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Miki Fujioka
Department of Biochemistry and Molecular Biology and the Kimmel Cancer Center, Thomas Jefferson University,
Miki.Fujioka@jefferson.edu

Guizhi Sun
Department of Biochemistry and Molecular Biology and the Kimmel Cancer Center, Thomas Jefferson University,
Guizhi.Sun@jefferson.edu

James B Jaynes
Department of Biochemistry and Molecular Biology and the Kimmel Cancer Center, Thomas Jefferson University,
james.jaynes@jefferson.edu

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Recommended Citation
Fujioka, Miki; Sun, Guizhi; and Jaynes, James B, "The Drosophila eve Insulator Homie Promotes eve Expression and Protects the Adjacent Gene from Repression by Polycomb Spreading." (2013). Department of Biochemistry and Molecular Biology Faculty Papers. Paper 69.
https://jdc.jefferson.edu/bmpfp/69

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The *Drosophila eve* Insulator Homie Promotes *eve* Expression and Protects the Adjacent Gene from Repression by Polycomb Spreading

Miki Fujioka, Guizhi Sun, James B. Jaynes*

Department of Biochemistry and Molecular Biology and the Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania, United States of America

Abstract

Insulators can block the action of enhancers on promoters and the spreading of repressive chromatin, as well as facilitating specific enhancer-promoter interactions. However, recent studies have called into question whether the activities ascribed to insulators in model transgene assays actually reflect their functions in the genome. The *Drosophila even skipped* (*eve*) gene is a Polycomb (Pc) domain with a Pc-group response element (PRE) at one end, flanked by an insulator, an arrangement also seen in other genes. Here, we show that this insulator has three major functions. It blocks the spreading of the *eve* Pc domain, preventing repression of the adjacent gene, **TER94**. It prevents activation of **TER94** by eve regulatory DNA. It also facilitates normal eve expression. When Homie is deleted in the context of a large transgene that mimics both *eve* and **TER94** regulation, **TER94** is repressed. This repression depends on the *eve* PRE. Ubiquitous **TER94** expression is “replaced” by expression in an eve pattern when Homie is deleted, and this effect is reversed when the PRE is also removed. Repression of **TER94** is attributable to spreading of the eve Pc domain into the **TER94** locus, accompanied by an increase in histone H3 trimethylation at lysine 27. Other PREs can functionally replace the *eve* PRE, and other insulators can block PRE-dependent repression in this context. The full activity of the *eve* promoter is also dependent on Homie, and other insulators can promote normal *eve* enhancer-promoter communication. Our data suggest that this is not due to preventing promoter competition, but is likely the result of the insulator organizing a chromosomal conformation favorable to normal enhancer-promoter interactions. Thus, insulator activities in a native context include enhancer blocking and enhancer-promoter facilitation, as well as preventing the spread of repressive chromatin.

Introduction

A variety of regulatory elements have evolved in higher eukaryotes to regulate gene expression. Cis-regulatory modules (CRMs, or enhancers) are bound by DNA-binding transcription factors that coordinate recruit coactivators and corepressors. Enhancers communicate with basal promoters at least in part through a looping out of intervening DNA, allowing them to act over large distances along a chromosome, or even in trans, with a promoter on another chromosome [1–4]. Enhancer activities are regulated by the chromatin environment, which is “managed” by both the enhancers themselves and other DNA elements such as Polycomb-group response elements (PREs) [5–8]. Further coordination of these activities is provided by elements such as insulators that affect chromosomal organization and conformation. Insulators harbor activities that can limit the range of action of enhancers and repressive chromatin, as well as facilitate long-range enhancer-promoter communication, depending on context [9–12].

Insulators typically show “barrier” function that prevents the spread of heterochromatin, as well as enhancer blocking activity, in model transgene assays [9–12]. Pairs of insulators can interact with each other to generate chromosomal loops between them. This has been postulated to create distinct functional domains that somehow prevent enhancer-promoter cross-talk between domains. Repressive chromatin structures include heterochromatin and Polycomb (Pc) chromatin, which constitutes a form of epigenetic transcriptional memory, stabilizing developmental fate choices, among other functions. Pc chromatin is maintained through the recruitment of Pc-group (PcG) gene products to PREs [5–8]. PREs can extend their influence outward to produce Polycomb domains that encompass multiple regulatory regions within a gene or a gene complex [13–17]. PREs can also synergize with each other in trans [18], and in some cases facilitate long-range enhancer-promoter communication [19]. Both Pc domains and mammalian X-inactivation involve the histone modification H3K27me3, catalyzed by Pc-repressive complex 2 (PRC2) [20–22].

The functions of PREs and insulators have been studied within *Drosophila* Hox genes [23–25]. There, functional chromatin domains are flanked by insulators, so that all the enhancers and PREs within a domain are coordinately regulated. Enhancers acting early in development (“initiators”) are spatially regulated to determine whether a domain will be active or not throughout the rest of development. They do this by inactivating PREs, so that...
Insulators are specialized DNA elements that can separate the genome into functional units. Many of the current thinking about these elements comes from studies done with model transgenes. Studies of insulators within the specialized Hox gene complexes have suggested that model transgenes can reflect the normal functions of these elements in their native context. However, recent genome-wide studies have called this into question. This work analyzes the native function of an insulator that resides between the Drosophila genes eve and TER94, which are expressed in very different patterns. Also, the eve gene is a Polycomb (Pc) domain, a specialized type of chromatin that is found in many places throughout the genome. We show that this insulator has three major functions. It blocks the spreading of the eve Pc domain, preventing repression of TER94. It prevents activation of TER94 by eve regulatory DNA. It also facilitates normal eve expression. Each of these activities are consistent with those seen with model transgenes, and other known insulators can provide these functions in this context. This work provides a novel and convincing example of the normal role of insulators in regulating the eukaryotic genome, as well as providing insights into their mechanisms of action.

