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# A chromatin insulator mediates transgene homing and very long-range enhancer-promoter communication

**Miki Fujioka, Xian Wu and James B. Jaynes***

Insulator sequences help to organize the genome into discrete functional regions by preventing inappropriate cross-regulation. This is thought to be mediated in part through associations with other insulators located elsewhere in the genome. Enhancers that normally drive *Drosophila even skipped* (*eve*) expression are located closer to the *TER94* transcription start site than to that of *eve*. We discovered that the region between these genes has enhancer-blocking activity, and that this insulator region also mediates homing of P-element transgenes to the *eve-TER94* genomic neighborhood. Localization of these activities to within 0.6 kb failed to separate them. Importantly, homed transgenic promoters respond to endogenous *eve* enhancers from great distances, and this long-range communication depends on the homing/insulator region, which we call Homie. We also find that the *eve* promoter contributes to long-distance communication. However, even the basal *hsp70* promoter can communicate with *eve* enhancers across distances of several megabases, when the communication is mediated by Homie. These studies show that, while Homie blocks enhancer-promoter communication at short range, it facilitates long-range communication between distant genomic regions, possibly by organizing a large chromosomal loop between endogenous and transgenic Homies.

**KEY WORDS: Insulator, Homing, Enhancer blocking, Epigenetics, Even skipped, *Drosophila***

## INTRODUCTION

Sequences that help to organize chromatin into functional domains can have a profound influence on gene regulation. Enhancers are capable of activating transcription across tens or hundreds of kilobases (kb) along a chromosome. Paradoxically, gene-rich genomic regions contain many genes within that distance, without known functional crosstalk. Insulator sequences have been identified that can prevent inappropriate enhancer-promoter (E-P) communication, helping to resolve this apparent paradox (Bushey et al., 2008; Dorman et al., 2007; Gaszner and Felsenfeld, 2006; Valenzuela and Kamakaka, 2006). Insulators are typically found between genes, or within complex loci such as the bithorax complex (BX-C) (Maeda and Karch, 2006; Maeda and Karch, 2007), where they act in combination with other sequences to orchestrate complex regulatory programs during development.

Underlying mechanisms appear to involve the formation of loops, possibly organizing chromatin into functionally isolated domains (Bushey et al., 2008; Dorman et al., 2007; Gaszner and Felsenfeld, 2006; Valenzuela and Kamakaka, 2006). The *scs* and *scs′* insulators (Udvardy et al., 1985) are each bound by distinct protein complexes (Gaszner et al., 1999; Hart et al., 1997; Zhao et al., 1995) that interact with each other, resulting in a chromosomal loop that encompasses the 87A7 *hsp70* genes (Blanton et al., 2003). The *gypsy* transposon exhibits enhancer-blocking activity (Geyer and Corces, 1992; Geyer et al., 1988; Modolell et al., 1983; Peifer and Bender, 1988) that requires the Suppressor of Hairy wing protein (Parnell et al., 2006; Ramos et al., 2006; Spana et al., 1988), as well as CP190 and Mod(mdg)4, which form a complex (Gause et al., 2001; Ghosh et al., 2001; Pai et al., 2004). Vertebrate insulators often bind the CTCF protein (Bell and Felsenfeld, 2000; Bell et al., 1999; Hark et al., 2000; Kanduri et al., 2000), and CTCF interacts with cohesins (Parelho et al., 2008; Wendt et al., 2008), an interaction that correlates with its enhancer-blocking activity.

Insulators can cooperate with other regulatory sequences. In the BX-C, gene activities in early embryos are differentially regulated, and these patterns of gene activity are maintained through Polycomb- and Trithorax-response elements (PREs and TREs, respectively) (Maeda and Karch, 2006). The regulatory regions *Mcp* (Busturia et al., 1997; Karch et al., 1994; Muller et al., 1999), *Fab-7* (Gyurkovics et al., 1990; Karch et al., 1994; Mihaly et al., 1997) and *Fab-8* (Barges et al., 2000; Zhou et al., 1999) each contain closely linked PRE/TREs and insulators. CTCF binds to *Mcp* and *Fab-8* (Holohan et al., 2007), whereas *Fab-7* binds other factors (Aoki et al., 2008; Schweinsberg and Schedl, 2004). CTCF can facilitate repressive interactions between an insulator and a promoter that involve Polycomb group (PcG) complexes (Li et al., 2008). In addition, promoter targeting sequences can overcome insulator activity to maintain an active state (Zhou and Levine, 1999), and promoter specificity sequences, such as the promoter-tethering element in *Abdominal-B* (Akbari et al., 2008), facilitate specific E-P interactions. Loop formation by insulators (Cleard et al., 2006) and PRE/TREs (Lanzuolo et al., 2007) may be an essential component of maintaining proper gene expression through development. The DNA-binding GAGA factor, which binds to many PRE/TREs, can also contribute to enhancer blocking (Belozerov et al., 2003; Ohtsuki and Levine, 1998; Schweinsberg et al., 2004; Schweinsberg and Schedl, 2004). Loop attachment sites may block the propagation along the DNA of chromatin modifications or protein complexes that enhance transcription (Bushey et al., 2008; Dorman et al., 2007; Gaszner and Felsenfeld, 2006; Valenzuela and Kamakaka, 2006). Much remains to be discovered concerning how chromosomal architecture affects gene expression, and how regulatory elements that control this architecture carry out their functions.

In *Drosophila*, it has been observed that some sequences cause transgenes to insert non-randomly in the genome, near the site of origin of those sequences. This transgene homing has been observed for regions of *engrailed* (Hama et al., 1990; Kassis, 2002; Kassis et

Department of Biochemistry and Molecular Biology and the Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA.

*Author for correspondence (jaynes@jci.tju.edu)

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al., 1992), *linotte* (Taillebourg and Dura, 1999), also known as *derailed*, and the BX-C (Bender and Hudson, 2000). The BX-C homing element may contain an insulator that separates two enhancer regions (Bender and Hudson, 2000), whereas for *engrailed*, the homing element is associated with a PRE (Kwon et al., 2009). Homed reporter transgenes have been seen to communicate with enhancers from the endogenous locus across several other genes (Devido et al., 2008; Hama et al., 1990; Kassis et al., 1992; Kwon et al., 2009).

The *Drosophila even skipped* (*eve*) locus has been particularly well characterized (Fujioka et al., 1999; Goto et al., 1989; Harding et al., 1989; Sackerson et al., 1999; Small et al., 1992; Small et al., 1996), including the identification of a PRE at its 3′ end (Fujioka et al., 2008; Oktaba et al., 2008). The 3′-adjacent gene, *TER94* (Leon and McKearin, 1999; Pinter et al., 1998; Ruden et al., 2000), is expressed in the syncytial blastoderm and, by embryonic stage 11, throughout the central nervous system (CNS; this study and Berkeley *Drosophila* Genome Project) (Tomancak et al., 2002). Several of the *eve* enhancers are close to *TER94*, yet *TER94* is not expressed in an *eve* pattern (or vice versa). We found that the region between these genes has enhancer-blocking activity. The same region also mediates transgene homing, and homed transgenes communicate with the endogenous *eve* enhancers. Long-range E-P communication occurs from as far away as 3300 kb. This E-P communication requires the insulator/homing region in the transgene, but not the PRE. Thus, regulatory interactions between enhancers and promoters can occur between linearly distant genomic regions, and such interactions can be mediated by sequences with insulator properties that also mediate transgene homing.

