Comparing Enhancer Action in *Cis* and in *Trans*

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ABSTRACT Studies from diverse systems have shown that distinct interchromosomal interactions are a central component of nuclear organization. In some cases, these interactions allow an enhancer to act in *trans*, modulating the expression of a gene encoded on a separate chromosome held in close proximity. Despite recent advances in uncovering such phenomena, our understanding of how a regulatory element acts on another chromosome remains incomplete. Here, we describe a transgenic approach to better understand enhancer action in *trans* in *Drosophila melanogaster*. Using phiC31-based recombinase-mediated cassette exchange (RMCE), we placed transgenes carrying combinations of the simple enhancer GMR, a minimal promoter, and different fluorescent reporters at equivalent positions on homologous chromosomes so that they would pair via the endogenous somatic pairing machinery of *Drosophila*. Our data demonstrate that the enhancer GMR is capable of activating a promoter in *trans* and does so in a variegated pattern, suggesting stochastic interactions between the enhancer and the promoter when they are carried on separate chromosomes. Furthermore, we quantitatively assessed the impact of two concurrent promoter targets in *cis* and in *trans* to GMR, demonstrating that each promoter is capable of competing for the enhancer's activity, with the presence of one negatively affecting expression from the other. Finally, the single-cell resolution afforded by our approach allowed us to show that promoters in *cis* and in *trans* to GMR can both be activated in the same nucleus, implying that a single enhancer can share its activity between multiple promoter targets carried on separate chromosomes.

IN an oversimplified view of the genome, each chromosome could be considered a linear arrangement of genic units, with each gene controlled solely by nearby *cis*-acting regulatory sequences. However, extensive analyses based on DNA fluorescence *in situ* hybridization (DNA FISH) and chromatin conformation capture (3C) have shown that the three-dimensional organization of the nucleus involves physical interactions between distant genomic regions, suggesting that gene regulation is more complex than this simplified model (reviewed by Gondor and Ohlsson 2009; Naumova and Dekker 2010). Further complicating our view, long-distance interactions are not limited to genomic regions on the same chromosomes (in *cis*), but are also observed between specific loci on separate chromosomes (in *trans*) (reviewed by Williams *et al.* 2010). Through genome-wide 3C-based approaches, extensive interchromosomal interactions have been identified in yeast (Duan *et al.* 2010), *Drosophila* (Sexton *et al.* 2012), mice (Zhang *et al.* 2012), and humans (Lieberman-Aiden *et al.* 2009) and are thus a component of nuclear organization in diverse organisms.

Long-distance intra- and interchromosomal interactions can result from colocalization of unlinked genomic regions to a common nuclear compartment, including loci undergoing active transcription or that bind insulator or Polycomb Group proteins (reviewed by Bantignies and Cavalli 2011; Bulger and Groudine 2011; Dean 2011). In some cases, long-distance interactions could reflect communication between regulatory sequences that will directly affect gene expression, as is the case when a distant enhancer loops toward a promoter to activate transcription (reviewed by Bartkuhn and Renkawitz 2008; Bulger and Groudine 2011). Interchromosomal interactions that directly affect gene expression have been demonstrated in several developmental contexts, including X-inactivation (Bacher *et al.* 2006; Xu *et al.* 2006), *Ifng* expression in naive T helper cells (Spilianakis *et al.* 2005), and the establishment of replication timing of imprinted loci (Sandhu *et al.* 2009) in mice, and *IFN-β* expression in response to virus infection in humans (Apostolou and Thanos 2008). In addition, physical interactions between chromosomes have been postulated to explain epigenetic phenomena such as repeat-induced point mutation in *Neurospora* (reviewed by Galagan and Selker 2004) and paramutation in

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maize (Chandler and Stam 2004). However, our ability to identify and catalog interchromosomal interactions has grown more quickly than our ability to determine their potential impact on gene expression. Thus, a central question remains: Is it common for sequences that regulate gene expression to communicate between chromosomes when they are physically juxtaposed?

Drosophila provides an excellent model system to better understand the potential impact of interchromosomal interactions on gene regulation. In *Drosophila*, homologous chromosomes are stably paired from end to end in virtually all cells of the organism (reviewed by McKee 2004). The potential for communication between *Drosophila* homologs was first postulated >100 years ago by Nettie Stevens (Stevens 1908) and was demonstrated genetically in 1954 by Ed Lewis, who coined the term transvection to describe the phenomenon of pairing-dependent gene regulation (Lewis 1954). Transvection has since been shown to influence many genes in *Drosophila* and can lead to either upregulation or silencing of gene expression by several mechanisms (reviewed by Duncan 2002; Kennison and Southworth 2002).

In one form of transvection, an enhancer on one homolog acts in *trans* to activate a promoter on a paired homolog, leading to upregulation of a linked gene. Enhancer action in *trans* is often uncovered as an unexpected case of intragenic complementation involving specific types of mutant alleles. For example, one mutant allele may carry a nonfunctional enhancer while another carries a compromised promoter; acting alone, neither allele can support gene expression, but when paired, the remaining functional enhancer and promoter interact in *trans* to activate transcription (Figure 1A). For many genes, enhancer action in *trans* is thought to produce reduced levels of transcription relative to activation in *cis*, as evidenced by the partial complementation of mutant phenotypes that result from *trans*-action (*e.g.*, Lewis 1954; Geyer *et al.* 1990; Leiserson *et al.* 1994). However, precise quantification of specific enhancer–promoter interactions in *trans* has been difficult due to the complexity of regulatory sequences surrounding most genes.

Despite decades of genetic studies of transvection, several questions regarding enhancer action in *trans* remain. First, is the capacity to act in *trans* a common property of enhancers? Only a handful of the thousands of enhancers encoded in the Drosophila genome have been demonstrated to act in *trans*, which could reflect that this is a rare property, but could also be a consequence of the specific types of alleles required to easily observe *trans* activity. Second, are specific sequences required to promote enhancer action in *trans*? Some examples of transvection at the Abdominal-B locus require a 10-kb sequence dubbed the transvection-mediating region (*tmr*) (Hopmann *et al.* 1995), and transgenic studies have shown that the *tmr* can positively affect pairing (Ronshaugen and Levine 2004). Similarly, long-distance interactions between enhancers and promoters in *cis* can be facilitated by promoter tethering sequences (Zhou and Levine 1999; Calhoun *et al.* 2002; Calhoun and Levine 2003; Lin 2003; Akbari *et al.* 2008; Fujioka *et al.* 2009; Kwon *et al.* 2009). It is as yet unclear whether similar sequences are required for any enhancer to act in *trans* or, alternatively, whether transvection between regulatory elements is a natural consequence once chromosomes are physically paired. Finally, in light of the extensive pairing in the *Drosophila* genome, how does an enhancer choose a target promoter when simultaneously presented with promoters in *cis* and in *trans*? For several genes, the presence of a promoter in *cis* reduces the strength of enhancer action in *trans* (Martinez-Laborda *et al.* 1992; Casares *et al.* 1997; Gohl *et al.* 2008), suggesting that promoters in *cis* and in *trans* compete for an enhancer's activity. Because these observations have been largely based on mutant phenotypes in whole animals, the cellular basis for this competition is not yet clear. Does an enhancer from each cell choose either the promoter in *cis* or the promoter in *trans*? Or can a single enhancer in one cell activate both targets?

Here we describe a transgenic approach to the study of enhancer action in *trans*. Notably, the use of transgenic reporters greatly simplifies the manipulation of regulatory sequences via the use of straightforward *in vitro* cloning techniques, permitting the construction of various "alleles" with which to better understand transvection. Our approach was informed by a prior experiment that used transposons carrying *yellow* alleles, including flanking regulatory sequences, that were designed to mimic mutations known to support enhancer action in *trans* at the natural *yellow* locus. That study showed the *Drosophila* genome to be generally permissive to transvection, demonstrating the feasibility of a transgenic approach to the analysis of enhancer action in *trans* (Chen *et al.* 2002). In the present study, our goal was to construct transgenes based on fluorescent reporters such as GFP and mCherry to facilitate quantification of gene expression and to provide cellular resolution of transgene activation. Using the eye-specific enhancer GMR, we first established that simple constructs containing different combinations of enhancer, promoter, and transcriptional unit can support enhancer action in *trans*, suggesting that special sequences are not required to mediate this form of transvection. Furthermore, we showed that GMR action in *trans* occurs in a variegated pattern in cells of the eye disc. Variegation was not seen when GMR acts in *cis*, even when the distance between the enhancer and the promoter was increased, suggesting that stochastic interactions between the enhancer and the promoter are specific to enhancer action in *trans*. Finally, we quantitatively assessed the impact of promoter competition on GMR function, supporting a model wherein promoters in *cis* and in *trans* will both compete for an enhancer's activity.