Author Summary

Insulators are specialized DNA elements that can separate the genome into functional units. Most of the current thinking about these elements comes from studies done with model transgenes. Studies of insulators within the specialized Hox gene complexes have suggested that model transgenes can reflect the normal functions of these elements in their native context. However, recent genome-wide studies have called this into question. This work analyzes the native function of an insulator that resides between the Drosophila genes eve and TER94, which are expressed in very different patterns. Also, the eve gene is a Polycomb (Pc) domain, a specialized type of chromatin that is found in many places throughout the genome. We show that this insulator has three major functions. It blocks the spreading of the eve Pc domain, preventing repression of TER94. It prevents activation of TER94 by eve regulatory DNA. It also facilitates normal eve expression. Each of these activities are consistent with those seen with model transgenes, and other known insulators can provide these functions in this context. This work provides a novel and convincing example of the normal role of insulators in regulating the eukaryotic genome, as well as providing insights into their mechanisms of action.

where initiators are active, later-acting enhancers can also be active. The main effect of deleting insulators in this context is to extend the influence of initiators to inactivate PREs in the adjacent domain, which allows its later-acting enhancers to be inappropriately active. However, phenotypic details suggest that in some cells, repressive chromatin may spread instead [26].

Genome-wide chromatin immunoprecipitation (ChiP) analysis of the locations of insulator binding proteins show a wide range of binding patterns [27-40]. In Drosophila, insulator proteins include dCTCF (CCCTC-binding factor), Mod(mdq)67.2, Su(Hw) (Suppressor of hairy wing), CP190 (centrosomal protein 190), BEAF32 (boundary element-associated factor 32), and Zw5 (Zeste-white-5). Recent genome-wide studies also implicate the mitotic spindle protein Chromator [41] and the nuclear lamina [30,37] in insulator function. In mammals, CTCF is associated with most known insulators [10,12,42-44]. CTCF targets in the regulation of β-globin [45,46] and the imprinted Igf2 and IHH loci [47-49]. Based on recent genome-wide studies, it has been suggested that insulator proteins bind at many sites that do not function as predicted by model transgene assays [34,36,38]. Transgenic dissection in a native context can help to determine their normal functions.

The even skipped (eve) locus is a well-defined Pc domain based on genome-wide analysis [13-17], and is regulated by PcG genes [50-54]. An insulator flanks its well-characterized regulatory region, which includes the eve PRE at its 3’ end [51,55]. Thus, this insulator is in a position to separate both positive and negative eve regulatory elements from the constitutively expressing neighboring gene TER94, and/or to prevent ectopic activation of eve by TER94 enhancers. This insulator was shown to have 3 distinct activities in model transgene assays. In addition to enhancer blocking, it causes homing of P-element transgenes to the endogenous eve neighborhood, for which it was nicknamed Homie (Homing insulator at eve). Furthermore, from within a several megabase region flanking endogenous eve, it causes long-range interactions of transgenic promoters with endogenous eve enhancers [55]. Genome-wide analysis showed that most known insulator proteins bind to the Homie region [27,33].

Homic shares properties with other insulators based on model transgene assays and, like many other putative insulators, is situated close to both a transcription start site (TSS) and a PRE. Thus, understanding Homic’s function in its native context can illuminate many of the mysteries that surround this enigmatic group of regulatory elements. In order to investigate its native function, we constructed a transgenic eve-TER94 locus that mimics the normal regulation of both genes. Using this artificial locus, we show that Homic functions as a PRE blocker to protect TER94 from repression due to spreading of the eve Pc domain. Heterologous insulators and PREs can substitute for Homic and the eve PRE, suggesting that limiting the range of PRE action is an important function of insulators generally. Homic also prevents the eve enhancers from activating TER94 in specific tissues. Furthermore, Homic facilitates normal eve expression by augmenting communication between the eve promoter and its 3′ enhancers, likely through a chromosomal looping mechanism.

Results

Insulators are generally considered to have two major functions. First, they can shield promoters from the effects of distal enhancers. Second, they can block the spread of repressive chromatin. Here, we investigate the roles that these activities play in the normal functions of an insulator (Homic) located between the ubiquitously expressed TER94 gene and the highly patterned eve gene. We find that blocking the spread of repressive Polycomb chromatin by Homic is critical for normal TER94 promoter expression. In addition to exhibiting the canonical insulator activities in a near-native context, we find that Homic facilitates certain aspects of normal eve expression, and we present a model for how this occurs.

Homic Shields the TER94 Promoter from eve PRE Activity

In order to analyze the function of the eve 3′ insulator Homic, we employed a pseudo-locus that contains all the regulatory DNA necessary for normal expression of both eve [56-59] and the 3′ adjacent gene TER94 [60-62]. This transgene extends from −6.4 to +11.3 kb relative to the eve TSS, from the 5′-most enhancer of eve to the 3rd exon of TER94. In addition to all of the eve enhancers, this region contains a characterized PRE [51] located just upstream (on the eve side) of Homic [55]. On the other side of Homic is the TER94 promoter and TSS, which are sufficient for ubiquitous expression, augmented by enhancers in the TER94 introns (data not shown). The eve coding region was replaced with lacZ coding DNA, and the 3rd exon of TER94 was fused with the EGFP coding region (Figure 1A). In this study, we make repeated use of a version of recombinase-mediated cassette exchange (RMCE) [63] that allows modified transgenes to be inserted in either orientation at pre-defined chromosomal landing sites. All aspects of transgene expression were consistent for both orientations and at multiple landing sites, with a few minor exceptions (as noted below).

In embryos, TER94 RNA is present ubiquitously at early blastoderm, and begins to fade around stage 10. Most of this RNA is maternally derived, but there is a ubiquitous zygotic contribution as well [see below]. At stage 10 and later, strong expression is also observed throughout the brain and central nervous system (CNS) [53]. TER94-GFP expression from our transgene simulates endogenous TER94 expression (Figure 1B, “intact ‘gene”). Although the level of expression varies somewhat with chromosomal location, the relative behavior of modified transgenes was consistent at each chromosomal location (compare Figure 1B and Figure S1).
Deletion of Homie caused a severe loss of early, ubiquitous expression driven by the TER94 promoter (Figure 1B “D Homie”, Figure S1). When the eve PRE was deleted in addition to Homie, the ubiquitous expression in embryos returned (Figure 1B “D Homie D PRE”, Figure S1). Deletion of the eve PRE alone did not affect the expression pattern (Figure 1B “D PRE”). These results show that the loss of ubiquitous expression from the TER94 promoter caused by deletion of Homie depends on the presence of the PRE. So, one function of Homie is to protect TER94 from PRE-dependent repression.

We also note that when Homie is removed, expression in an eve-like pattern is seen (Figure 1B “ΔHomie”, Figure S1A). This indicates that without Homie, eve enhancers can access the TER94 promoter. We investigate this effect further below.