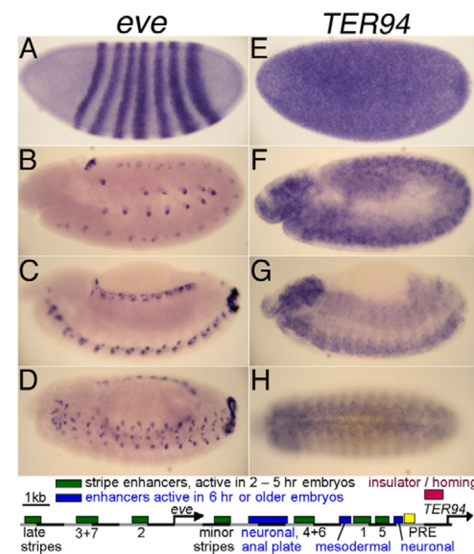


**Fig. 1. The *eve* and *TER94* expression patterns are distinct.** Staged *Drosophila* embryos were subjected to in situ hybridization against *eve* (**A-D**) or *TER94* (**E-H**). Stage 4 (A,E) and stage 11 (B,F) embryos, and lateral (C,G) and ventral (D,H) views of stage 13 embryos. Bottom panel: map of the *eve* locus and 3′ border region. The start sites and direction of transcription of *eve* and *TER94* are shown as arrows. Green and blue boxes, *eve* enhancers; yellow box, a PRE (Fujioka et al., 2008); red box, the core insulator/homing element.

## MATERIALS AND METHODS

### Plasmid construction and transgenic analysis

All sequence coordinates in this study are relative to the transcription start site of *eve* (+1) (Frasch et al., 1988), unless otherwise stated. Details of eZ, hZ, eZ46-15W and eZAR-MeW constructs, and derivatives of pCfhL, are available on request. For the ΦC31-RMCE (Bateman et al., 2006) attP target plasmid, two attP sequences derived from pUAST-P2 (Bateman et al., 2006) were inserted (in opposite orientations) into eZRR11K, flanking *eve-lacZ* and *mini-white*. To replace transgenic attP-flanked targets, various donor regions (described in the Results section) were cloned into attBΔ2 (Fujioka et al., 2008), a modified piB-GFP plasmid (Bateman et al., 2006). RMCE events were identified by loss of *mini-white*-dependent eye color, and confirmed by PCR.

P-element insertion sites were identified by inverse PCR (Ochman et al., 1988; Huang et al., 2000) and homed sites were confirmed using one PCR primer from the flanking genomic sequence and one from within the P-element. Transgenesis (Fujioka et al., 2000; Rubin and Spradling, 1982), in situ hybridization and antibody staining (Fujioka et al., 1999) were performed as previously described.

## RESULTS

### Enhancer blocking by the *eve-TER94* border region

Although some *eve* enhancers are closer to the *TER94* promoter than to the *eve* promoter, the expression patterns of the two genes are distinctive (Fig. 1). *TER94* is expressed ubiquitously in embryos, whereas *eve* is expressed in a discrete pattern in several tissues. Based on this, we tested whether the border region acts as an enhancer-blocking insulator. We began by examining the region from +8.4 to +10.5 kb (SR105) in three enhancer-blocking assay constructs. One of these constructs (eZ46-15W) contains *eve* early-stripe enhancers (for stripes 4+6, 1 and 5), an *eve*-promoter-*lacZ* reporter gene (eZ), and the *mini-white* gene (W), with the two promoters divergently transcribed. The stripe 4+6 enhancer is proximal to the *eve*-promoter-*lacZ* reporter, and the stripe 1 and 5 enhancers are proximal to *mini-white*. Transgenes carrying this construct showed expression of both reporter genes in all four stripes, even when a 2 kb stretch of phage λ DNA was inserted between the two stripe enhancers (Fig. 2A,B). By contrast, when SR105 was placed between the enhancers, *lacZ* was expressed strongly in stripes 4 and 6, but only very weakly (five out of seven lines) or not at all (two out of seven lines) in stripes 1 and 5, whereas *mini-white* was expressed in the complementary pattern (Fig. 2C,D), showing that SR105 has enhancer-blocking activity. Some variation in blocking activity with the site of insertion is expected based on studies with other insulators (Belozerov et al., 2003; Majumder and Cai, 2003).

Next, we tested whether this enhancer-blocking region works with heterologous elements, using a standard vector (pCfhL). This vector consists of the *fushi tarazu (ftz)* neuronal enhancer proximal to a heat shock promoter-*lacZ* reporter gene, and the *ftz* 7-stripe element proximal to *mini-white* (Hagstrom et al., 1996). When either SR105 (three lines) or a smaller element of 1.3 kb (R105, from +9.2 to +10.5 kb; eight lines) was inserted between the two enhancers, each activated the proximal reporter gene much more strongly than the distal one (Fig. 2E-H). Again, we observed some minor variation in the strength of enhancer blocking with the site of insertion (not shown), but in each case, the element specifically reduced expression driven by the distal enhancer. Thus, this enhancer-blocking insulator can function with heterologous E-P combinations.

Finally, we tested enhancer-blocking activity at later embryonic stages using a construct (eZAR-MeW) with the *eve* anal plate ring (AR) enhancer proximal to the same *eve*-promoter-*lacZ* reporter (eZ) described above for eZ46-15W, and the *eve* mesodermal enhancer proximal to *mini-white*. When SR105 was placed between these two enhancers, *lacZ* was expressed in the AR but not the

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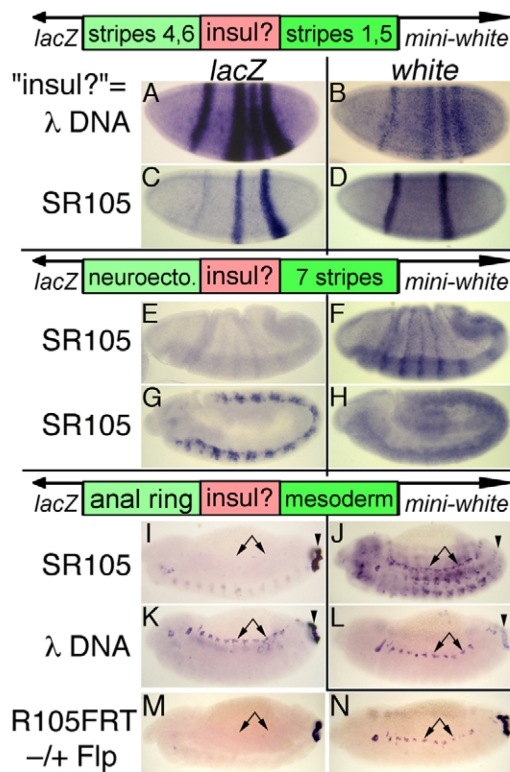