Materials and Methods

Fly stocks and husbandry

Flies carrying the recombinase-mediated cassette exchange (RMCE) target cassette P[attP.w+.attP] at positions 37B and 53F were previously described (Bateman and Wu 2008); the

target line 38F is an insertion of the same target cassette at polytene band 38F. All insertions are intergenic. Flies were maintained at 25° on standard *Drosophila* cornmeal, yeast, sugar, and agar medium with *p*-hydroxybenzoic acid methyl ester as a mold inhibitor (Morris *et al.* 1998).

Plasmid construction

The RMCE donor vector piB-GFP was previously described (Bateman *et al.* 2006). To create GMR-GFP carrying the Complete^{gfp} construct, the GMR enhancer was amplified by PCR from the plasmid pGMR (a gift from J. Settleman), using primers 5xGMR1 and 5xGMR2 carrying additional *Cla*I recognition sequences at their 5′ ends (note that two additional cloning steps below make use of the same primers that instead carry *Bam*HI or *Asc*I sites), and subcloned into pcr2.1 using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). The enhancer was then digested from pcr2.1 and inserted upstream of the minimal *hsp70* promoter in piB-GFP using *Cla*I.

To create GMR-lacZ carrying the Complete^{lacZ} construct, we first digested piB-GFP with *Xba*I and *Hin*dIII to remove all promoter, GFP, and UTR sequences. We then isolated a 4.6-kb *Spe*I-*Hin*dIII fragment carrying the minimal *hsp70* promoter, the β-galactosidase open reading frame, and the SV40 3′-UTR from the plasmid pCaSpeR-hs43-lacZ and cloned it into the remaining piB-GFP backbone to create piB-lacZ (consisting of the lacZ transgene flanked by phiC31 attB sequences to allow for insertion into RMCE sites). The GMR enhancer was then cloned into a *Bam*HI site upstream of the *hsp70* minimal promoter.

To create the Promoterless construct, a 4.5-kb *Sal*I fragment consisting of the β-galactosidease open reading frame and the SV40 3′-UTR but omitting the minimal *hsp70* promoter was isolated from pCaSpeR-hs43-lacZ. This fragment was cloned into the *Sal*I-digested backbone of piB-GFP, producing piB-lacZ(P-), carrying a promoterless lacZ transgene flanked by attB sites. Next, we introduced a minimal *hsp70* promoter flanked by loxP sites that could be removed *in vivo* by treatment with the Cre recombinase. We amplified the *hsp70* promoter from piB-GFP using loxTATA1 and loxTATA2, subcloned this fragment into pcr2.1 using a TOPO-TA kit, and then liberated the loxP-promoter-loxP fragment from pcr2.1 using *Hin*dIII and inserted it into a unique *Hin*dIII site upstream of the lacZ gene in piB-lacZ(P-) to create piB-LTL-lacZ. Finally, the GMR enhancer was inserted into a *Bam*HI site upstream of the loxP-promoter-loxP cassette to create GMR-LTL-lacZ. Once integrated into flies via RMCE (see below), the final Promoterless transgene was created by removing the promoter between the two loxP sites via crosses to flies carrying the Cre recombinase on the balancer CyO (Siegal and Hartl 1996). Promoter deletions were verified by PCR using primers Pry1 and RNXG2.

To create GMR-mCherry carrying the Complete^{mC} construct, we first used gene splicing by overlap extension (SOEing) (Horton *et al.* 1990) to combine an mCherry ORF from pmCherry (Clonetech) with the minimal *hsp70* promoter. Briefly, the *hsp70* promoter was amplified from piB-GFP using cherrySOE1_for and cherrySOE2_3RC_rev primers, and the mCherry ORF was amplified using cherrySOE3_2RC_for and cherrySOE4_rev. The two products were combined in a PCR reaction containing cherrySOE1_for and cherrySOE4_rev, and the resulting product was subcloned into pcr2.1 via TOPO-TA cloning. The *hsp70*-mCherry fragment was then digested from pcr2.1 using *Bam*HI and *Xba*I and cloned into *Bam*HI/*Xba*I-digest piB-GFP(-S) (a piB-GFP derivative generated by religation after *Xba*I/*Spe*I digestion, removing *Bam*HI, *Spe*I, and *Xba*I sites from downstream of the 3′-UTR) to create piB-mCherry, carrying the *hsp70* promoter, an mCherry ORF, and the SV40 3′-UTR flanked by attB sites. Finally, the GMR enhancer was inserted into the *Bam*HI site upstream of the promoter.

To create GMR-λ3-GFP carrying the Complete$^{+3.0}$ construct, a 3.0-kb fragment that has previously been demonstrated to lack insulator activity (Chung *et al.* 1993; Singer *et al.* 2009) was amplified from phage lambda DNA using primers lambda3kb_Bam_3 and lambda3kb_BamAsc_5, and the resulting fragment was subcloned into pcr2.1 via TOPO-TA cloning. Next, the GMR enhancer flanked with *Asc*I sites was cloned into the *Asc*I site at one end of the lambda fragment. Finally, the GMR-lambda fragment was liberated with *Bam*HI and cloned into the *Bam*HI site upstream of the promoter in piB-GFP(-S).

Site-specific transformation via RMCE

All constructs were integrated into RMCE target sites as previously described (Bateman and Wu 2008), using a genomic source of the phiC31 integrase (Groth *et al.* 2004; Bischof *et al.* 2007). Following integration, the orientation of insertion was assessed using primers lac4 or 3′Pend1, which are complementary to the 5′ and 3′ *P*-element ends flanking the RMCE cassette, respectively, and RNXG9, which is complementary to the SV40 3′-UTR. All constructs were analyzed in the same orientation with the enhancer or promoter near the 3′ *P*-element end, with the exception of the Complete$^{+3.0}$ construct, which generated only one insertion in the opposite orientation.

Tissue preparation, immunostaining, and microscopy

Eye-antennal discs were dissected from wandering third-instar larvae in 1× phosphate-buffered saline (PBS) and fixed in 4% formaldehyde (Electron Microscopy Sciences) in PBS for 20 min. Discs were then rinsed three times in PBS, 0.1% Triton X-100 (PBST). For direct visualization of fluorescent reporters, discs were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) or Fluoromount G with DAPI (Southern Biotech). For immunostaining, discs were blocked in 4% normal goat serum (NGS) in PBST for 1 hr and then incubated in primary antibodies overnight at 4°. GFP was visualized with a polyclonal rabbit anti-GFP (Invitrogen) diluted 1:2000, and Elav was visualized using a concentrated supernatent of mouse monoclonal antibody elav-9F8A9 (Developmental Studies Hybridoma Bank) at 1:400. After

primary antibody incubation, discs were rinsed and then washed 2 × 20 min in PBST, blocked in 4% NGS/PBST for 1 hr, and then incubated for 1 hr in secondary antibodies, goat anti-mouse Cy3 (Jackson ImmunoResearch) at 1:250 and goat anti-rabbit AlexaFluor-488 (Invitrogen) at 1:2000. Discs were then washed 4 × 20 min in PBST and mounted in Vectashield with DAPI or Fluoromount G with DAPI.

For low-magnification imaging, discs were visualized on an Olympus BX51, using a Media Cybernetics Evolution VF color camera. For higher-magnification imaging, samples were visualized using a Zeiss Axioplan 2 microscope with a 510 Meta confocal laser scanning system, with optical sections collected at 0.7-μm increments. At least 10 discs of each genotype were analyzed; all discs with the same genotype showed similar patterns of expression.

Quantification of variegation

To quantify the degree of variegation in GFP *cis*- and *trans*-activation, confocal *z*-stacks of immunostained discs were first max-projected into a single-plane image. Using ImageJ imaging software, a small elliptical region of interest (ROI) was placed over cell R4 of each ommatidial cluster of the five posterior-most rows and the mean GFP fluorescence was measured for each R4 cell. Next, the mean background fluorescence, determined by averaging the mean fluorescence of 10 ROIs in nonfluorescent regions of the disc, was subtracted from each measurement. Finally, each background-corrected measurement was converted to a relative intensity by expressing it as a percentage of the highest-intensity cell in the field of view.

Reverse transcription and quantitative PCR

For each sample, eye-antennal discs were dissected from 10 wandering third-instar larvae (20 discs total) and frozen at −80°. Tissue homogenization, genomic DNA elimination, and RNA purification were carried out using a QIAGEN RNeasy plus kit according to the manufacturer's protocol. Briefly, discs were suspended in RLTplus buffer, passed through a 25-gauge needle eight times, and then spun through a qiashredder spin column. The lysate was then spun through a gDNA eliminator spin column, and the flow-through was transferred to an RNeasy spin column for RNA purification. Generation of cDNA was performed using a QIAGEN QuantiTect Reverse Transcription Kit according to the manufacturer's protocol, including pretreatment with gDNA Wipeout Buffer to eliminate any remaining traces of genomic DNA.