Homie Blocks eve PRE Activity in Ovaries

Early, ubiquitous expression of TER94 comes from maternally deposited RNA, based on its early appearance and the fact that TER94 is expressed strongly in developing oocytes [60–62]. This was confirmed by staining for transgene expression in the absence of a maternal contribution, which is much weaker at early stages than the maternally derived signal (Figure S2 “intact t’gene”; compare to Figure 1B, Figure S1). Since TER94-GFP RNA is deposited maternally, we examined expression in ovaries. TER94 mRNA is present in both the germline, including nurse cells, and somatic epithelial follicle cells [60–62]. No eve expression in ovaries has been reported. In our transgenic lines, strong TER94-GFP expression was seen at all stages of oogenesis (Figure 2 “intact t’gene”, Figure S4) in both germline and somatic epithelial cells (Figure S3 “intact t’gene”). However, the level depended to some extent on chromosomal location (compare Figure 2 and Figure S4). In each case, expression was severely repressed when Homie was deleted (Figure 2 “ΔHomie”, Figure S4). As was seen in embryos, it was restored when the PRE was also deleted (Figure 2 “ΔHomie ΔPRE”, Figure S4). These data confirm that in ovaries, Homie is required for TER94 promoter activity, due to its blocking of PRE-dependent repression.

Figure 1. Homie shields the TER94 promoter from eve PRE activity. A: map of the eve-TER94 transgene. The 3rd exon of the TER94 protein coding region is fused to that of GFP, while the eve coding region is replaced by that of lacZ. Otherwise, the transgene consists of the entire genomic region from −6.4 kb to +11.3 kb relative to the eve TSS, and includes all of the eve enhancers, plus TER94 enhancers located within its first two introns. The locations of the major eve PRE (“eve PRE”), 3’ insulator (“Homie”), early embryonic stripe enhancers (numbered), and late embryonic enhancers (labeled) are shown as colored boxes. B: embryonic expression, at the indicated stages, of GFP RNA, driven by the TER94 promoter, from the transgene shown in A (top row “intact t’gene”), or the same transgene modified by deletion of either the insulator alone (2nd row “ΔHomie”), the PRE alone (bottom row “ΔPRE”), or both (3rd row “ΔHomie ΔPRE”), each inserted at attP landing site 95E5, visualized by whole-mount in situ hybridization. Note that the normal, ubiquitous expression (mimicking TER94) is changed to resemble the eve pattern (shown for comparison at the bottom) by deletion of Homie, while further deletion of the PRE restores TER94-like expression. Deletion of the PRE alone does not noticeably affect the embryonic pattern.
doi:10.1371/journal.pgen.1003883.g001
The eve PRE Is Redundant in Embryos

Since most of the ubiquitous TER94-GFP RNA seen in early embryos is maternally derived, we tested whether zygotic expression from a paternally-derived transgene is affected by Homie deletion. In this assay, non-transgene-carrying (yw) female flies are crossed with transgene-carrying males, so that there is no maternal GFP RNA in the progeny. Two chromosomal locations were analyzed. In both cases, GFP expression was reduced when Homie was deleted (Figure S2).

Because of the relatively low level of expression, we quantified GFP RNA using RT-PCR. Embryos from three timed collections were analyzed: 2–3 hr. (stages 5–6) and 4–6 hr. (stages 9–11) after egg deposition, and stages 13–15. The effect of Homie deletion paralleled those described above for both ovaries and embryos, in that expression was repressed. However, unlike in ovaries, when both Homie and the PRE were deleted, TER94-GFP expression remained repressed at all stages examined (Figure S2 and data not shown). This is consistent with the idea, confirmed below, that another PRE in the eve locus substitutes in embryos (but not in ovaries) for the eve 3’ PRE. In fact, the eve promoter-proximal region has PRE-like properties [51] (see Discussion).

Other Drosophila Insulators Block eve PRE Action

Are the functions of Homie seen in our assays unique, or are they shared among insulators? In order to test this, we replaced Homie with other known insulators. As a negative control, a 500 bp stretch of λ phage DNA was tested. It had no effect on repression of the TER94 promoter by the eve PRE (Figure 3A “ΔHomie”). In contrast, other characterized Drosophila insulators can substitute for Homie to block repression. gypsy (Figure 3A, B), Fab-7, scs’ (Figure 3A), and Fab-8 (Figure 3B) each prevented TER94 promoter repression. Although in one orientation, scs did not work (Figure 3A, “+ scs”), it did work in the opposite orientation (Figure 3A, “+ scs(inv)”). Fab-8 and gypsy showed a minor directionality in their effectiveness (not shown). Restoration of GFP expression is somewhat weaker for scs’ and scs(inv) than for gypsy and Fab-7, indicating that they only partially block eve PRE action. Despite differences in efficiency, blocking of PRE action in this context is a shared property of insulators.

Heterologous PREs Repress TER94 in the Absence of an Insulator, and Homie Blocks this Repression

In order to test whether the repression of TER94 by the eve PRE is due to some unusual property associated with this PRE, we replaced it with other known PREs. We tested both the bxd PRE and an en PRE for the ability to substitute for the eve PRE in ovaries, in the context of a Homie-deleted transgene. In both

Figure 2. Homie blocks PRE action in ovaries. Fluorescence and GFP RNA levels in ovarioles from fly lines carrying the indicated transgenic reporters (described in Figure 1), or no transgene (“no t’gene”). Note the strong fluorescence from TER94 promoter-driven GFP with the intact transgene (“intact t’gene”) at all stages of oogenesis (which proceeds from left to right within each string of ovarioles), and that this is lost when Homie is deleted (“ΔHomie”). Remarkably, strong GFP expression is restored when both Homie and the PRE are deleted (“ΔHomie ΔPRE”), indicating that in the absence of Homie, the PRE is responsible for repression of TER94-GFP. Strong expression is also seen when only the PRE is removed (“ΔPRE”). The graph at the bottom shows, on a log scale, the results of quantitation in triplicate (averages with standard deviations) of GFP RNA from ovaries of lines carrying the indicated transgenes (see Materials and Methods). Note that GFP RNA levels decrease more than 50-fold when Homie is deleted, and are restored by additional deletion of the PRE.

doi:10.1371/journal.pgen.1003883.g002
PRE Blocking by Insulators

A

ΔHomie

+ gypsy

+ Fab-7

+ scs

+ scs(inv)

+ scs'

B

ΔHomie

+ gypsy(inv)

+ Fab-8

C

ΔHomie ΔPRE

+ bxd PRE

+ en PRE

D

ΔPRE

+ bxd PRE

+ en PRE
cases, repression was seen at a comparable level to that seen with the eve PRE (Figure 3C, compare to Figure 3A,B “ΔHomie”), indicating that TER94 repression is due to a property shared by PPREs.