**Fig. 2. The region between *eve* and *TER94* has enhancer-blocking activity.** Top diagram: enhancer-blocking test vector (eZ46-15W) for **A-D**. The tested sequence (red box marked 'insul?') is flanked by two distinct enhancers (green boxes), upstream of divergently transcribed reporter genes (*lacZ* and *mini-white*). Middle (pCfhL) and lower (eZAR-MeW) diagrams, test vectors for **E-H** and **I-N**, respectively, have the same design, with different enhancers. The 'insul?' test sequence is shown on the left for each row: either λ phage DNA (negative control), SR105 or R105 (the latter flanked by FRT sites). SR105 and R105 were in the same orientation relative to the direction of transcription of *mini-white* as they normally are relative to that of *eve* (and *TER94*). Transgenic embryos carrying these constructs were subjected to in situ hybridization against *lacZ* or *white*. (A-D) Stage 5 embryos carrying eZ46-15W. (E-H) Stage 6 (E,F) or stage 11 (G,H) embryos carrying pCfhL. (I-N) Stage 13 embryos carrying eZAR-MeW. Arrows and arrowheads indicate positions of mesodermal and AR expression, respectively. The lateral ectopic expression in J is not relevant for this assay, and is possibly due to a position effect. With R105 flanked by FRT sites as 'insul?', *lacZ* was driven only by the proximal AR enhancer (M). When R105 was removed by FLP-mediated recombination in vivo, activation by the distal mesodermal enhancer is seen (N, arrows).

mesoderm (Fig. 2I), whereas *mini-white* was expressed in the mesoderm but not the AR (Fig. 2J). By contrast, when λ DNA was inserted between the two enhancers, both reporters were expressed in both patterns (Fig. 2K,L). Thus, the directional enhancer-blocking activity of SR105 is also functional at later stages of embryogenesis.

## Enhancer blocking depends on the continued presence of the element

When either SR105 (data not shown) or the smaller R105 sequence (Fig. 2M) was flanked by FLP recombination target (FRT) sites (Golic and Lindquist, 1989) in the eZAR-MeW construct, enhancer-blocking activity was the same as for SR105. After subsequent removal of the element through FLP-mediated recombination, *lacZ* expression was observed in both tissues (Fig. 2N). This rules out the possibility that the apparent enhancer blocking is due to transgenes targeted to genomic sites that inactivate one reporter gene but not the other.

## Enhancer blocking conferred by a 600 bp element

We dissected the insulator first of all by deleting from each end. The region from +9.2 to +10.0 kb (Fig. 3, R100) retained activity. Then, internal deletions of ~100 bp were made (Fig. 3). We saw three distinct levels of activity. Whereas some regions blocked E-P communication completely in both directions, others blocked the interaction between the AR enhancer and *mini-white* completely, while only partially blocking *lacZ* expression in the mesoderm. This partial blocking could be further quantified based on the developmental stage at which mesodermal β-galactosidase (β-gal) accumulated to detectable levels. Strong blocking caused a delay until embryonic stages 13-14, whereas weak blocking allowed an earlier appearance of mesodermal β-gal (at stages 11-12). Furthermore, there was some variation in activity with the site of insertion, as detailed in Fig. 3.

Deletion of subregions A-E (see Fig. 3) individually caused little or no reduction in activity. By contrast, deletion of all five subregions together strongly reduced activity (ΔAE, Fig. 3). Deletion of region F (ΔF) resulted in only weak activity in most lines. Nonetheless, two non-overlapping regions, one containing region F (ΔAE) and the other not (ΔFH), both showed partial activity (Fig. 3). Thus, multiple small regions contribute to activity.

R100 contains the *TER94* start site and, with some other insulators, a region near a transcription start site is needed for enhancer blocking (Avramova and Tikhonov, 1999; Kellum and Schedl, 1992; Kuhn et al., 2004). Here, subregion G contains the start site, and although it might contribute to enhancer blocking, it is not required (ΔGH, Fig. 3). ΔGH ends 45 bp upstream of the *TER94* start site, yet retains strong activity. Thus, although it seems likely that the functional region overlaps with *TER94* regulatory sequences, transcription initiation within the insulator is apparently not required for enhancer blocking. This conclusion is reinforced by the fact that subregion A-F (ΔGH), which does not include the *TER94* start site, retains considerably more activity than subregion F-H (ΔAE), which spans the start site, and includes more than 100 bp of upstream sequence.

## The 3′ end of the *eve* locus contains a homing element; homed transgenes engage in long-range enhancer-promoter communication

While investigating activities in the border region between *eve* and *TER94*, we created a transgene carrying sequences from +7.9 to +11.3 kb driving the *eve*-promoter-*lacZ* reporter (RR11K, Fig. 3). This region contains a single *eve* enhancer (Fujioka et al., 1999), the *eve* 3′ PRE (Fujioka et al., 2008) and the 5′ portion of *TER94* (FlyBase) (Tweedie et al., 2009). Even though this construct contains only one *eve* enhancer, which is active only in the RP2 and a/pCC cells of the CNS, many lines carrying it nonetheless showed β-gal expression in a full *eve* pattern (Fig. 4A-D). Inverse PCR revealed that these transgenes had inserted in the chromosomal neighborhood of the *eve* locus (within 130 kb of *eve*, green arrowheads in Fig. 5A). We see this phenomenon only when transgenes are inserted in the *eve* neighborhood. Thus, the transgenic promoter is communicating with endogenous *eve* enhancers over large distances, across a number of other genes (Fig. 5A). The *eve* locus has been analyzed for enhancer activities in our lab using hundreds of transgenic lines with the same reporter (Fig. 4, middle