Quantitative PCR was carried out on a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA). For each reaction, cDNA was diluted 1:5 in water and combined with SYBR green PCR mastermix (Applied Biosystems) and primers. GFP cDNA was amplified using primers GFPRT_1 and GFPRT_2, and mCherry cDNA was assayed using primers cherryRT_1F and cherryRT_1R. Primers RP49-58F and RP49-175R targeting the housekeeping *rp49* cDNA (Moon *et al.* 2008) were used as an internal reference. Each reaction was performed in triplicate, and each genotype was assayed using at least three independent cDNA samples from separate crosses. Relative levels of transcript were calculated via the $\Delta\Delta Ct$ method, using StepOne software. Unpaired *t*-tests were performed using GraphPad Software's online *t*-test calculator (www.graphpad.com).

Primer sequences

Primers used in this study are indicated below in 5′ to 3′ orientation:

5XGMR1: GATCCCCCTAGAATCCCAAA
5XGMR2: TATACTCCGGCGCTCTTTTC
loxTATA1: AAGCTTATAACTTCGTATAGCATACATTATACGA AGTTATAAGAGCGCCGGAGTATAAATAG
loxTATA2: AAGCTTATAACTTCGTATAATGTATGCTATACGA AGTTATCTGCAGATTGTTTAGCTTGTTCAG
RNXG2: CAGCTGCGCTTGTTTATTTG
Pry1: CCTTAGCATGTCCGTGGGGTTTGAAT
cherrySOE1_for: ATGTCGAGGTCGACGGTATC
cherrySOE2_3RC_rev: ATGGTGGCGACCGGTACTCTTCTTT CTCGGTAACTTGTTGA
cherrySOE3_2RC_for: TCAACAAGTTACCGAGAAAGAAGA GTACCGGTCGCCACCAT
cherrySOE4_rev: CGGCGCTCAGTTGGAAT
lac4: ACTGTGCGTTAGGTCCTGTTCATTGT
3′Pend1: GTCGGCAAGAGACATCCACT
RNXG9: GTGGTTTGTCCAAACTCATCAA
GFPRT_1: ATTCTCGTGGAACTGGATGG
GFPRT_2: AGCTTTCCAGTGGTGCAGAT
cherryRT_1F: CCCGCCGACATCCCCGACTA
cherryRT_1R: CTGGGTCACGGTCACCACGC
RP49-58F: TACAGGCCCAAGATCGTGAAG
RP49-175R: GACGCACTCTGTTGTCGATACC.

Results

Our goal was to better understand enhancer action in *trans* and its relationship to expression in *cis* by comparing these modes of gene activation under controlled conditions. We reasoned that a transgenic approach would be advantageous, permitting us to easily manipulate the identities of the transgene's components, to vary simple parameters such as deletions and relative distances between regulatory regions, and to accurately quantify levels of gene expression. We chose to focus our analysis on the simple enhancer GMR, which consists of five tandem binding sites for the transcription factor Glass and drives tissue-specific expression of reporter constructs in the photoreceptors, or R cells, posterior to the morphogenetic furrow of the developing eye (Moses and Rubin 1991).

To assess *cis* expression, we created a "Complete^{gfp}" reporter carrying GMR, the *hsp70* minimal promoter, and an open reading frame encoding GFP fused to the SV40 3′-UTR. To address *trans* expression, we designed "Enhancerless" and "Promoterless" transgenes that were modeled after alleles demonstrating transvection at other *Drosophila* genes, with the former construct lacking an enhancer and the latter lacking a promoter

(Figure 1). Additionally, we designed the Enhancerless construct to encode GFP and the Promoterless construct to encode β-galactosidase to differentiate potential expression of one transgene from the other. Importantly, neither the Enhancerless nor the Promoterless transgene is capable of reporter gene expression in isolation. We anticipated that pairing of the two nonfunctional transgenes would permit GMR to activate the *hsp70* promoter in *trans*, producing GFP fluorescence specifically in developing R cells.

We began by integrating our transgenes into an RMCE target site at polytene band 53F (Bateman and Wu 2008). As expected, the Complete^{gfp} reporter produced robust GFP expression in mature retinal cells posterior to the morphogenetic furrow of third-instar eye discs, whereas neither the Enhancerless nor the Promoterless transgene alone produced detectable GFP (Figure 2A, data not shown). Next, we established crosses to produce progeny carrying paired Enhancerless and Promoterless constructs in *trans* to one another at 53F. Excitingly, we observed GFP fluorescence specifically in the photoreceptors of the developing retina, consistent with our prediction if GMR were to function in *trans*. To quantify relative levels of expression, we isolated RNA from whole eye-antennal discs expressing GFP under the control of GMR in *cis* or in *trans* and prepared cDNA for quantitative RT-PCR. We found that total levels of GFP transcript in the eye-antennal disc were strongly diminished when GMR acted in *trans*, with 2.2% [95% confidence interval (C.I.), 2.0–2.3%] of transcript levels relative to discs where GMR acted in *cis* (Figure 2B).

To ensure that our observations were representative of other genomic regions, we integrated the same constructs into RMCE targets located at polytene bands 37B and 38F and observed nearly identical patterns of expression in both *cis* and *trans* (Figure 2A and Supporting Information, Figure S1). Additionally, quantitative RT-PCR showed that relative levels of GFP transcripts from constructs inserted at 37B were similar to those from transgenes at 53F, with 1.7% [95% C.I., 1.6–1.8%] of total GFP transcript in eye discs where GMR acts in *trans* relative to those where activation is in *cis* (Figure 2B). Notably, expression in *trans* was dependent on pairing of Enhancerless and Promoterless transgenes, as flies carrying an Enhancerless transgene at 53F and a Promoterless transgene at 37B produced no detectable fluorescence (data not shown). We conclude that the enhancer GMR, and therefore the transcription factor Glass that is responsible for its expression, is capable of activating the *hsp70* promoter in *trans*. Furthermore, as our transgenes carry only minimal enhancer and promoter elements in addition to exogenous reporter sequences, we infer that, once homologous chromosomes are paired, GMR and the *hsp70* promoter are capable of communicating in *trans* without the need for additional sequences (see *Discussion*).

GMR action in trans is variegated

In eye discs where GMR acts in *trans*, we noted that the pattern of GFP expression was uneven, or variegated, in cells

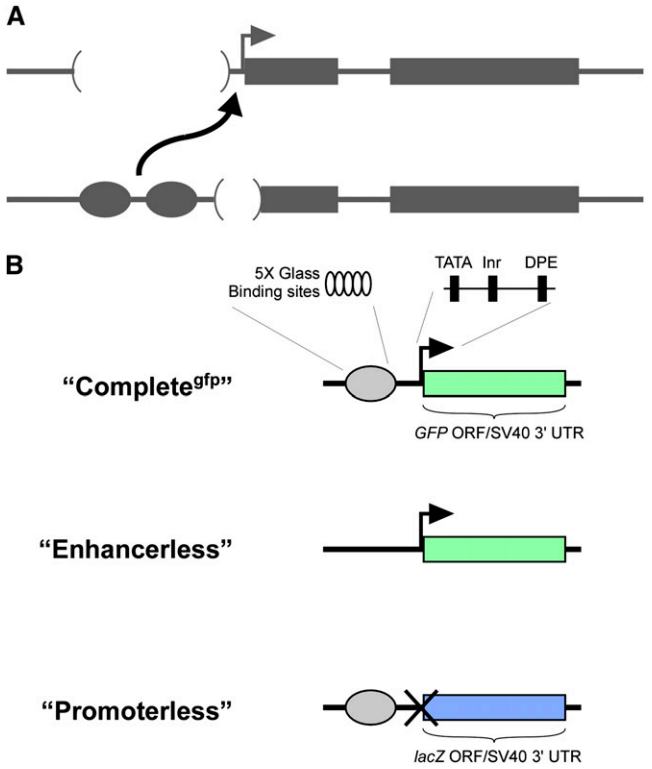

Figure 1 Schematic view of constructs used to establish a transgenic system for transvection. (A) A schematic of typical alleles that show transvection at endogenous genes. In the top allele, the enhancers (circles) are deleted, while in the bottom allele, the promoter (bent arrow) is deleted. When paired, the remaining enhancers of the bottom allele act in *trans* (curved arrow) on the intact promoter of the top allele. Boxes represent exons, and straight lines represent chromosomal DNA. (B) Schematic view of transgenic constructs inspired by the alleles in A. Complete^{gfp} carries the GMR enhancer (gray oval), consisting of five tandem Glass binding sites, the *hsp70* promoter (black bent arrow), which has TATA, Inr, and DPE core promoter elements, and a transcriptional region consisting of a GFP ORF fused to the SV40 3′-UTR (green box). The Enhancerless construct is identical to Complete^{gfp} with the omission of GMR sequences. The Promoterless construct carries GMR, with no promoter (indicated by an uppercase X), and replaces the GFP ORF with that of lacZ (blue box). All three constructs are flanked by phiC31 attB sites to allow for site-specific transformation via RMCE.