We also tested whether Homie can prevent repression by heterologous PPREs. To do so, we replaced the eve PRE with either the bxd PRE or the en PRE. In both cases, Homie blocked their action on the TER94 promoter, and the resulting expression was like that of the wild-type transgene (Figure 3D). This shows that Homie can block repression by a variety of PPREs. Taken together, these results suggest that insulators block PRE-dependent repression generally. Thus, the commonly occurring arrangement of PPREs flanked on one side by insulators [31] is likely to function to provide a sharp transition in chromatin structure.

Homie Prevents Spreading of the eve Polycomb Domain

The eve locus is a Pc domain, associated with both Polycomb and the characteristic histone modification H3K27me3 [13–17]. We asked whether the repression of TER94-GFP in the ΔHomie transgene is accompanied by spreading of this Pc domain over the TER94-GFP region. Indeed, in ovaries, we found that H3K27me3 was increased in the TER94-GFP region when Homie was deleted (Figure 4A,B). Additionally removing the PRE reversed this effect almost completely (Figure 4A,B), indicating that spreading of H3K27me3 depends on the eve PRE. Thus, when TER94-GFP is repressed, H3K27me3 is increased, and when this repression is reversed, H3K27me3 levels return to normal. This suggests that Pc domain spreading is likely to be responsible for the repression.

In embryos, as in ovaries, H3K27me3 spreads into the TER94-GFP region when Homie is deleted (Figure 4C,D). However, in contrast to ovaries, additionally removing the PRE does not reverse the effect (Figure 4C,D), suggesting that there is redundancy between this PRE and other PPREs in embryos. This redundant activity may be provided by the eve upstream promoter region [31], or by uncharacterized PPREs within the eve locus. Again, recalling that the eve PRE is redundant in embryos for repression of TER94-GFP in the absence of Homie (Figure S2), there is a striking correlation between spreading of the Pc domain and repression of the TER94 promoter.

Homie Shields the TER94 Promoter from eve Enhancers

Intriguingly, when Homie is deleted, the loss of ubiquitous TER94-GFP expression is accompanied by weak expression in an eve pattern (Figure 1B “ΔHomie”, Figure S1A). With the intact transgene, early stripe expression of TER94-GFP driven by eve enhancers might be obscured by early ubiquitous expression, so we cannot rule out that eve enhancers are working on the TER94 promoter at early stages. In fact, eve-like stripe expression from the transgenic TER94 promoter is seen at one chromosomal landing site when the intact transgene is heterozygous and paternally derived, so that there is no maternal contribution (Figure S2B “intact t’gene”). However, eve-like mesodermal, CNS, and anal plate ring (APR) expression seems clearly to be caused by deletion of Homie (Figure 1B “ΔHomie”), stages 11 and 15; Figure S1A “ΔHomie”, stage 13), because with the intact transgene, ubiquitous expression in these tissues is low, yet no such eve-like expression is seen. Furthermore, these later-stage aspects of eve expression are not seen with a paternally derived, intact transgene (Figure S2). Therefore, the data suggest that one of Homie’s functions is to prevent interaction between the TER94 promoter and eve enhancers. Accompanying the recovered ubiquitous expression when the PRE is also deleted, expression in an eve pattern is lost (Figure 1B “ΔHomie ΔPRE”), stages 11 and 15; Figure S1A “ΔHomie ΔPRE”, stage 13). This loss of mesodermal, CNS, and APR expression of TER94-GFP caused by additional deletion of the PRE indicates that the PRE not only represses ubiquitous TER94 promoter activity, but also facilitates communication between the eve enhancers and the TER94 promoter in the absence of Homie (see Discussion).

Homie Facilitates eve 3’ Enhancer Action on the eve Promoter

We then tested whether Homie affects eve promoter activity. To do this, we monitored transgenic lacZ expression, which is driven by the eve promoter (Figure 3, Figure S3A,B). When Homie is removed, there is a reduction in expression driven by enhancers located 3’ of the eve coding region. Interestingly, these are the eve enhancers located between Homie and the eve TSS. Comparing “ΔHomie” with the intact transgene at stage 5 (Figure 5 left column), we see that stripes 1, 4, 5, and 6 are weakened relative to stripes 2, 3, and 7. A similar reduction of expression is seen at later stages, where mesodermal, CNS, and APR expression are weakened by deletion of Homie (Figure 5 middle and right columns). This effect is seen at all transgene landing sites tested (Figure 5A, Figure S3A,B), although it varies in strength with the direction of transgene insertion (data not shown). Despite these differences, we consistently see significant disruptions of normal eve expression when Homie is removed.

It seemed possible that the effects of removing Homie on eve promoter activity were caused by the relief of enhancer blocking, which then might allow eve enhancers access to the TER94 promoter. The resulting promoter competition might reduce eve expression. Alternatively, removing Homie might cause the loss of a chromosome conformation that favors eve enhancer-promoter interactions. This possibility is suggested by the ability of Homie to promote the activation by endogenous eve enhancers of a transgenic eve promoter located up to several megabases away.
To distinguish between these possibilities, we performed two sets of experiments. First, we tested whether expression from the TER94 promoter occurs in a pattern that matches the loss of expression from the eve promoter when Homie is deleted. We found that this is not the case. Rather, TER94 expression in an eve striped pattern does not show a difference among the stripes (Figure 1B, Homie, compare to Figure 5). Second, we directly tested the promoter competition hypothesis by deleting the TER94 promoter in addition to Homie. Removing the potentially competing promoter did not restore normal eve expression (Figure 5B). While we cannot rule out competition with endogenous promoters, promoter competition seems unlikely to be the primary cause of the eve pattern disruptions that result from removal of Homie. The facilitation of eve promoter activity by

