2 stem loops was purified from pbBlueScript-24x MS2SL-stable (Bertrand et al., 1998) by digesting with BamHI and SpeI restriction sites. A DNA fragment containing dsRed and loxP site was amplified from pHD-DsRed-attP (addgene #51019) using primers (5'-GGG GGC ATA TGG GAT CTA ATT CAA TTA GAG A-3') and (5'-TCT TTA CTA GTA CCG GTT AAG ATA CAT TGA TGA G-3'), and digested with NdeI and Spel. The resulting fragment was inserted between the NdeI and Spel sites of the plasmid.

**pBS-ftz5'-PP7-loxP-dsRed-loxP-ftz3'**

A DNA fragment containing 24x PP7 stem loops was purified from pbBlueScript-24x PP7 (Fukaya et al., 2016) by digesting with BamHI and BglII. The resulting fragment was inserted into the unique BamHI site in pBS-loxP plasmid. Subsequently, a DNA fragment containing dsRed and loxP site was amplified from pHD-DsRed-attP (addgene #51019) using primers (5'-GGG GGC ATA TGG GAT CTA ATT CAA TTA GAG A-3') and (5'-TCT TTA CTA GTA CCG GTT AAG ATA CAT TGA TGA G-3'), and digested with NdeI and Spel. The resulting fragment was inserted between the NdeI and Spel sites of the plasmid.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All the image processing methods and analysis were implemented in MATLAB (R2016b, MathWorks).

**Nuclei segmentation and tracking**

For each time point, maximum projections were obtained for all 21 z sections per image. His2Av-mRFP and His2Av-eBFP2 were used to segment nuclei for single-color imaging and dual-color imaging, respectively. Nuclei-labeled channel images were pre-processed with Gaussian filtering, top-hap filtering, and adaptive histogram equalization, in order to enhance the signal-to-noise contrast. Processed images were converted into binary images using a threshold value obtained from Otsu’s method. The number and the position of separate components within a frame was obtained, where each component serves as a mask for individual nuclei. Since nuclei hardly move past 5 min into nc 14, nuclei tracking was obtained by finding the component with minimal movement across these frames (5 min into nc 14 to the onset of gastrulation).

**Recording MS2 and PP7 signals**

Maximum projections of raw images were used to record fluorescent intensities. Using nuclei segmentation as a mask for each nucleus, fluorescence intensities within each nucleus were extracted. After subtracting the background nuclear signal, the signal of MS2 and PP7 transcription foci was determined by taking an average of the top three pixels with the highest fluorescence intensity within each nucleus.
3D tracking of MS2 and PP7 alleles

The entire 21 z stack images were used to track the three-dimensional position of the active MS2 and PP7 transcription foci within a nucleus. For each nucleus, x, y, and z coordinates of the pixel with the maximum MS2 or PP7 signal was determined respectively using raw image files. The MS2 and PP7 foci coordinates were then transformed with respect to the center of each corresponding nucleus. The resulting x, y, and z resolution is 0.277 μm, 0.277 μm, and 0.5 μm per pixel, respectively. The distance between MS2 and PP7 alleles was measured only when the MS2-lacZ and PP7-lacZ are both transcriptionally active. MS2 and PP7 alleles were considered to be paired when the average distance between the two alleles was less than 1 μm (Figures 3H, S3E, and S6B). In Figures 3, S3F, and S6B, pairing duration was determined by measuring the time during which the allelic distance was less than 1.5 μm.

Analysis of coordinated transcription

For each of the MS2 and PP7 trajectories from a given nucleus, the Pearson correlation coefficient was calculated. In the transvection assay, the correlation coefficients within the time window between the first and the last time point of active MS2 transcription were measured. When MS2 and PP7 reporter genes were individually placed under the control of separate enhancers, the correlation coefficients were measured using the MS2 and PP7 trajectories during the period of 5 min into nc 14 to the onset of gastrulation. To compare the histograms between two datasets, the distribution of correlation coefficients was determined by normalizing to the number of total analyzed nuclei. In Figures 5E and S7C, the statistical significance of the difference between two distributions of correlation coefficients was examined using a two-sided Mann-Whitney-U test.

Measurement of cumulative fraction of active nuclei

The time of activation was determined as the first time frame wherein MS2 fluorescence intensity exceeded a threshold value. To determine the threshold value, maximum fluorescence intensity throughout the entirety of nc 14 was measured for each embryo. Subsequently, 15% of the maximum fluorescence intensity was calculated and averaged among replicates. This value was used as the threshold for each genotype. Nuclei were considered as active if MS2 signal exceeded the threshold for longer than 2 min. The presumptive mesoderm region was defined as a domain containing ~17-18 cell widths centered to the ventral most nuclei, which corresponds to endogenous sna expression domain. The very first cells that undergo gastrulation were determined as the ventral most nuclei. The same measurement methods were used in Figures 1, 2, 6, S1, and S2. In all these figures, error bars represent the standard error of the mean.

False coloring of active nuclei

Active nuclei were determined by possessing MS2 signal above a threshold value as described in the previous section. The set of nuclei that were active at any point during the ~50 min period of nc 14 was then identified per embryo. Using their segmentation mask, these active nuclei were then colored and layered over the raw His2Av-eBFP2 image at the time point of ~30 min into nc 14.

Characterization of transcriptional bursting

The amplitude was measured by taking the peaks of each transcriptional burst after smoothing with the local regression (LOESS) method. A transcriptional burst was defined as a change in fluorescence intensity where the local dynamic range (i.e., distance between peak and trough) was at least twice the dynamic range between the global and local minima (i.e., distance between baseline and trough). When a nucleus had above-baseline transcriptional activity at the onset (5 min into nc14) or end (onset of gastrulation) of the analysis, that burst was excluded, due to uncertainty in the detection of its associated amplitude. A two-sided Mann-Whitney-U test was performed to determine the statistical significance of the difference between two distributions of burst amplitude shown in Figure S7D.

DATA AND SOFTWARE AVAILABILITY

Original imaging data of in situ hybridization assay has been deposited to Mendeley Data (https://doi.org/10.17632/3ff644zs6s.1).