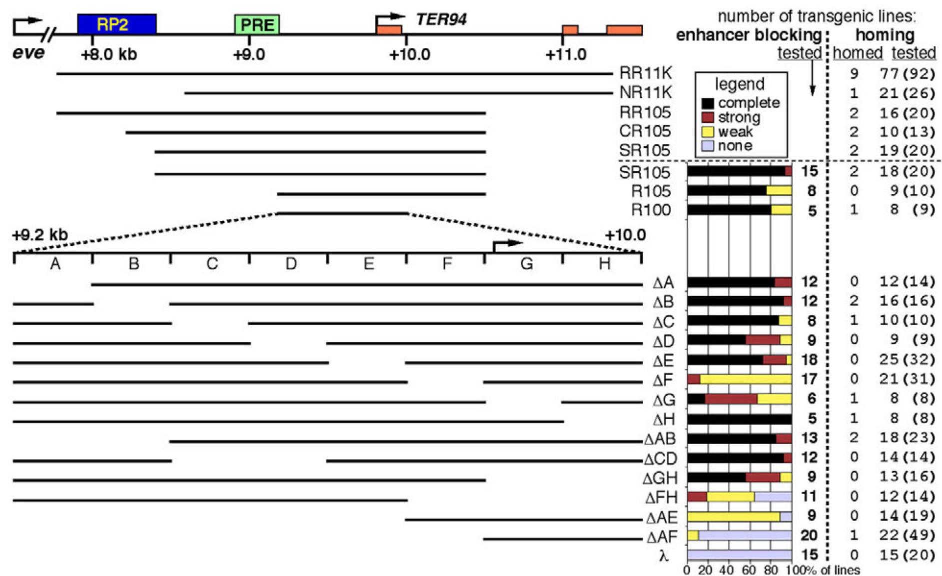

**Fig. 3. Enhancer-blocking and homing activity conferred by the *eve-TER94* border region.** Diagrams on the left represent the *eve-TER94* border region (top), and subregions (line diagrams below) tested for enhancer-blocking and homing activity. Distances (in kb) are relative to the *eve* transcription start site. Green box, a PRE; orange boxes, *TER94* exons; blue box, RP2 enhancer. R100 was subdivided into eight ~100 bp regions (A-H), and these were deleted individually or in combination, as indicated. Columns on the right list the number of independent transgenic insertion lines tested for either enhancer-blocking activity or homing, as well as the number of lines found to be homed. Enhancer blocking: below the dotted line, the region diagrammed on the left was present in an eZAR-MeW transgene, analyzed as shown in Fig. 2I-N. Colored bars indicate the percentage of lines showing enhancer blocking, with either complete, strong, or weak activity (see key). Those with 'complete' activity showed full enhancer blocking in both directions, i.e. neither *lacZ* nor *mini-white* expression driven by the distal enhancer was detected. Those lines in which *lacZ* expression driven by the distal (mesodermal) enhancer led to detectable *lacZ* levels only at later stages (embryonic stages 13-14) and showed no *mini-white* expression driven by the distal (AR) enhancer were considered to have 'strong' activity. Those in which mesodermal *lacZ* accumulated to detectable levels by stage 11-12 but showed no AR *mini-white* expression were counted as having 'weak' activity. Those that showed both early mesodermal *lacZ* and AR *mini-white* expression were considered to have no activity ('none'). In four of the SR105 lines (out of 15), the element was between the *eve*-promoter-*lacZ* reporter and the two enhancers. Each of these lines showed no *lacZ* expression, which was counted as complete enhancer blocking (*mini-white* was driven by both enhancers, as expected). Homing: under 'homed' is the number of independent insertions carrying the region on the left that were within 200 kb of *eve*. Under 'tested' is the number of lines stained for β-gal expression; the total number of lines established is in parentheses. Above the dotted line, the region diagrammed on the left was present upstream of an *eve*-promoter-*lacZ* reporter in a P-element transgene, except for 11 of the 19 SR105 lines, which carried the *hsp70-lacZ* reporter (one of each was homed). Most of the homed lines were discovered to be homed because they expressed β-gal in an *eve* pattern. The locations of these and of a subset of the 'non-homed' insertions were determined by inverse PCR and sequencing. Overall, 57 eZ, 7 *hsp70-lacZ* and 77 eZAR-MeW insertions that were found by genetic mapping to be on the second chromosome were localized in this way (listed in Table S1 in the supplementary material). As not all of the second chromosome lines were localized by inverse PCR, the number of homed lines might be an underestimate, i.e. there might be additional homed lines among those that do not express β-gal in an *eve*-like pattern. In most cases, untested lines were not stained because they were not healthy. In other cases, a subset of healthy lines was chosen at random for analysis. Details of insulator/homing regions in eZ constructs: in most cases, the 5′ end of the region (i.e. the end that is normally closer to *eve*) was oriented towards the *eve-lacZ* reporter; exceptions are noted below. RR11K lines (which contain eZ constructs) include both attP lines (4 out of 49 established lines found to be homed) and non-attP lines (5/43 homed). The non-attP RR11K lines include: the wild-type element in the orientation described above (3/10 homed); the wild-type element in the opposite orientation (0/5 homed); and modified elements with sequence alterations within the PRE, which is not required for either enhancer blocking or homing (Fujioka et al., 2008) (2/28 homed). NR11K includes lines with the region in the normal orientation (0/9 homed) and lines with the inverted orientation (1/17 homed). RR105 include a homed transgene with an eGFP reporter, which expresses GFP in an *eve*-like pattern (1/10 homed).

diagram), but we have never before observed this phenomenon. This suggested that both homing activity and long-range E-P communication were conferred by the region from +7.9 to +11.3 kb, which also contains the insulator.

## The homing/insulator element can mediate strong E-P communication from over 170 kb away

To further investigate long-range E-P communication, *lacZ* reporter-carrying transgenes without *eve* stripe, mesodermal or AR enhancers were used (listed above the dashed line in Fig. 3). Out of 171 lines obtained with these constructs, 143 were examined for an *eve*-like pattern throughout embryogenesis, and 13 showed such a pattern (Fig. 4A-D). All 13 were localized by inverse PCR to between ~140 kb upstream and 180 kb downstream of *eve* (Fig. 5A, narrow green and blue arrowheads). We refer to lines inserted within this neighborhood of *eve* as 'homed'. An additional 44 of these lines that were inserted on the second chromosome but did not show an *eve*-like pattern were localized by inverse PCR, and three of them were found to be homed (narrow yellow and red arrowheads, Fig. 5A). One of these showed β-gal expression only in the AR (CR105), whereas the other two showed no *eve*-like expression. One of these two carried the *hsp70* basal promoter-*lacZ* reporter (SR105), and the other carried *eve*-promoter-*lacZ* (RR11K).

In the 13 homed lines that show an *eve*-like *lacZ* pattern, expression begins around stage 5, with the early *eve* stripe pattern, and is followed by later *eve*-like expression in both the mesoderm and AR (Fig. 4A-