Figure 4. Homie blocks spreading of the eve Pc/H3K27me3 domain into the adjacent gene, TER94. ChIP assays were used to quantify the association of H3K27me3 with the endogenous eve and TER94 coding regions, and with the transgenic eve-lacZ and TER94-GFP coding regions. A, B: ovaries were dissected and subjected to ChIP analysis, as described in Materials and Methods. In A is graphed the averages and standard deviations from triplicate PCR assays using primer pairs specific to each of the 4 coding region indicated along the bottom, and for the transgenic lines indicated ("intact t'gene" carries the entire transgene diagrammed in Figure 1A; "ΔHomie" is the same transgene with Homie deleted; "ΔHomie ΔPRE" has both Homie and the PRE deleted). Either H3K27me3-specific antibodies or non-specific IgG were used to precipitate cross-linked chromatin, as indicated in the inset key. In B is graphed the same data ("trial 2" in the inset key), and two other trials ("trial 1" and "trial 3") using independent chromatin preparations (see Materials and Methods), normalized to the H3K27me3-specific signal from the eve coding region for each transgenic line. C, D: embryos (2–20 hr. after egg deposition) were collected and subjected to ChIP analysis, as in A and B above. Note that the specific signals from the transgenic eve-lacZ coding region do not change significantly when Homie is deleted, while the transgenic TER94-GFP coding region shows an increase in H3K27 trimethylation when Homie is deleted, correlating with its repression.

doi:10.1371/journal.pgen.1003883.g004
Homie may therefore be due to its ability to organize specific chromosomal loops, possibly with the eve promoter (see Discussion). Interestingly, heterologous insulators are able to restore normal eve enhancer-promoter interactions to different degrees (Figure S5C and data not shown), roughly in parallel to their abilities to restore PRE blocking (Figure 3A,B). For example, gypsy restores normal eve promoter activity, while scs does not (Figure S5C). The abilities of heterologous insulators to perform this function may be due to interactions between them and a region of the eve locus that normally interacts with Homie.

Discussion

A Transgenic eve-TER94 Locus to Assess PRE and Insulator Activity

The eve 3′ insulator, Homie, was shown previously to have three activities: P-element transgene homing, enhancer blocking, and facilitation of long-range enhancer-promoter communication between endogenous eve enhancers and a transgenic promoter [55]. We sought to address how these activities relate to Homie’s normal function. Both eve and TER94 are essential genes, and eve is highly dose-dependent, making it problematic to manipulate the
endogenous locus. Therefore, we constructed a transgene that contains these genes in their normal configuration. Both the eve and TER94 coding regions were replaced with reporter genes to monitor promoter activity. This transgene simulates the expression pattern of both genes, when inserted at several different chromosomal sites. We used this system to manipulate both Homie and the nearby PRE, to assess their normal functions.

Homie Protects the TER94 Promoter from Spreading of PRE-dependent Repressive Chromatin

A major finding of this study is that Homie is required to prevent PRE-dependent repression of the TER94 promoter. Removal of Homie causes a near-complete loss of the normally ubiquitous TER94 promoter activity. Although Homie is close to the TER94 promoter, its removal does not affect the promoter directly. Rather, removing Homie allows eve enhancers to drive the TER94 promoter in an eve pattern (Figure 1). Furthermore, additional removal of the nearby PRE restores ubiquitious expression. This restoration is complete in some instances (e.g., Figures 1, 2), although it is incomplete in others (e.g., with a paternally-derived transgene in embryos, Figure S2). A simple explanation for the lack of complete restoration in some circumstances is that PRE activity varies in different tissues, and the eve 3’ PRE is partially redundant with other PREs at some times in development.

Ubiquitous expression of TER94 in early embryos, as well as some of the later ubiquitous CNS expression, is due to maternally loaded RNA. Consistent with this, expression in ovaries is robust, and, like early embryonic expression, is strongly repressed without Homie (Figures 2, S4). Accompanying repression in both ovaries and embryos, trimethylation of H3K27 at TER94-GFP is strongly increased when Homie is removed (Figure 4). Thus, without Homie, the eve Pc domain spreads into the adjacent gene, apparently shutting down expression.

Homie is bound in vivo by most known insulator binding proteins, including Su(Hw), CP190, Mod(mdg4)67.2, BEAF32, CTCF, and GAF [27,33]. In a previous study, depletion of CTCF by RNAi in a cultured cell line caused a reduction in H3K27me3 levels throughout the eve locus [36]. The authors suggested that depleting CTCF altered the activity of insulators flanking eve, which led to a decrease in H3K27me3. In contrast, we found that deletion of Homie did not cause a significant reduction in H3K27me3 levels in the eve-lacZ region of our pseudo-locus, either in embryos or in ovaries (Figure 4). There could be several possible reasons for this discrepancy, including the cell types assayed, and indirect effects of depleting CTCF.

Tissue Specificity and Redundancy of PRE Activity

With removal of Homie, the spreading of H3K27me3 in ovaries is reversed by deletion of the PRE (Figure 4A,B). However, in embryos, this spreading is only partially reversed (Figure 4C,D). A simple explanation for this is that additional PRE activity within the eve locus comes into play in embryos. Consistent with this, the eve promoter-proximal region has PRE-like properties. It causes pairing-sensitive silencing of mini-white in transgenes that carry it [51], a property associated with most known PREs. Furthermore, it has consensus binding sites for several PRE-associated DNA binding proteins [8,51], and it shares with the eve 3’ PRE the ability to support positive epigenetic maintenance of enhancer activity from embryos to larval stages in eve-positive neurons [51].

Perhaps the clearest evidence for redundant PRE activity within the eve locus is that the level of H3K27me3 at the eve-lacZ coding region is not significantly reduced when the 3’ PRE is deleted. This is true in both embryos and ovaries. In contrast, spreading of the Pc domain into TER94 in ovaries requires the 3’ PRE (Figure 4). Our data are consistent with the idea that PREs are the nucleation point for spreading of the H3K27me3 mark, and that PRE activity is regulated, so that PREs are differentially active in different tissues.

Furthermore, because there may be a dynamic balance between active and repressive chromatin, maintaining a boundary between them may have different requirements at different chromosomal locations, and at different times in development. Insulators that are not required to maintain a boundary in one cell type may be required for that function in other cells, or at specific times in development, as previously suggested [34]. One reason for such differences may be regulated PRE activity.