across the eye disc (Figure 2A). The variegated expression was unlikely to result from a position effect as has been observed for some transgenic insertions near heterochromatic regions, because we noted a similar pattern for *GMR* expression in *trans* at all three genomic locations tested, whereas GMR action in *cis* produced no variegation. Furthermore, we found no evidence that the variegated expression produced when GMR acts in *trans* was altered by reduction of mRNA encoding the heterochromatin protein HP1, nor did we observe any evidence of variegated eye pigmentation for insertions of *mini-white* or a *white* transgene under the control of the *hsp70* promoter in the same location (Figure S2, Figure S3, and data not shown). Thus, the variegated pattern appears to be specific to *GMR* action in *trans*.

To better understand the variegated phenotype, we used confocal microscopy to produce high-magnification three-

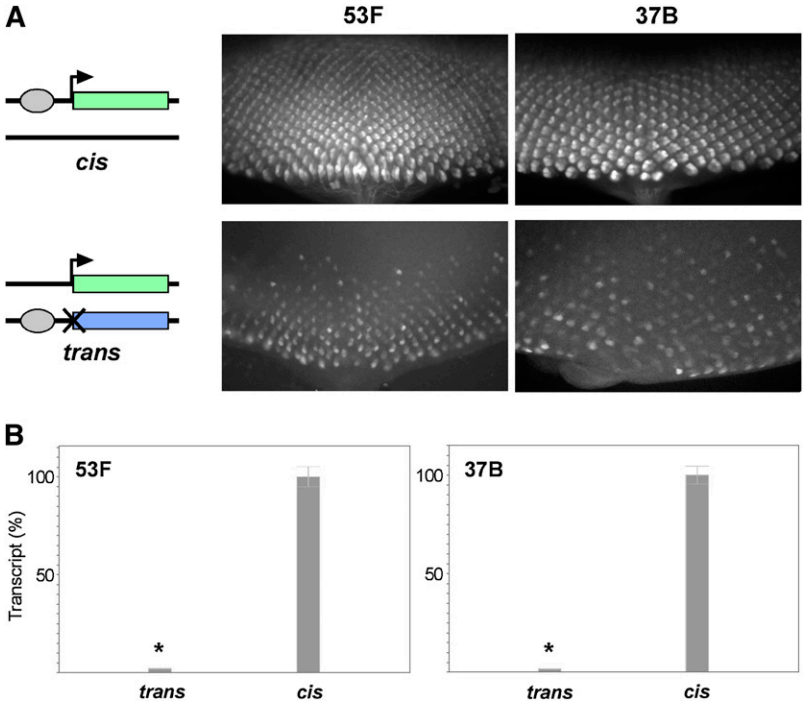

Figure 2 GMR activates the *hsp70* promoter in *trans*. (A) Schematics of genotypes and images of GFP expression in third-instar eye discs. Top row, GFP fluorescence resulting from *cis*-activation by an insertion of the Complete^{gfp} on one homolog. Bottom row, GFP fluorescence from *trans*-expression of GFP in flies with the Enhancerless construct on one homolog and the Promoterless construct on the other. Transgenes were placed at an RMCE target at polytene position 53F (left) or 37B (right). The posterior of the eye disc is oriented to the bottom of each image. (B) Relative quantification of GMR activation of GFP in *cis* and in *trans* by quantitative RT-PCR. Expression in *cis* was defined as 100% for both 53F and 37B. Error bars indicate 95% confidence intervals. $*P < 0.05$ (*t*-test).

dimensional *z*-stacks of developing retinal cells expressing GFP under the control of GMR in *cis* and in *trans* (Figure 3). The retinal photoreceptors undergo a stereotyped pattern of development as the morphogenetic furrow passes from posterior to anterior across the eye disc, resulting in a repeating pattern of ommatidial clusters, each possessing eight photoreceptor subtypes, R1–R8. All subtypes are highlighted using antibodies targeting Elav, a neuronal-specific cell marker (Robinow and White 1988) (Figure 3). In discs where GMR acts in *cis*, GFP fluorescence was evident in all of the R cells of each ommatidial cluster, with the highest levels of expression in R3 and R4. When GMR acts in *trans*, expression in R3 and R4 once again predominated, but some of these cells expressed GFP strongly, some weakly, and some undetectably.

To quantify the variegation, we assessed the relative GFP fluorescence intensity specifically in R4 cells of the posteriormost five rows of ommatidial clusters in eye discs expressing GFP in *cis* and in *trans* (see *Materials and Methods*) and plotted the data in percentile plots. For transgenes at position 53F, expression in *cis* showed a near-normal distribution, with 28.3% of R4 cells having less than half the fluorescent intensity of the brightest cells in the image and no cells having <20% of the intensity of the brightest cells (Figure 4B). In contrast, expression in *trans* showed a strong shift in the distribution of relative intensities, with 83.1% of R4 cells showing less than half the fluorescent intensity of the brightest cells; among these, 7.7% of cells showed no detectable fluorescence (defined as <1% of highest-intensity cells), which could reflect either weak expression that was below our limit of detection or a complete lack of enhancer activity. Analysis of transgenes inserted at 37B produced nearly identical results, with 83.4% of R4 cells that expressed GFP in *trans* showing less than half the fluorescent intensity of the brightest cells and 15.9% of cells showing no detectable fluorescence. Thus, GMR action in *trans* results in weak expression in the majority of R4 cells, with sporadic "jackpot" events resulting in bright fluorescence in a small proportion of the cell population.

Variegation is not reproduced by a distant enhancer in cis

We reasoned that the weak and variegated expression produced by GMR action in *trans* might be related to changes in the distance between the enhancer and the promoter when the two communicate in *trans* relative to their interactions in *cis*. Indeed, in our Complete^{gfp} transgene, the enhancer and promoter are separated by only 50 bp, which likely facilitates efficient enhancer–promoter communication. In contrast, we presumed that when GMR was carried on a separate chromosome from the promoter, the two would be less likely to lie in close physical proximity; thus, one possible explanation for the variegated pattern of GFP activation is that the increased distance between enhancer and promoter leads to stochastic activation of the promoter as the probability of interaction is decreased.

As a test of whether a simple increase in distance between GMR and the *hsp70* promoter can account for the variegated expression in *trans*, we created a variant of our Complete transgene wherein the GMR enhancer is separated from the *hsp70* promoter by 3.0 kb of DNA derived from the lambda phage genome ("Complete$^{+3.0}$" transgene), thereby increasing the distance between enhancer and promoter in *cis* relative to our initial Complete^{gfp} construct. After integrating the Complete$^{+3.0}$ construct into position 53F, we assessed total transcript levels by quantitative RT-PCR. Similar to the