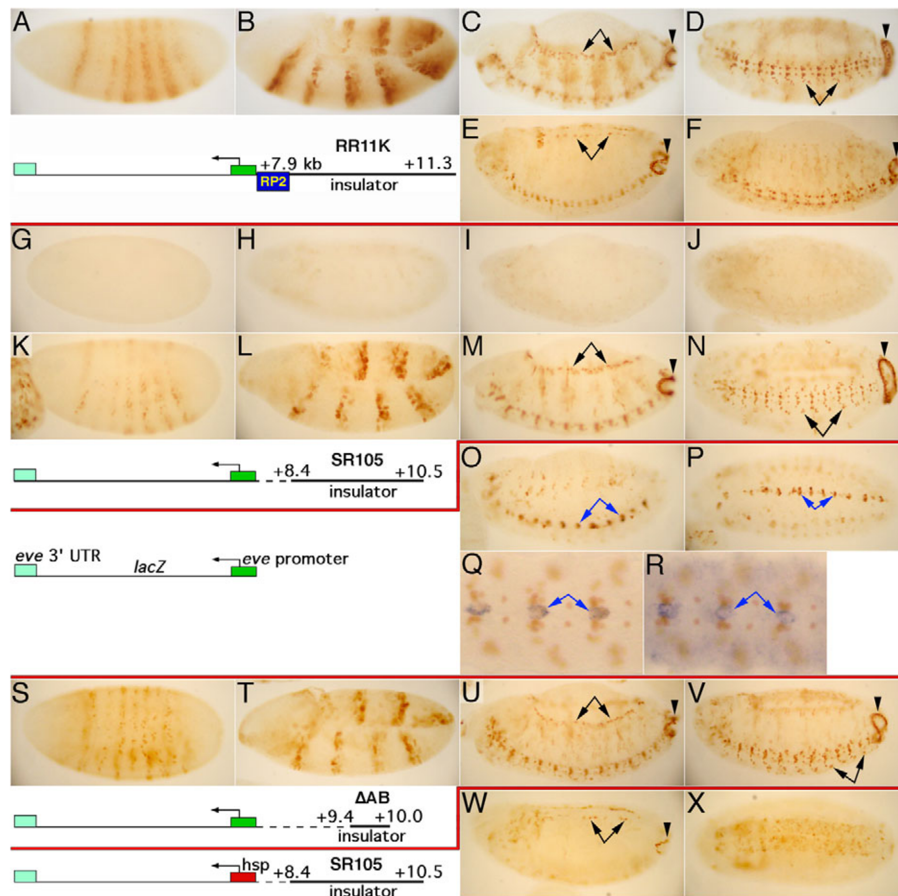


**Fig. 4. The *eve-TER94* insulator is required for, and the eve promoter contributes to, long-range E-P communication.** Staged embryos were subjected to anti-β-gal staining, except Q and R, which show anti-Eve staining (orange) with either *lacZ* (Q) or *CG1623* (R) RNA in situ hybridization (blue). (**A-D**) RR11K-I (see Table S1 in the supplementary material; diagram below A,B) inserted in the third exon of *TER94*. Note *eve*-like stripe (A,B), mesodermal (arrows in C), AR (arrowheads in C and D) and CNS (D) expression. Expression in cells near the midline is from the RP2+a/pCC enhancer (transgenic and/or endogenous), and expression away from the midline, indicated by arrows in D, is from the endogenous *eve* U/CQ and EL enhancers (compare with Fig. 1C,D). (**E**) attP-eZRR11K-II, inserted at –1652 kb (relative to the *eve* start site; see Table S1 in the supplementary material). Note that although AR expression is robust (arrowhead), mesodermal expression is weaker (arrows; it becomes clearly detectable only at this slightly later stage relative to C,M and U). (**F**) attP-eZRR11K-NN, inserted at –3322 kb (see Table S1 in the supplementary material). Note that AR expression is robust (arrowhead) but that, unlike in D, off-midline CNS expression is not clearly detectable. (**G-J**) SR105 with the *eve*-promoter-*lacZ* reporter (diagram below K,L) inserted on chromosome 1. Note that there is no *eve*-like expression, showing that the *eve* insulator and promoter do not have the ability to drive expression unless they communicate with endogenous enhancers. (**K-N**) The original attP-RR11K insertion at –142 kb (attP-eZRR11K-X, which showed indistinguishable expression) was replaced by SR105 with the *eve*-promoter-*lacZ* reporter (diagram below K,L). This and other replacements also differed from the original insertion in not having the *mini-white* gene. Note the *eve*-like expression, as seen in A-D. (**O-R**) The same attP insertion was replaced by the *eve*-promoter-*lacZ* reporter without a homing/insulator element (diagram to the left of Q). Note the lack of *eve*-like expression, showing that the insulator is required for long-range E-P communication. Instead, lateral and midline CNS expression (blue arrows) in non-*eve*-expressing cells is seen. This was confirmed by double staining for Eve protein and *lacZ* RNA (Q, shown at higher magnification). The same cells express the gene into which the transgene is inserted (R), showing that without the insulator, the reporter acts as a typical enhancer trap. (**S-V**) The same attP insertion was replaced by the *eve*-promoter-*lacZ* reporter with the 600 bp ΔAB region (diagram below S,T; see Fig. 3). Note that expression is like that in A-D and K-N, showing that this minimal insulator supports long-range E-P communication. (**W**,**X**) The same –142 kb attP insertion was replaced by SR105 with the *hsp70-lacZ* reporter (diagram to the lower left of W). Note that expression in the mesoderm (arrows) and AR (arrowhead) is weaker, and that expression in the CNS (X) is similar to that of *TER94* (compare with Fig. 1H). Early stripe expression was barely detectable (not shown).

D). Endogenous *eve* is also expressed in RP2, a/pCC, CQ/U and EL neurons. Although most of these transgenic insertions (RR11K and RR105; Fig. 3) carry the RP2+a/pCC enhancer (so that expression in these neurons cannot be attributed to the endogenous enhancer), even those that do not carry this enhancer (NR11K, CR105 and SR105; Fig. 3) express β-gal in these neurons. Furthermore, expression of β-gal is also seen in CQ/U and EL neurons, which is attributable only to communication with endogenous *eve* enhancers. This expression is often weaker and delayed in its appearance relative to endogenous *eve* expression. Overall, most transgenic insertions within the 'homed' region communicate with all of the endogenous *eve* enhancers, with some variation in the strength of the interaction. All of those insertions that communicate carry the *eve*-promoter-*lacZ* reporter, whereas the one that carries *hsp70-lacZ* (described above) does not. This suggested that promoter specificity might contribute to long-range E-P communication, an idea that we test below.

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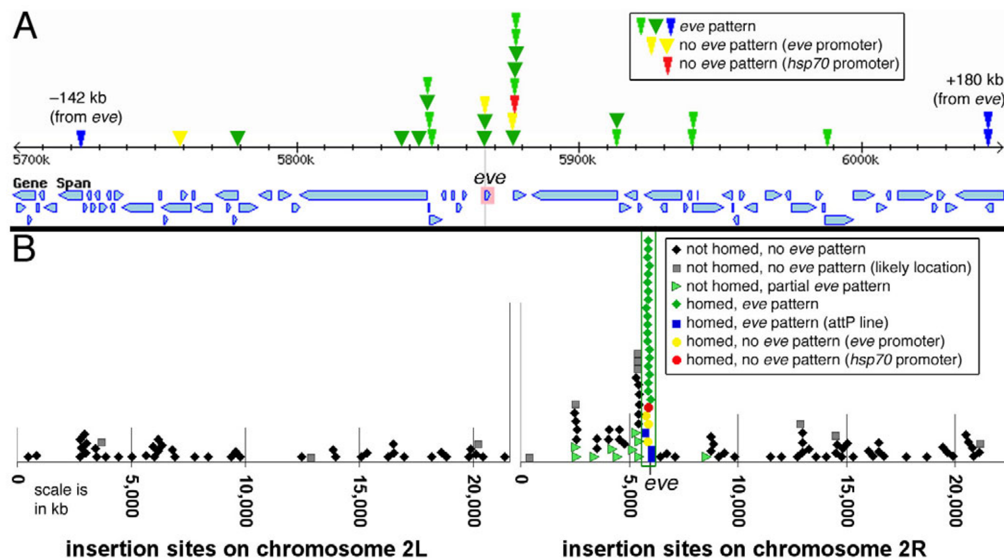