In some cases, spreading of repressive chromatin can be stopped by an active promoter [11,64]. In the case of the TER94 promoter, although it is robustly expressed, particularly in ovaries, this is not sufficient to stop the spreading of H3K27me3 in the absence of a insulator. This contrasts with the suggestion from recent genome-wide studies in both cultured cells and Drosophila that insulator protein function is generally not required to prevent spreading of H3K27me3 into active genes, or to maintain most normal gene expression [34,36,30]. Because many insulator proteins bind to overlapping sets of sites, it is likely that there is considerable redundancy in their function. Thus, knocking out any one of them may not reveal the full function of a majority of their binding sites.

Enhancer Action from Within a Pc Domain

It is intriguing that the eve locus is a Pc domain with well-defined boundaries that flank its extensive regulatory regions. Within chromosomal domains of the Drosophila bithorax complex (BX-C), active enhancers prevent the establishment of repressive Pc-dependent chromatin in early embryos. Conversely, in tissues where such repressive chromatin has been established, such as in parts of the CNS and imaginal discs, later-acting enhancers are repressed [25]. Do similar mechanisms operate within the eve locus? Extensive dissection of eve regulatory DNA has not identified enhancers that can drive expression outside the normal eve pattern, arguing against such a close analogy with the BX-C. However, in PcG mutants, eve is ectopically expressed throughout the late-stage embryonic CNS [50,54], showing that PcG proteins do negatively regulate eve, as they do the Hox complexes.

In our previous studies of eve PRE activity, we found that in a transgenic context, both the 3’ PRE and the PRE-like eve promoter region could facilitate positive maintenance of an eve CNS enhancer from embryonic to larval stages, as well as prevent ectopic expression in cells that normally do not express eve [51]. Unlike maintenance elements [63] in the BX-C, the eve 3’ PRE was found to require the DNA binding PcG protein Pleiohomeotic rather than Trithorax-group members for positive maintenance [51]. In this study, we also see evidence of a positive effect of the eve 3’ PRE on enhancer activity. In this case, it facilitates TER94-GFP expression in an eve pattern when Homie is removed (Figure 1B). eve-like mesodermal and CNS expression are seen when Homie is removed, but are not seen when both Homie and the PRE are removed. One possible explanation for this is that eve enhancers have evolved to function within a Pc domain, and they may be better able to activate the TER94 promoter when the Pc domain spreads over it. In this view, PREs facilitate both the on state and the off state, yet the chromatin may be differently modified in the two cases. This model is similar to the “integration model” proposed for how heterochromatin can have a positive effect on the expression of genes that normally reside within it [66].
Homie’s Activity Relative to that of Other Drosophila and Mammalian Insulators

Homie sits adjacent to the eve 3’ PRE, an arrangement that is reminiscent of some boundaries in the BX-C. The mammalian homologs of eve, evx1 and evx2, are located at the 3’ end of the HOX-A and HOX-D clusters, respectively, suggesting that the ancestral eve locus was part of a Hox cluster [67]. Consistent with conservation of the eve insulator-PRE relationship, recent studies identified an enhancer-blocking activity between evx2 and Hoxd13 [68], and a PRE in the HOX-D cluster [69]. The presence of a PRE near an insulator, with a promoter on the other side, may indicate a functionally important boundary between active and repressive chromatin domains.

Previous studies showed that within the BX-C, neither scs nor gypsy could functionally replace Fab-7 [70], indicating that there are different classes or strengths of insulators. In these cases, the primary effects of insulator deletion was ectopic activation, due to early acting enhancers (“initiators”) “turning off” PREs throughout a chromatin domain delineated by insulators [26,71]. In our system, the major effect of insulator deletion is the spreading of the eve Pc domain, reminiscent of the shielding of transgenic reporter genes from repressive effects at some insertion sites [18,72–75]. Despite the differences in normal function, BX-C insulators can replace Homie in our assay, indicating some degree of universality in insulator function as a PRE blocker. However, our assays did reveal differences in effectiveness in carrying out this function. Specifically, scs showed slightly weaker activity than either gypsy, Fab-7, or Fab-8, while the activity of scs was highly orientation-dependent (Figure 3).

Homie Blocks Enhancer-promoter Cross-talk, While Facilitating Enhancers that Lie Between It and the eve Promoter

Deletion of Homie results in expression of TER94-GFP in an eve pattern. In fact, the eve early embryonic stripe enhancers may access the TER94 promoter even when Homie is present, because with a paternal-only transgene, we sometimes see eve-like stripe expression from TER94-GFP (Figure S2B “intact t’gene”). However, at later stages of embryogenesis, we do not see eve-like expression in either the mesoderm, CNS, or APR unless Homie is deleted. Therefore, one of Homie’s functions is to prevent communication between the TER94 promoter and eve enhancers.

Deletion of Homie, but not deletion of the PRE, also reduced eve-lacZ expression driven by the eve 3’ enhancers (Figures 5, S5). We considered the possibility that because the TER94 promoter has access to eve enhancers in the absence of Homie, the resulting promoter competition might reduce eve promoter activity. However, in ΔHomie lines where we see TER94 expressed in eve stripes, there is no apparent bias in expression toward the 3’ enhancers (Figures 1B, S1A), arguing against this possibility. Furthermore, at later embryonic stages, eve promoter activity is reduced when both Homie and the PRE are removed (in mesoderm, CNS, and APR, which are all the tissues where eve is expressed at these stages, Figure 5A), but this is not accompanied by TER94-GFP expression in an eve pattern (Figures 1B, S1A). Finally, when the TER94 promoter is removed along with Homie, pattern disruptions persist (Figure 5B). While we cannot rule out competition with other promoters in the genome, these lines of evidence together suggest that promoter competition is unlikely to be responsible for this effect.

A second possible explanation for the reduction in eve 3’ enhancer-promoter communication when Homie is deleted is that a 3-dimensional (3-D) conformation that allows the eve promoter to better access the 3’ enhancers is stabilized by the presence of Homie. One possible conformation is a loop between the eve promoter region and Homie (Figure 6). Although we have not tested this directly, evidence consistent with this model is that activation of promoters, including the eve promoter, by downstream Gal4 binding sites can be facilitated by heterologous insulators in a model transgene assay [76]. This possible pairing of Homie with the eve promoter region would result in a loop that would bring the 3’ enhancers in closer proximity to the promoter. Such a model is similar to that proposed for the 3-D organization of regulatory regions upstream of the Abd-B gene [25]. If such loops are anchored to large clusters of insulator proteins, perhaps within insulator bodies, this may serve as a 3-D barrier that separates distinct chromatin domains, and occludes interactions between regulatory elements located on opposite sides of the insulator. At the same time, otherwise distant elements can be brought close together, facilitating specific enhancer-promoter contacts, particularly if those elements are brought to the same side of the 3-D barrier.