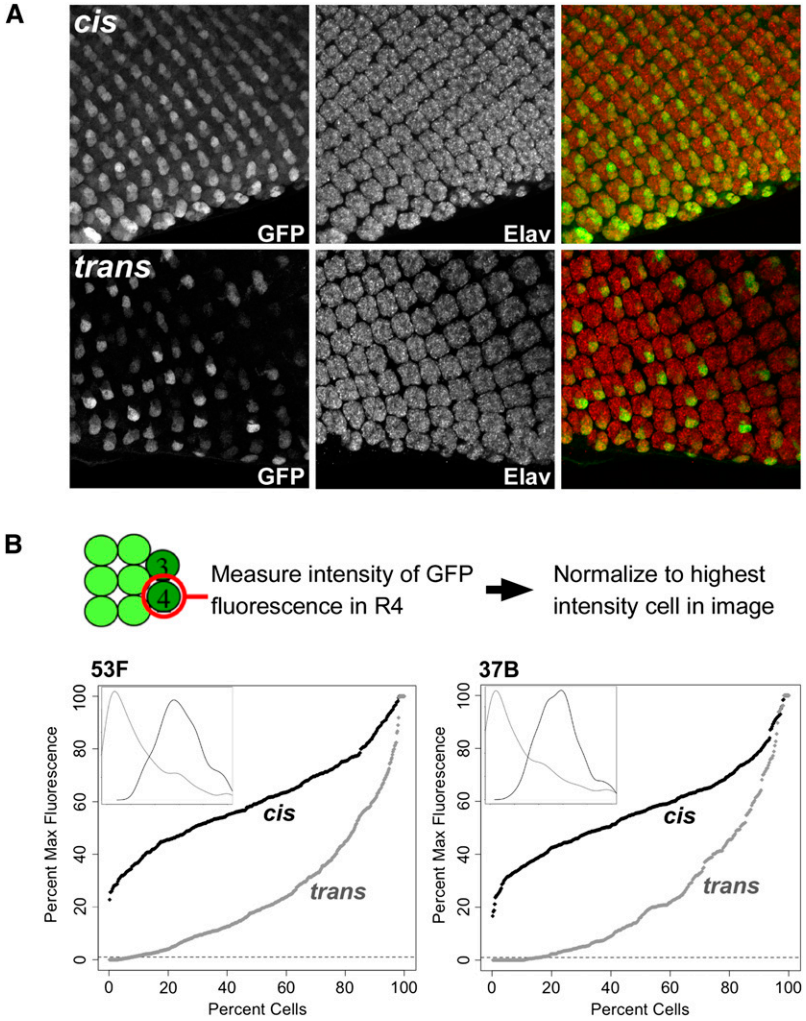

Figure 3 GMR action in *trans* is variegated. (A) Max-projected confocal *z*-stacks showing GMR-driven expression of GFP in *cis* (top) or in *trans* (bottom). Constructs are identical to those in Figure 2 and are integrated at 53F. GFP is visualized by immunostaining, and ommatidial clusters are shown by Elav immunostaining. GFP expression is highest in cells R3 and R4 in both genotypes, with *trans*-activation showing variegated expression. Posterior is oriented to the bottom of each image. (B) Quantification of relative fluorescent intensities. Top, rough schematic of cell positions in each ommatidial cluster; intensities of GFP fluorescence were measured for each R4 cell in the posterior-most five rows and normalized to the brightest cell of the image. Percentile plots show data for expression in *cis* (black) and in *trans* (gray) at 53F ($n = 427$ cells from four discs for *cis* and 532 cells from five discs for *trans*) and 37B ($n = 467$ cells from five discs for *cis* and 321 cells from four discs for *trans*). Dashed line represents a threshold of 1% of the maximum intensity, below which expression is not detectable. Insets show density plots of the same data, demonstrating the approximately normal distribution from expression in *cis* and strong leftward shift from expression in *trans*.

case when GMR acts in *trans*, we observed a reduction in transcript levels to 15.2% (95% C.I., 12.8–18.1%) relative to the original Complete^{gfp} construct (data not shown), consistent with the notion that increasing the distance between enhancer and promoter decreases the efficiency of transcriptional activation. However, when we examined discs carrying Complete$^{+3.0}$ by microscopy, the pattern of GFP did not appear variegated; instead, we observed a "delayed" pattern of expression where GFP fluorescence was primarily observed in the posterior-most ommatidial clusters that were the earliest to commit to photoreceptor cell fates (Figure 4A).

To quantify this effect, we assessed the relative fluorescent intensities of R4 cells in the five posterior-most ommatidial rows as above; in this case, we isolated data from each row in separate box plots to better dissect changing levels of expression between early- and late-committing cells (Figure 4B). In discs where GMR acts in *trans*, we saw great variation in all rows; notably, some cells in the newly committed rows are among the brightest cells, whereas cells with undetectable levels of fluorescence persist in the longest-expressing rows. In contrast, discs where GMR acts at a distance in *cis* show a strong trend for increased expression over time, with all nonexpressing cells limited to the newly committed rows and all of the brightest cells found in the early-committed cells. Importantly, the distributions of relative intensities in the posterior-most row are significantly different between the two genotypes ($P = 3.7 \times 10^{-9}$, Welch's *t*-test). In sum, our data indicate that increasing the distance between *GMR* and the *hsp70* promoter in *cis* has a negative impact on transcriptional activation, but does not lead to variegation.

Promoter targets in cis and in trans compete for GMR enhancer activity

In the experiments above, the GMR enhancer was provided with just one target *hsp70* promoter provided either in *cis* or in *trans*. We next assessed how GMR would respond when given a choice between promoters in *cis* and in *trans* concurrently. First, we created a new Complete construct encoding β-galactosidase instead of GFP (Complete^{lacZ}), which is identical to the Promoterless construct except for the addition of a functional *hsp70* promoter. After targeting Complete^{lacZ} to RMCE sites at 53F and 37B, we verified that it was capable of robust expression of β-galactosidase in *cis* (Figure S4). We then placed the Complete^{lacZ} construct in *trans* to the

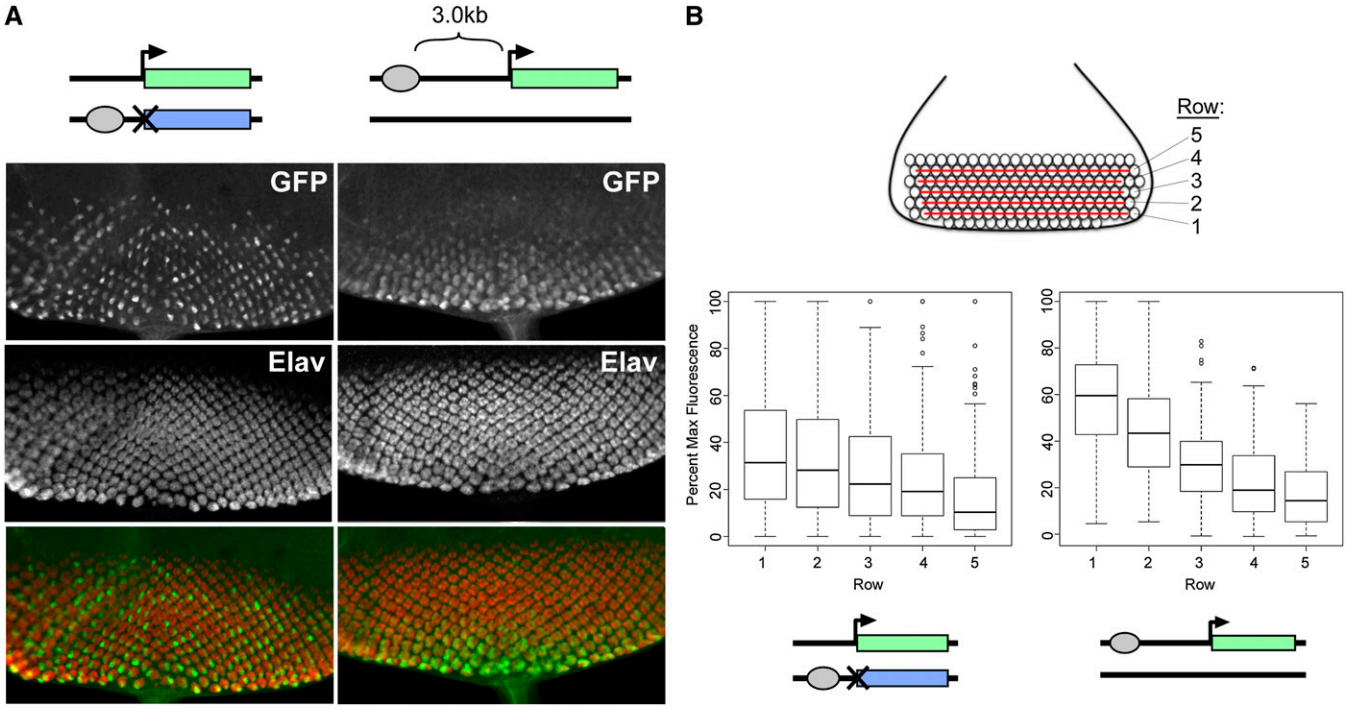

Figure 4 Increasing enhancer–promoter distance in *cis* delays GFP expression, but does not lead to variegation. (A) Discs where GMR acts in *trans* (left) or is separated from the *hsp70* promoter by 3 kb in *cis* (right). GFP is visualized by immunostaining, and ommatidial clusters are shown by Elav immunostaining. The distant enhancer in *cis* does not produce the variegated pattern of GFP fluorescence; rather, GFP expression is highest in the posterior-most cells that were the earliest to commit to the R cell fate and lowest or undetectable in the newly committing cells. (B) Quantification of relative R4 cell fluorescent intensities for GMR action in *trans* and action from a distance in *cis*. Schematic shows the positions of row 1 (posterior-most, longest committed) through row 5 that label the *x*-axis in the box plots below. Left, GMR action in *trans* (n = 984 cells from nine discs); right, GMR action in *cis* with 3 kb separation from the promoter (n = 458 cells from six discs). Outliers are indicated by white circles; note the presence of cells at 0% and at 100% of the maximum fluorescence in all rows for *trans*-action, but not for *cis*-action. A significant difference in relative intensities is observed between row 1 cells expressing GFP in *cis vs.* in *trans* by either parametric (*t*-test) or nonparametric (Mann–Whitney) statistical tests.