**Fig. 5. Insertion sites of transgenes carrying the insulator/homing region.** (**A**) Genetic map covering 350 kb of chromosome 2R, from 5700 to 6050 kb. The transcription start site of *eve* (at 5867 kb on this scale) is marked with a vertical gray line. Transcription units (as seen in FlyBase) are shown as blue boxes. Insertion sites of homed lines are indicated by narrow arrowheads for eZ and *hsp70-lacZ* constructs (above the dotted line in Fig. 3), and wide arrowheads for eZAR-MeW constructs (below the dotted line in Fig. 3). Colors indicate the following: green, transgenes expressing β-gal in an essentially complete *eve* pattern; blue, attP RR11K insertions that also show an *eve*-like pattern; red, an SR105 transgene with an *hsp70-lacZ* reporter not expressed in an *eve*-like pattern; yellow (near the red arrowhead), CR105 with an *eve*-promoter-*lacZ* reporter that gave expression only in the AR; yellow (just upstream of the *eve* start site), an attP-RR11K insertion that does not give an *eve*-like β-gal expression. (**B**) Distribution of insertion sites of eZ and eZAR-MeW transgenes on the second chromosome. The region shown in A is bracketed by two vertical green lines. Note that insertion sites are concentrated near the *eve* locus, and transgenes that communicate with endogenous *eve* (green arrowheads) are up to 3300 kb away. Most other insertion lines within this region (all but three of the black diamonds) carry the AR and mesodermal *eve* enhancers (eZAR-MeW), obscuring potential long-range interactions (see text). Gray squares correspond to the most likely locations of transgenes where inverse PCR-derived sequences gave somewhat ambiguous results (see Table S1 in the supplementary material).

## Transgene clustering outside the 'homed' region

The tendency of the homing element to induce transgene insertion in a larger region of chromosome 2R might be observed as an increased frequency of insertion on the entire second chromosome. Based on a random sampling of transgenes not carrying this region from previous studies in our laboratory, the second chromosome insertion frequency is 42% (based on 485 lines). By contrast, even when homed lines are excluded, 47% of transgenes carrying the homing region inserted on the second chromosome (211 out of 446 lines obtained). As can be seen in Fig. 5B, many of these insertions occurred just centromere proximal from the 'homed' region, and there are also a number of other regions where several insertion sites are clustered. Within these clusters on the second chromosome, there are five regions (outside the 'homed' region) where two or more insertions occurred within 2.5 kb, suggesting that the homing element might be tethering to regions other than the *eve-TER94* locus. It will be interesting to determine whether these regions harbor insulators.

It is also noteworthy that within the 'homed' region, four insertions are within 2 kb of each other near the promoter of the *Mef2* gene, ~20 kb upstream of the *eve* promoter (Fig. 5A; see Table S1 in the supplementary material). Such regions might bind protein complexes that interact with the homing element, facilitating transgene insertion and also organizing chromosomal architecture in developing tissues.

## The homing/insulator element can mediate E-P communication from 3300 kb away

In addition to homed insertions, there is also clustering of insertions in a larger region of chromosome 2R, mostly centromere proximal from *eve*. Within this larger region, many lines showed communication with the endogenous *eve* AR and mesodermal enhancers (Fig. 5B). Out of the aforementioned 143 lines examined for *eve*-like β-gal expression, ten that were not homed (green triangles, Fig. 5B) showed AR expression (Fig. 4E,F), and in some cases *eve*-like mesodermal expression (Fig. 4E), but not stripe or CNS (CQ/U and EL neuronal) expression. Two of these lines carried *hsp70-lacZ*, and eight carried *eve*-promoter-*lacZ*. At least nine of these ten lines were inserted on chromosome 2R, within 3400 kb of *eve* (but outside the 'homed' region); the other line could not be localized by inverse PCR. Among these 143 lines, three others were also found to be within this distance of *eve*, but did not show β-gal expression with any aspect of the *eve* pattern. Insertions on chromosomes 1, 2L and 3 were also examined, and none showed *eve*-like expression (Fig. 4G-J).

The other insertions in this region (shown in Fig. 5B as black diamonds) carried the AR and mesodermal enhancers (in the context of the eZAR-MeW enhancer-blocking construct), preventing the determination of whether they communicate with the endogenous *eve* enhancers. Conversely, the possibility of long-range E-P communication resulted in ambiguity as to whether transgenic *eve*-promoter-*lacZ* reporters inserted in this part of the genome were driven by transgenic or endogenous enhancers. Therefore, these lines were not considered in the analysis of enhancer blocking.

## The insulator mediates homing

The transgenes constructed for the dissection of enhancer-blocking activity were also tested for homing activity and long-range E-P communication (Figs 3 and 5). We found that there is a close correlation among the required regions for these activities. Thus, the 600 bp ΔAB construct, which retains clear enhancer-blocking activity,

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is also sufficient for homing and long-range E-P communication, as two homed lines were obtained with this construct (out of 18 tested, Fig. 3), and each showed communication with the endogenous *eve* enhancers (Fig. 4S-V). Furthermore, out of 210 transgenes tested for homing that carry all or part of the R100 insulator (R100 and those below it in Fig. 3), nine (4.3%) were homed.

These data show that the *eve* 3′ border region confers three distinct activities, enhancer blocking, homing and long-range E-P communication. The analysis described below further addresses the extent to which these activities are related.

### The homing/insulator element mediates long-range E-P communication

To analyze long-range E-P communication further, we used ΦC31 recombinase-mediated cassette exchange (RMCE). In this system, an attB-carrying insertion plasmid can replace an existing attP insertion in a target line, allowing modified elements to be compared in the same chromosomal environment (Bateman et al., 2006; Groth et al., 2004). To create homed target sites, an attP cassette carrying the *eve*-promoter-*lacZ* reporter and the homing region from +7.9 to +11.3 kb (RR11K; top diagram in Fig. 4) was inserted by conventional P-element transformation. Two resulting lines inserted at –142 and +180 kb (Fig. 5A, blue arrowheads) were used to dissect the requirements for long-range E-P communication. These lines showed β-gal expression in an *eve*-like pattern throughout embryogenesis (similar to Fig. 4K-N; data not shown). When each of these insertions was replaced by one that lacked the homing/insulator element, β-gal expression in an *eve*-like pattern was completely lost (Fig. 4O,P). Instead, expression was observed in non-*eve*-expressing cells of the CNS, suggesting that the transgenic reporter is responding to non-*eve* enhancers near the insertion site. This was verified for both insertion sites (Fig. 4Q,R; see Fig. S1 in the supplementary material; data not shown). Thus, the transgenic enhancer-blocking element is required for long-range communication with endogenous *eve* enhancers.