Implications for Other Insulators and PREs throughout the Genome

The activities of Homie and the eve PRE are largely interchangeable with those of other insulators and PREs, respectively, in our assay system. Previous studies showed that Homie and the eve PRE have the canonical properties of insulators...
and PREs when tested in other contexts [51,55]. Thus, our results are likely to be applicable to many such elements throughout the genome. In particular, a common function of insulators is likely to be to limit the action of PRE-dependent repressive chromatin.

Genome-wide studies using RNAi to knock down specific insulator proteins suggested that insulators may not typically be required in their normal context either to block enhancer-promoter cross-talk or to prevent the spread of repressive chromatin [34,36]. Our results suggest that Homie is critically important in its normal context for just such activities, functionally separating the loci on either side. Importantly, other insulators function in place of Homie. This suggests that the activities of insulators defined in model transgene assays do in fact correspond to their normal functions. In particular, as with Homie and the TER94 promoter, the tendency of insulator proteins to cluster just upstream of promoters suggests that one of their typical functions is to shield basal promoters from the effects of upstream CRMs, especially PREs. Further, our finding that insulators facilitate enhancer-promoter communication in this context suggests that their ability to organize chromosomal conformations that augment appropriate transcription is also likely to be a common mode of endogenous insulator function.

Materials and Methods

Plasmid Construction and Transgene Production

The eve-TER94 locus construct ("intact t'gene" in figures) was created as follows (detailed sequence coordinates are given in Figure S6). DNA from −6.4 kb to +166 bp relative to the eve TSS was fused to the lacZ coding region. The S′ end of the lacZ coding region was fused to DNA from +1.3 to +11.4 kb, which includes the eve poly-A signal, and extends into the 3rd exon of TER94. This was joined with the EGF coding region, followed by the poly-A signal of α-tubulin. The entire construct was placed between two inverted attB sequences [63,77]. The following deletions were then made in this construct: from +8.4 to +9.2 kb for ΔPRE, from +8.4 to +9.7 kb for ΔHomie ΔPRE, and from +9.2 to +9.7 kb for ΔHomie. To test promoter competition between eve and TER94, DNA from −7.4 to +8.6 kb relative to the eve TSS was used, with the eve coding region replaced by that of lacZ as described above. This construct does not contain the TER94 promoter.

Replacements of Homie with either heterologous insulators or phage λ DNA were created using the ΔHomie construct, and adding DNA fragments corresponding to gypsy [78], Fab-7 [79–82], Fab-8 [83,84], ms [85,86], scs′ [85,86], or λ DNA (see Figure S6 for details). For testing repression activity of heterologous PREs, either the engiined 181PRE [87] or the bsd PRE [88] were inserted into the ΔHomie ΔPRE construct at the site of deletion. For testing Homie activity against these PREs, either the eve PRE or the bsd PRE were inserted into the ΔPRE construct at the site of deletion.

All transgenic lines were made using φC31 recombinase-mediated cassette exchange (RMCE) [63]. Three alternative attP target sites were used, at cytological locations 93E5, 74A2, and 30B3. The direction of each insertion was determined by PCR. Both directions were analyzed if obtained. Some variations with insertion site were found, as described in Results.

Analysis of Gene Expression in Embryos and Ovaries

Embryos were collected at time points described in figure legends, and subjected to in situ hybridization using DIG-labeled anti-sense RNA probes against either lacZ or GFP. Expression patterns were visualized by alkaline phosphatase-conjugated anti-DIG with BCP and NBT as substrates (Roche Applied Science). GFP expression was detected by fluorescence microscopy in ovaries dissected from 1–2 day-old females. In some cases, expression was also detected using anti-GFP antibody staining (Roche Applied Science), analyzed by confocal microscopy (Zeiss) of material in DAPI-containing mounting medium.

Chromatin Immunoprecipitation

Ovaries were dissected from 2–3 day-old females. Fifty ovaries were cross-linked in 1.8% formaldehyde in PBS for 10 min. After sonication so as to produce a peak near 500 bp in the DNA fragment size distribution, isolated chromatin was immunoprecipitated with anti-H3K27me3 (EMD Millipore), and with rabbit IgG (Jackson ImmunoResearch) as a negative control. Precipitated chromatin samples were collected using ProteinG magnetic beads (EMD Millipore). Immunoprecipitated DNA samples were dissolved in 20-50 μl TE, and 1 μl was used for each PCR reaction. Either duplicate or triplicate samples were analyzed by real-time PCR (Life Technologies, StepOnePlus), using SYBR Green Master Mix with ROX dye (Roche Applied Science). Data analysis were analyzed with StepOne software (Life Technologies), using the standard curve method. Standard deviations were calculated using Excel software (Microsoft). Embryo ChIP analysis was described previously [51], except that results were quantified by real-time PCR, as described above for ovary analysis.

Specific ChIP signals were determined by subtracting the average non-specific IgG signal from the average α-H3K27me3 signal, with standard deviations combined by adding. Errors bars for species-specific signals relative to that of endogenous eve were determined by adding the relative errors in quadrature; that is, by taking the sum of the squares of the relative standard deviations (the standard deviations divided by their respective averages) to give the square of the relative standard deviation of the ratio.

The following primers were used: TCCAGTCCCGGATAACCTCTTGTGAAC and TGGTAGAATCTCCTTCTGCAGCGACGAGCGCUATGATCATCTGCCTGGTT for the endogenous eve coding region, TGAAGCAGGCGCTGTTAATCTTTCAT and TTTGGACATGTATCCTCCGGTCCGGTT for the endogenous TER94 coding region, GCTGTGGCGGATAATGGTCCCAAAGC and TACTGGACGAAAGCGGCTGCCGATAT for the transgenic eve-lacZ coding region, and TGGCGGATGTTAAGTTCACCTTG for the transgenic TER94-GFP coding region.