Enhancerless GFP construct and once again observed variegated GFP expression in the eye disc, indicating that GMR is capable of activating a promoter in *trans* in the presence of a promoter in *cis* (Figure 5A). Next, we prepared cDNA from discs carrying the Enhancerless construct in *trans* to either the Promoterless or the Complete^{lacZ} construct and quantified relative levels of GFP transcript between the two genotypes via quantitative RT-PCR (Figure 5B). At both 53F and 37B, we observed a near twofold reduction in GFP transcript levels in flies carrying the Enhancerless transgene paired with Complete^{lacZ} relative to those with the Promoterless construct [50.1% (95% C.I., 45.8–56.4%) and 66.4% (95% C.I., 59.1–74.7%) at 53F and 37B, respectively], consistent with the model that the promoter in *cis* competes for the enhancer's activity and thereby interferes with GMR's ability to act in *trans*.

Our quantitative data suggest that GMR acts on both promoters in *cis* and in *trans* in flies carrying paired Enhancerless and Complete^{lacZ} constructs. We reasoned that this could happen in one of two ways: first, that the single copy of GMR in each photoreceptor would exclusively act on one promoter, with different cells choosing the promoter either in *cis* or in *trans*. Alternatively, the enhancer in each cell could have the capacity to activate both promoters, resulting in expression from *cis*- and *trans*-promoters in the same nucleus. To distinguish between these possibilities, we created a variant of our complete construct encoding the red fluorescent reporter mCherry (Complete^{mC}) so that when Complete^{mC} was paired with the Enhancerless construct, we could easily observe expression in *cis* via red fluorescence and expression in *trans* via green fluorescence. In flies carrying Complete^{mC} alone at 53F, we observed prominent red fluorescence in all R3 and R4 cells with lower levels of fluorescence in other cells of each ommatidial cluster, similar to the expression of GFP observed from Complete^{gfp} alone (data not shown). We therefore focused our analysis on R3 and R4 where expression could be confidently scored. When Complete^{mC} was paired with the Enhancerless construct at 53F, we never observed R3 or R4 cells exhibiting green fluorescence in the absence of red fluorescence (0/~400 cells), indicating that the promoter in *cis* was not ignored in favor of the promoter in *trans* (Figure 6A). Rather, many R3 and R4 cells expressed both mCherry and GFP, indicating that a GMR enhancer in a single cell can activate promoters in *cis* and in *trans*. The lack of "green-only" cells was true even for the newly committed photoreceptors where the fluorescent reporters were first detectable (n = 56), implying either that promoters in *cis* and in *trans* are activated simultaneously by one enhancer or that potential

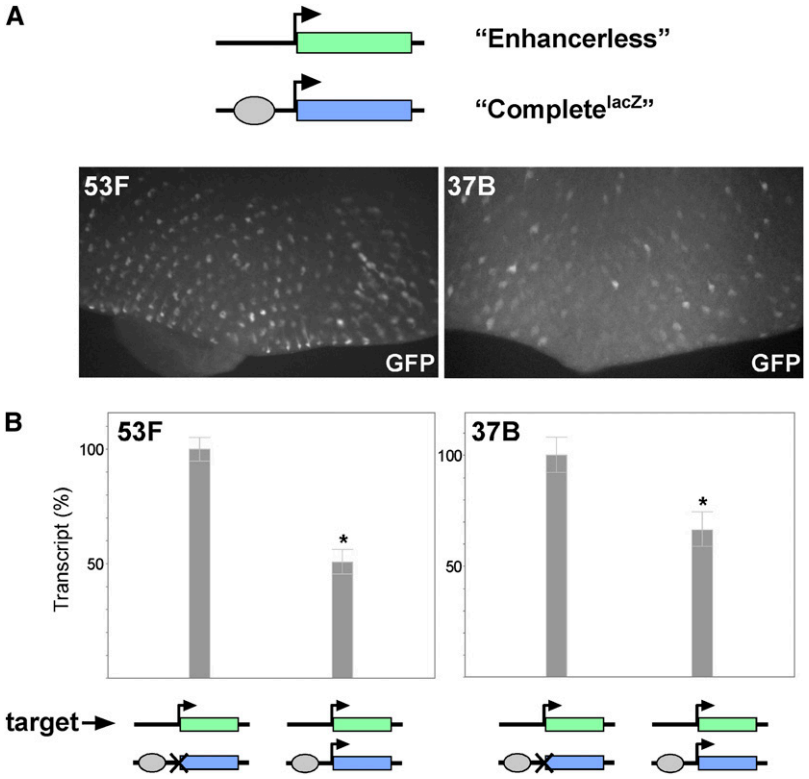

Figure 5 A promoter in *cis* decreases GMR action in *trans*. (A) Top, schematic showing the Enhancerless construct paired with Complete^{lacZ}. This combination of paired alleles produces variegated GFP fluorescence when integrated at 53F or 37B. (B) Relative quantification of GMR activation of GFP in *trans* with and without a promoter in *cis* by quantitative RT-PCR. For both 53F and 37B, total levels of GFP transcript generated from the Enhancerless construct are decreased in discs with an intact promoter in *cis* to GMR (right column) relative to those without a promoter in *cis* (defined as 100%, left column). Error bars indicate 95% confidence intervals. *$P < 0.05$ (*t*-test).

switching of the enhancer between promoters occurs rapidly (see *Discussion*).

The data above support a model where promoters in *cis* and in *trans* will compete for the activity of a single GMR enhancer when they are paired. As a final test of this model, we assessed whether the presence of a promoter in *trans* could affect GMR activation of a promoter in *cis*. To do so, we quantified levels of mCherry transcript generated from the Complete^{mC} construct either alone or when paired with the Enhancerless construct. Consistent with a competitive role for the promoter in *trans*, we observed a significant reduction in mCherry transcript levels generated in *cis* when the Enhancerless construct was paired with Complete^{mC} (83.3%; 95% C.I., 80.7–85.9%) relative to levels from the Complete^{mC} construct alone (Figure 6B). To ensure that the change in mCherry expression in *cis* was not due to possible topological changes when Complete^{mC} is or is not paired to another transgene, we also assessed mCherry transcript levels in flies carrying paired Complete^{mC} and Complete^{gfp} transgenes, where the latter differs from the Enhancerless construct only by the addition of GMR. Notably, pairing with Complete^{gfp} did not decrease transcript levels from Complete^{mC}; in fact, we observed a significant increase in mCherry transcripts by 14.5% (95% C.I., 10.0–19.4%) (Figure 6B), suggesting that the paired enhancers could augment expression from the Complete^{mC} promoter, perhaps via the concurrent action of one enhancer in *cis* and the other in *trans* (see *Discussion*). In sum, our data support a model wherein, in each cell, an enhancer has the capacity to share its activity between promoters in *cis* and in *trans*, with an overall bias toward the promoter in *cis*.

Discussion

Using a transgenic approach, we have demonstrated several key aspects of enhancer action in *trans*. First, we showed that the enhancer GMR, which had never before been tested for the ability to act in *trans*, can activate the *hsp70* promoter on a paired homolog, thereby adding a new enhancer–promoter pair to a growing list of those that can communicate in *trans*. Second, because our transgenes carried only a simple enhancer, minimal promoter, and reporter sequences derived from other organisms, it is unlikely that putative transvection-mediating sequences are required for an enhancer to act in *trans* once homologs are paired. Third, because our approach provided single-cell resolution, we were able to show that GMR action in *trans* results in a variegated pattern of expression across the cells of the eye disc. Fourth, by simultaneously presenting GMR with multiple promoter targets, we showed that the enhancer can activate promoters in *cis* and in *trans* within the same cell. Finally, the use of fluorescent reporters permitted us to precisely quantify relative levels of gene expression in the presence of varying regulatory components and to demonstrate that promoters in *cis* and in *trans* will each compete for the activity of an enhancer.