We next tested whether smaller regions with insulator activity support long-range E-P communication. When we exchanged in the SR105 insulator/homing region, the *eve*-like β-gal pattern was maintained (Fig. 4K-N). In this version of RMCE, either direction of insertion is possible (Bateman et al., 2006). Although one direction of the modified insertion at –142 kb showed increased ectopic β-gal expression in some cells (see Fig. S1 in the supplementary material), in no case were the *eve* aspects of pattern affected. Thus, SR105 fully supports long-range E-P communication. We also exchanged both insertions with one carrying the minimal 600 bp insulator (ΔAB, Fig. 3). The β-gal pattern was not affected (Fig. 4S-V; see Fig. S1 in the supplementary material), showing that the minimal insulator is sufficient for long-range E-P communication.

### Promoter specificity in long-range communication with *eve* enhancers

To test whether the transgenic *eve* promoter contributes to long-range E-P communication, a cassette carrying *hsp70*-*lacZ* (along with the SR105 insulator/homing element) was exchanged into both attP target sites. Compared with the same cassette carrying *eve*-promoter-*lacZ*, the early striped pattern was severely weakened (see Fig. S1 in the supplementary material; data not shown), and AR and mesoderm expression were delayed and at a reduced intensity (Fig. 4W; see Fig. S1 in the supplementary material). Moreover, in the CNS, β-gal was expressed ubiquitously, which is similar to the expression of *TER94* (Fig. 4X, compare with Fig. 1H). These data indicate that, although the insulator/homing region is required for long-range E-P communication, the transgenic *eve* promoter also contributes to the efficient communication with endogenous *eve* enhancers. These data further suggest that the *eve* promoter communicates preferentially with *eve* enhancers over those of *TER94*. However, as described above, we have also seen that this basal *hsp70* promoter can communicate with the endogenous *eve* AR and mesodermal enhancers over much greater distances (Fig. 4E,F), when present in a transgene with the insulator/homing element. Thus, the insulator/homing element is the primary determinant of long-range E-P communication, and the *eve* promoter contributes to its strength and enhancer preference.

## DISCUSSION

### An insulator separates *eve* and *TER94*

Some of the *eve* enhancers are close to the *TER94* promoter, yet they do not activate *TER94*. Although *TER94* is expressed nearly ubiquitously in embryos, it is expressed only at a low level in the mesoderm and anal plate, where *eve* expression is high in a subset of cells, making it unlikely that *eve* enhancers acting on *TER94* would be masked by this expression (Fig. 1). Therefore, something isolates *TER94* from *eve* enhancers (and probably vice versa). Indeed, the region between the 3′-most *eve* regulatory element, a PRE, and the *TER94* transcription start site has the properties of an enhancer-blocking insulator (Figs 2 and 3). It exhibits directional enhancer blocking in transgenes carrying *eve* enhancers in combination with either the *eve* promoter region or heterologous promoters, as well as between heterologous enhancers and promoters.

### The role of the *TER94* promoter in enhancer blocking

We dissected this insulator region in the context of transgenes carrying two different enhancers between divergently transcribed reporter genes (Fig. 2I-N). Some deletion mutants were still able to block the AR enhancer from activating the *mini-white* reporter, while allowing the *eve* mesodermal enhancer to activate the *eve*-promoter-*lacZ* reporter across the mutant insulator (Fig. 3). This might result from a relatively weak interaction between the *eve* AR enhancer and the heterologous *mini-white* promoter, which suggests a degree of specificity of *eve* enhancers for their cognate promoter. This mechanism also contributes to long-range E-P communication mediated by the insulator, as discussed below. Furthermore, the recently discovered presence of an insulator at the 3′ end of *mini-white* (Chetverina et al., 2008) might contribute to stronger enhancer blocking in this direction.

We first narrowed enhancer-blocking activity down to an 800 bp sequence (R100, Fig. 3) that spans the 5′ end of *TER94*. Further dissection showed that the start site of *TER94* is not required (ΔGH, Fig. 3). This makes it unlikely that transcriptional interference (Martianov et al., 2007; Mazo et al., 2007) makes a strong contribution to our results, although it could be significant in some cases, such as for ΔF, which retains the *TER94* start site. Notably, region F, extending from ~150 to 45 bp upstream of this start site, seems particularly important for enhancer blocking. A similar situation pertains to the well-studied insulators *scs* and *scs′* (Avramova and Tikhonov, 1999; Geyer, 1997; Kellum and Schedl, 1992; Kuhn et al., 2004). Perhaps some promoter regions induce a chromatin configuration that blocks the progression of activating complexes or chromatin modifications, through which enhancers communicate with target promoters.

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### The *eve-TER94* insulator and homing activity

The region between *eve* and *TER94* also induces transgene homing. About 7% of transgenes carrying this region (27 out of 380 lines tested) inserted within 180 kb of *eve*. Among 27 homed lines, eight inserted within 1.5 kb of the endogenous insulator (Fig. 5), suggesting that homing involves direct tethering, possibly through a homophilic protein complex formed on the element in the germline, where transgenic insertion occurs. We call the responsible element Homie, for homing insulator at *eve*.

Although it is more difficult to dissect the region required for homing than it is to dissect the region required for enhancer blocking (due to the number of transgenic insertions required to validate a negative result), there is a clear correlation between these activities. Of the 210 transgenes tested for homing that carry all or part of the 800 bp R100 insulator (Fig. 3), nine of them (4.3%) were homed, even though the 'homed' region is less than 0.4% of the genome. Protein-protein interactions among insulators, when they occur in the germline, might lead to transgene homing.

### The *eve* PRE and homing activity

In previous studies of the *eve* 3′ region, we produced hundreds of lines that carried the *eve* PRE, yet we did not observe homing. Therefore, the *eve* PRE is not sufficient for homing. Furthermore, as the minimal homing element does not contain the PRE, this PRE is not required for either homing activity or long-range E-P communication. However, the *engrailed* homing region has PRE activity (Kwon et al., 2009), indicating that some PREs may engage in homotypic interactions that facilitate homing. Consistent with this, long-range interactions among PREs were seen in the BX-C (Lanzuolo et al., 2007). Furthermore, the *engrailed* PRE may also facilitate long-distance E-P communication (Devido et al., 2008).

### An extensive genomic region is a target for Homie-mediated homing and very long-range E-P communication

The *eve*-promoter-*lacZ* reporter in a homed transgene is usually expressed in a full *eve* pattern, showing communication with all of the endogenous *eve* enhancers from as far away as 180 kb, and across a number of other genes (Fig. 4; Fig. 5A). Beyond the homing target region, there is a tendency for Homie-carrying transgenes to insert on chromosome 2R, particularly centromere proximal from *eve* (Fig. 5B; additionally, Table S1 in the supplementary material lists the locations of all these mapped transgenic inserts). We have not referred to these insertions as 'homed', mainly to distinguish them from transgenes that pick up a full *eve* pattern of expression. However, they usually (9 out of 12) pick up a partial *eve* pattern. Intriguingly, Homie-carrying transgenes inserted as far as 3300 kb away (Fig. 5B; see Table S1 in the supplementary material), are capable of interacting with the endogenous *eve* AR and mesodermal enhancers (Fig. 4E,F). Previous indications of long-range E-P interactions mediated by transgenic insulators have come from the genetic and phenotypic analysis of transvection (Kravchenko et al., 2005) and related regulatory interactions (Hendrickson and Sakonju, 1995; Hopmann et al., 1995; Sipos et al., 1998).