RT-PCR

Total RNA was purified from either five pairs of ovaries from 2–3 day-old females or 10–20 μl of dechorionated embryos for each data point, using a RNA purification kit (Roche Applied Science). RNA was eluted in 50–100 μl elution buffer and stored at −80°C. cDNA was synthesized using the Transcripter first strand cDNA synthesis kit (Roche Applied Science), and quantified by real-time PCR as described above. A constitutively expressed RNA, RpL32 (a.k.a. RPM49), was used to normalize GFP RNA levels. The primers listed above for TER94-GFP were used for GFP, and AAGCCCAAAGGTATGCAGACAGA and TGGACAGAATTGTGCACCGTCCG for the transgenic eve-lacZ coding region.

Supporting Information

Figure S1 Homie shields the TER94 promoter from eve PRE activity. A, B: similar effects on TER94 promoter activity are seen at two chromosomal landing sites (different from that used in Figure 1), where deletion of Homie causes a loss of ubiquitous
activity, and further deletion of the PRE restores ubiquitous expression. Some differences are seen at the landing site shown in B, mostly attributable to a lower expression level from all three transgenes, which makes the eve-like expression (visible in the other lines when Homie is deleted) difficult to detect. The attP sites used are at cytological locations 74A2 (A) and 30B5 (B). A longer staining reaction time than that used in B, “ΔHomie”, showed both expression in stripes and in eve-expressing cells of the mesoderm (not shown). (TIF)

Figure S2 When the transgenic eve-TER94 locus is heterozygous and paternally derived, embryonic TER94-GFP expression is repressed in the absence of Homie as it is in ovaries, but this repression is not dependent on the 3′ PRE. Expression of GFP RNA in the transgenic lines indicated on the left from two different transgene landing sites (in A and B) is visualized at 3 different embryonic stages (indicated along the top) by in situ hybridization. The levels of GFP RNA (extracted from 2–3 hr-old embryos), quantified in triplicate, are shown on the right (averages with standard deviations, see Materials and Methods). A: In the top 3 rows and in the graph, expression is exclusively zygotic (mothers did not carry a transgene; the paternal transgene is at the same insertion site, 95E5, as that shown in Figure 1), while the bottom row shows, for comparison, maternal plus zygotic expression (both mothers and fathers were homozygous for the transgene) when Homie is deleted. Note that without Homie, the expression pattern is similar (albeit weaker) in heterozygotes as in homozygotes, consistent with the maternal contribution being repressed in the absence of Homie. This similarity includes eve-like stripes at all 3 stages, as well as eve-like mesodermal expression (dots in the dorsal-most part of each segment near the center of the germ-band extended embryo at stage 11). The quantitation shown in the graph suggests that ubiquitous zygotic expression is repressed when Homie is deleted. However, unlike for maternally derived expression (as shown in Figures 1, S1, and 2), this repression is not dependent on the PRE. This is consistent with there being other PREs that are active in embryos, possibly within the transgenic eve locus (see Results and Discussion). B: zygotic expression from a second transgene landing site (cytological location 74A2). Note that at this landing site, eve-like stripe expression is present from the intact transgene, indicating that some eve enhancers can work on the TER94 promoter when Homie is present, at least at some landing sites. Quantitation, shown in the graph, indicates that overall zygotic expression is reduced when Homie is deleted, consistent with repression of ubiquitous expression driven by TER94 enhancers present in the transgene. As with the other landing site, this repression is not dependent on the PRE, but may be due to redundant PRE activity. (TIF)

Figure S3 TER94-GFP is expressed throughout oogenesis in both germ cells and somatic epithelial follicle cells. Ovarioles dissected from female flies (carrying transgenic TER94-GFP) in the context of the transgene diagrammed in Figure 1A inserted at cytological location 95E5 aged 24–48 hours after eclosion were stained using antibodies to GFP (red) and DAPI (blue). Individual egg chambers were optically sectioned using confocal microscopy. Imaging of the surface (left column) shows mostly ovarian follicle cells, while imaging of the interior (right column) shows ovarian follicle cells at the periphery, and nurse cells and the oocyte in the central region. Note that GFP is strongly expressed in all cells from the intact transgene (top row), but not significantly above background in any cells when Homie is deleted from the transgene (bottom row). (TIF)

Figure S4 Homie blocks PRE action in ovaries. Fluorescence in ovarioles from transgenic lines carrying the indicated transgenes, at two different chromosomal insertion sites (left column, cytological location 74A2; right column, 30B5), distinct from that shown in Figure 2 (corresponding to those shown in Figure S1). Note the same trend, wherein deletion of Homie causes severe repression of fluorescence from transgenic TER94-GFP, while additional deletion of the PRE causes restoration of TER94 promoter activity. The graph at the bottom shows, on a log scale, the results of quantitation, as in Figure 2, of GFP RNA from ovarioles of the line shown above it in the same column. Note that GFP RNA levels decrease about 1000-fold when Homie is deleted, and are partially restored (about 200-fold) by additional deletion of the PRE. (TIF)

Figure S5 Homie facilitates eve 3′ enhancer action on the eve promoter. Expression of lacZ RNA driven by the eve promoter from the transgene diagrammed in Figure 1A and its derivatives was monitored by in situ hybridization. A, B: results from two different transgene landing sites (cytological locations 74E2 and 30B5, respectively), distinct from that shown in Figure 5A. Representative embryos at three stages are shown. Note that when Homie is deleted (“ΔHomie”), stripes 1, 4, 5, and 6 are weakened relative to stripes 2, 3, and 7 (left column, stage 5). This effect is still seen when both Homie and the PRE are deleted (“ΔHomie ΔPRE”), and occurs at both landing sites. In addition, mesodermal expression at stage 11 (middle column), as well as CNS and APR expression at stage 13 (right column), are weakened when Homie is deleted. These effects also persist when both Homie and the PRE are deleted, and are seen at both landing sites. C: results from two of the Homie-replacement lines shown in Figure 3. Note that gypsy, but not scs, facilitates 3′ enhancer activity. (TIF)

Figure S6 Construct details. In A are listed the details of construction of the eve-TER94 pseudo-locus transgene and its primary derivatives, as described in Materials and Methods. In B are listed the details of modifications to that construct, as described in Materials and Methods, Results, and figure legends. (TIF)

Acknowledgments
We thank our anonymous reviewers for excellent suggestions on improving the manuscript.

Author Contributions
Conceived and designed the experiments: MF JBJ. Performed the experiments: MF GS JBJ. Analyzed the data: MF JBJ. Wrote the paper: MF JBJ.

References