Although we expected GMR action in *trans* to produce lower levels of transcript than those resulting from expression in *cis*, we were surprised at the large divergence between the two modes of activation, with an ~50-fold difference detected at two different loci. Importantly, due to the variegated pattern of expression in discs where GMR acts in *trans*, the levels of GFP transcript assessed by quantitative RT-PCR represent

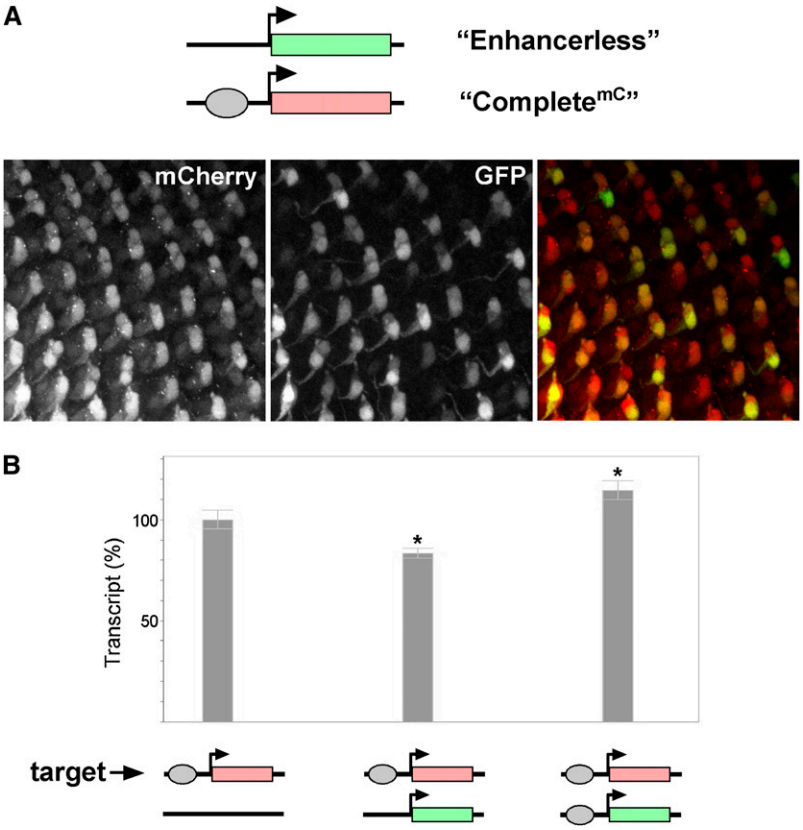

Figure 6 Promoters in *cis* and in *trans* compete for enhancer activity. (A) Top, schematic showing the Enhancerless construct paired with Complete^{mC}. GMR activates mCherry expression in *cis* in all R3 and R4 cells, including those expressing GFP, indicating that GMR can act in *cis* and in *trans* within the same cell. (B) Relative quantification of GMR activation of mCherry in *cis* via quantitative RT-PCR. Expression is reduced in the presence of a promoter in *trans* to 83.3% of transcript levels generated from the Complete^{mC} construct alone. In contrast, pairing with a Complete^{gfp} construct increases mCherry transcript levels by 14.5% relative to Complete^{mC} alone. Transcript levels from Complete^{mC} alone are defined as 100%. Error bars indicate 95% confidence intervals. $*P < 0.05$ (*t*-test) in comparison to the Complete^{mC} construct alone.

an average of mRNA from cells with little to no detectable expression and those with robust expression. It is possible that aspects of our transgenes could be optimized for higher expression in *trans*; for example, we designed our transgenes to encode different proteins to differentiate expression in *cis* and in *trans*, and the lack of homology over the coding regions may adversely affect the overall strength of *trans*-activation. However, our results may be consistent with existing data from the *yellow* locus. Here, promoter mutations show that full phenotypic complementation can be achieved with 11% of wild-type levels of *yellow* transcript generated in *cis*, implying that enhancer action in *trans*, which shows only partial complementation, contributes an even lower amount of transcript (Morris *et al.* 2004). In contrast, quantitative analyses of transvection at the genes encoding *Gpdh* and *Men* suggest that their enhancers can contribute up to 100% of normal *cis*-activity via enhancer action in *trans*, but that strength of *trans*-activity varies greatly with allele structure (Gibson *et al.* 1999; Lum and Merritt 2011). Thus, it is likely that local chromosomal environment and enhancer identity are important factors in determining the strength of an enhancer's *trans*-activity.

GMR action in trans is variegated

When GMR acts in *trans*, the majority of R4 cells express GFP at low or undetectable levels, with occasional jackpot cells producing robust fluorescence. The variegated pattern is specific to *trans* action; we saw no evidence of variegation for any transgenic construct where a reporter was controlled by an enhancer in *cis*, including a construct where GMR was separated from the *hsp70* promoter by 3 kb. The latter construct, Complete$^{+3.0}$, resulted in diminished levels of target gene expression relative to Complete^{gfp}, suggesting reduced transcriptional activation with increasing distance between the enhancer and the promoter as previously described (Dobi and Winston 2007). Although Complete$^{+3.0}$ did not show evidence of variegation, in other cases, long-distance *cis*-interactions between enhancers and promoters may produce variegated expression, as previously reported for a transgenic insertion into the engrailed locus (Kwon *et al.* 2009). It should be noted that some topological aspects of expression in *cis* may be distinct from those required for enhancer action in *trans*, complicating straightforward comparisons of expression from the Complete$^{+3.0}$ transgene to that from paired Enhancerless and Promoterless constructs; for example, the intervening sequence between an enhancer and a promoter in *cis* must bend to permit enhancer–promoter contact (reviewed by Bulger and Groudine 2011), but no such intervening sequence exists between enhancers and promoters carried on different chromosomes. Thus, aspects of enhancer action in *trans*, including local topology and patterns of gene expression, are distinct from long-distance interactions in *cis*.

Variegated and/or stochastic gene expression has been observed in other cases of interchromosomal gene regulation. For example, introduction of an ectopic human β-globin locus control region (LCR) into transgenic mice leads to interactions with and upregulation of the endogenous

β-globin genes, which are encoded on a different chromosome (Noordermeer *et al.* 2011). In this system, single-cell analysis showed that only a small number of cells have elevated levels of endogenous β-globin mRNA, suggesting stochastic activation of the native β-globin promoter. Similarly, expression of IFN-β in response to viral infection in humans is stochastic, with only a subset of the cells of a population showing detectable transcript (Zawatzky *et al.* 1985). A recent analysis has led to a model wherein IFN-β expression depends on interchromosomal associations between multiple unlinked loci, which occur only in a subset of cells (Apostolou and Thanos 2008). In the case of GMR action in *trans*, cell–cell variability in the proximity of GMR and the *hsp70* promoter may account for the variegated pattern of expression.

Because the majority of prior studies of transvection have focused on developmental phenotypes affecting adult structures, it is not yet clear whether cell–cell variability in expression will be common to other *Drosophila* genes that undergo transvection. One prior study used immunostaining to compare levels of *Ubx* expression directed by the *Contrabithorax*^{1} (*Cbx*^{1}) regulatory insertion in *cis* and in *trans* (Castelli-Gair *et al.* 1990). While this study demonstrated that expression in *trans* was limited to a reduced population of wing disc cells relative to expression in *cis*, it is not clear whether this change reflects variegation or some other patterning change. It is likely that variables including local pairing dynamics, efficiencies of promoter activation and transcriptional elongation, and mRNA or protein stability will strongly influence whether variegation will be a consequence of enhancer action in *trans*.

Cis- and trans-promoters compete for enhancer activity

Our data show that GMR is biased toward a promoter in *cis* relative to a promoter in *trans*, as evidenced by its relative strength of action in *cis vs. trans* as well as the strong reduction in *trans*-activation when a *cis*-promoter is present. Given that the sequences of the promoters in our experiments were identical, this bias likely reflects the closer proximity of the promoter in *cis*, consistent with other studies relating distance to promoter competition (Dillon *et al.* 1997; Kmita *et al.* 2002). We also found that a promoter in *trans* negatively affects expression from a promoter in *cis*, supporting a competitive role for the *trans*-promoter to the detriment of the *cis*-promoter. To our knowledge, this represents the first demonstration of a negative impact on expression in *cis* by a promoter in *trans*. We speculate that other transgenic insertions of strong promoters may attract the activity of compatible enhancers located nearby on a paired homolog, analogous to their recognized ability to trap enhancer activity in *cis* (O'Kane and Gehring 1987).