We directly tested the requirement for Homie in long-range E-P communication using ΦC31-RMCE to compare transgenes with and without this region at the same chromosomal insertion site. Removal of Homie resulted in complete loss of the *eve* pattern. The same results were obtained at two different landing sites, at opposite ends of the homing region (Fig. 5A, blue arrowheads). Communication of distant 'shadow' enhancers with promoters across several intervening genes has recently been proposed, based upon bioinformatics-based identification of functionally conserved enhancer regions with no other apparent target promoters (Hong et al., 2008). Our results suggest that for such distant enhancers to communicate effectively, they may need promoter-targeting and/or promoter-tethering sequences (Akbari et al., 2008; Zhou and Levine, 1999), and that some of these sequences might also act as insulators, generating a chromosomal architecture that facilitates functionally important interactions while preventing deleterious ones.

How does Homie mediate such long-range E-P communication? Both preferential insertion and the ability to pick up a partial *eve* pattern from long range could be explained by a homologous tethering mechanism (Bantignies et al., 2003; Vazquez et al., 2006), if we assume that this region of 2R is in relative proximity to the *eve* locus within a chromosome territory (Cremer and Cremer, 2001), both in the germline and in the developing AR and mesoderm. Homologous tethering might stabilize a functional E-P interaction, which in turn might facilitate transcription initiation through a combination of mechanisms, including targeting to regions of active transcription within the nucleus (de Laat and Grosveld, 2003; Fraser, 2006; Simonis and de Laat, 2008).

### Promoter specificity in long-range E-P communication

We used RMCE to test the role of promoter specificity in long-range communication. Exchanging a basal *hsp70* promoter for the *eve* promoter caused a complete loss of communication with some endogenous *eve* enhancers but not others. The communication that remained was with the AR and mesodermal enhancers, the same ones that often communicate with either the *eve* or *hsp70* promoters in transgenes inserted up to 3300 kb away (Fig. 5B; see Table S1 in the supplementary material). The ability of these enhancers to communicate at a much longer range than others might indicate relatively stable E-P interactions that can survive entropic forces tending to randomize their positions in the nucleus. Alternatively, the interactions of these enhancers might be specifically facilitated by Homie.

Another indication of the effects of promoter specificity in long-range E-P communication is that when the *eve* promoter was replaced by that of *hsp70*, β-gal reporter expression in the CNS changed from an *eve*-like pattern to one similar to that of *TER94* (Fig. 4X). Although it is possible that this *TER94*-like expression is driven by enhancers located near the insertion site, it is clear that which enhancers are targeted by the transgenic promoter depends in part on promoter specificity. Similar influences have recently been found on E-P communication at the *engrailed* locus (Kwon et al., 2009).

### Reconciling insulator with long-range facilitator functions

How can Homie act as an insulator and also mediate long-range communication? The key may lie in the details of the resulting chromosomal architecture. Precedence for this idea comes from the phenomenon of insulator bypass, in which the enhancer-blocking activity of a single insulator can be negated by placing a second insulator between the enhancer and promoter (Cai and Shen, 2001; Muravyova et al., 2001). This phenomenon is consistent with data from those homed insertions that lie just downstream of endogenous Homie. In these cases, both the transgenic and endogenous Homies are interposed between the *lacZ* reporter and the endogenous enhancers that drive its expression. Our data also show that the apparent bypass of endogenous Homie does not require that transgenic Homie lies between the interacting enhancer and

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promoter. In one case, the transgenic promoter lies between the two Homies, with the interacting enhancers on the outside. We propose that Homie has directionality, so that the two copies of Homie line up in parallel with each other within a wall-like structure. In the cases where both Homies are between the interacting enhancer and promoter, the Homies are inverted in orientation, whereas in the other case they are in the same orientation. In both cases, their lining up in parallel would tend to place the interacting enhancer and promoter on the same side of this wall-like structure, facilitating their communication. By contrast, a single copy of Homie would tend to block communication between sequences on either side, by placing them on opposite sides of the structure. Similar effects of insulator directionality have been seen for the *Fab-8* and *Mcp* insulators (Kyrchanova et al., 2007; Kyrchanova et al., 2008).

## Insulator directionality in facilitating long-range communication

In most homed lines, we did not see *mini-white* expression in an *eve* pattern. This might be due to the *mini-white* promoter being relatively weak and/or less compatible with *eve* enhancers than is the *eve* promoter, or even the *hsp70* promoter, which also often picked up AR or mesodermal enhancer activity from great distances (facilitated by Homie). Intriguingly, however, although in most of the transgenes carrying Homie its 5′ end was oriented toward the *lacZ* reporter, in one line (inserted at +46 kb), this orientation was reversed, and in that line *mini-white* was expressed in the *eve* pattern. Thus, it is possible that Homie directionality, through the mechanism described above for insulator bypass, might play a role in determining whether or not a weak E-P interaction is facilitated.

## What is the normal function of Homie?

There are two likely possibilities for how Homie functions in the regulation of *eve* and *TER94*. The first is that it simply prevents *eve* enhancers from activating *TER94*, and also prevents *eve* from being expressed broadly in the CNS like *TER94*, which would probably cause mis-specification of neurons (Broihier and Skeath, 2002). Another, not mutually exclusive, possibility is that Homie works in conjunction with the nearby PRE to orchestrate functionally appropriate chromosomal architectures during development. Known insulators in the BX-C are each situated near a PRE (Maeda and Karch, 2006), and these PRE-insulator regions interact with promoters in several contexts (Cleard et al., 2006; Lanzuolo et al., 2007). Our data suggest a similar interaction with the *eve* promoter region, based on the fact that three of our homed lines are inserted within the *eve* promoter region. Such an interaction might help enhancers from the 3′ end of the *eve* locus communicate with the *eve* promoter, while also preventing inappropriate interaction with *TER94* enhancers. One motivation for such a model is that in mutants for the PcG gene *polyhomeotic*, *eve* is ectopically expressed throughout the CNS (Smouse et al., 1988), which is reminiscent of normal *TER94* expression. Thus a loss of PcG repression, acting through the PRE, might disrupt the normal insulator function that prevents inappropriate activation of *eve*. This suggests that the functions of the PRE and Homie are coordinated during development, allowing the PRE to maintain either an activated or repressed state of *eve* in different cells (Fujioka et al., 2008), while maintaining the functional isolation of *eve* from *TER94*.

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**Supplementary material**
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