Prior genetic analyses have shown that, for many genes, enhancer action in *trans* is reduced in the presence of a promoter in *cis* (Martinez-Laborda *et al.* 1992; Casares *et al.* 1997; Gohl *et al.* 2008). Our data are consistent with the model that this reduction results from an enhancer sharing its activity between the two promoters. An apparent exception occurs at the well-studied *yellow* gene, where the presence of a functional promoter in *cis* abolishes all evidence of enhancer action in *trans*, a phenomenon termed "*cis*-preference" (Geyer *et al.* 1990; Morris *et al.* 1998, 1999). While this may reflect a novel mechanism, it is possible that *cis*-preference results from a highly skewed competition between promoters in *cis* and in *trans* such that *trans*-interactions, while permitted, are so reduced that the few transcripts resulting from enhancer action in *trans* have no impact on phenotype. According to this reasoning, the ratio of enhancer activity between promoter targets in *cis* and in *trans*, and thus the overall availability of an enhancer to act in *trans*, may vary among different enhancers and/or loci. Excitingly, these concepts can be easily tested using a transgenic system similar to that used here.

Our study demonstrates that a single enhancer can activate promoters in *cis* and in *trans* in the same cell. Due to the time delay required for detection of fluorescence and the perdurance of GFP and mCherry following translation, we were unable to determine whether GMR periodically switches from one target to the other over the time that we assayed or, alternatively, whether transcription is actively promoted simultaneously from both promoters in those cells expressing reporters in *cis* and in *trans* to the enhancer. We assayed relative fluorescent intensities of newly committed R4 cells when fluorescence was first detectable and found no evidence that the intensity of one channel was negatively correlated with that of the other channel (data not shown), suggesting that, if switching takes place, it must do so on a relatively short timescale. An analysis using reagents and strategies better suited to short-term dynamics, including observations of nascent transcripts (Kosman *et al.* 2004) or the use of destabilized fluorescent reporters (Li *et al.* 1998), may help to differentiate between these possibilities.

Finally, we note that our quantitative data show a significant elevation of expression from our Complete^{mC} reporter when paired with the Complete^{gfp} transgene that carries its own enhancer and promoter, suggesting a possible synergistic effect when "Complete" genes are paired. This synergism could result from the activities of both enhancers upon the promoter of the Complete^{mC} construct, as would be predicted if both enhancers acted on promoters in *cis* and in *trans* simultaneously. It is also possible that the nearby Complete^{gfp} construct increases the likelihood of both transgenes residing in a transcription factory, which may lead to elevated expression levels (reviewed by Bulger and Groudine 2011; Dean 2011). If the phenomenon of increased expression from paired genes is common to other enhancer–promoter interactions in *Drosophila*, it could represent a means to augment transcriptional activation across the genome when all chromosomes are paired, ensuring robust responses to environmental cues. Notably, a similar phenomenon has been reported in cells from a human renal oncocytoma, where extensive pairing between the q arms of chromosome 19 is correlated with elevated expression from genes along the length of that arm (Koeman *et al.* 2008), suggesting that increased gene expression resulting from pairing also occurs in other systems.

Transvection as a model for interchromosomal interactions

The study of transvection in *Drosophila* enjoys a rich history of genetic and cytological studies from some of the pioneers of genetics. Our recognition of the prevalence of interchromosomal interactions has continued to grow in other model systems, expanding the possibilities of potential effects on gene regulation resulting from interactions in *trans*. While the mechanisms that identify and pair homologs in *Drosophila* are not yet fully understood, they are likely to differ from many of the forces that bring together nonhomologous loci in other systems such as vertebrates. However, irrespective of the pairing mechanism, once chromosomes are brought in close proximity, the potential for *trans*-interactions between regulatory sequences is likely similar in different organisms, particularly given the strong evolutionary conservation of mechanisms for gene regulation in *cis*. Transgenic analyses of transvection in *Drosophila* have the potential to address the capacity of diverse enhancers and promoters to interact in *trans* and allow precise quantification of subtle effects on gene expression. Furthermore, the varying dynamics of pairing at different genomic locations in the *Drosophila* genome (Fung *et al.* 1998) afford the ability to contrast effects on transvection when chromosomes are stably or poorly paired. Thus, detailed analyses of transvection have the potential to inform our understanding of interchromosomal interactions in diverse systems.

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Note added in proof: In this issue of *GENETICS*, Mellert and Truman (pp. 1129–1141) describe an independent study of enhancer action in *trans* between transgenes inserted using site-specific transformation methods. In agreement with our data, they demonstrate that promoters in *cis* and in *trans* to an enhancer will compete for the enhancer's activity, with a bias toward the promoter in *cis*. Furthermore, they report that enhancer action in *trans* is limited to a smaller group of cells relative to activation in *cis*, with evidence of stochastic activation in *trans* for several enhancers.

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GENETICS

Supporting Information
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Comparing Enhancer Action in *Cis* and in *Trans*

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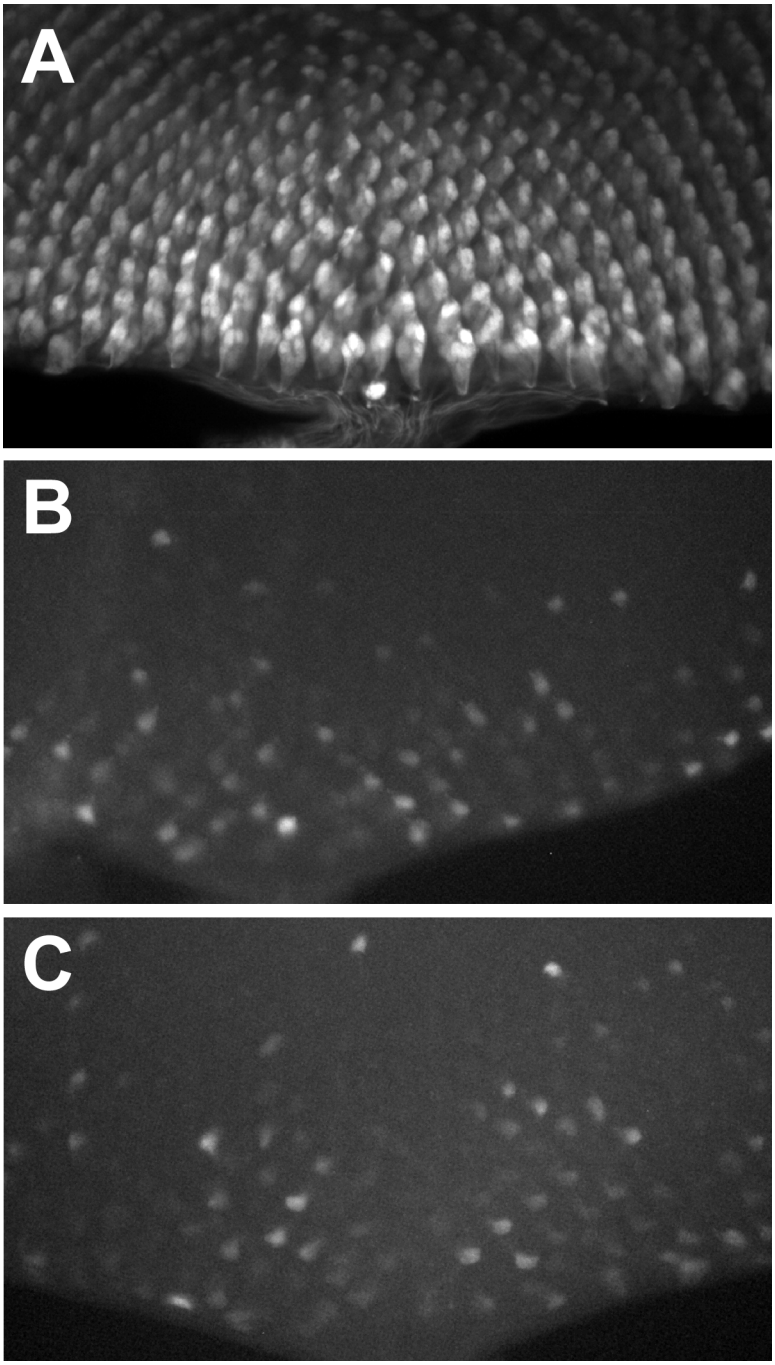

Figure S1 GMR acts in *trans* at polytene position 38F. GFP fluorescence is shown for discs where GMR acts in *cis* (A) or in *trans* (B-C). The constructs carried are (A), Complete^{gfp} alone; (B), Enhancerless construct paired with an insertion of a precursor to the Promoterless construct with a compromised promoter (see below); (C), Enhancerless paired with Complete^{lacZ}, which carries an intact promoter. In (B), the precursor is identical to the Promoterless construct except that the hsp70 promoter flanked by loxP sites has not been excised (See Materials and Methods). Quantitative RT-PCR shows that the precursor construct expresses *lacZ* in *cis* to a level of ~5% of Complete^{lacZ} (data not shown), indicating that the loxP sites compromise the function of the promoter. We therefore consider the expression in (B) to be analogous to that of Enhancerless paired with Promoterless.

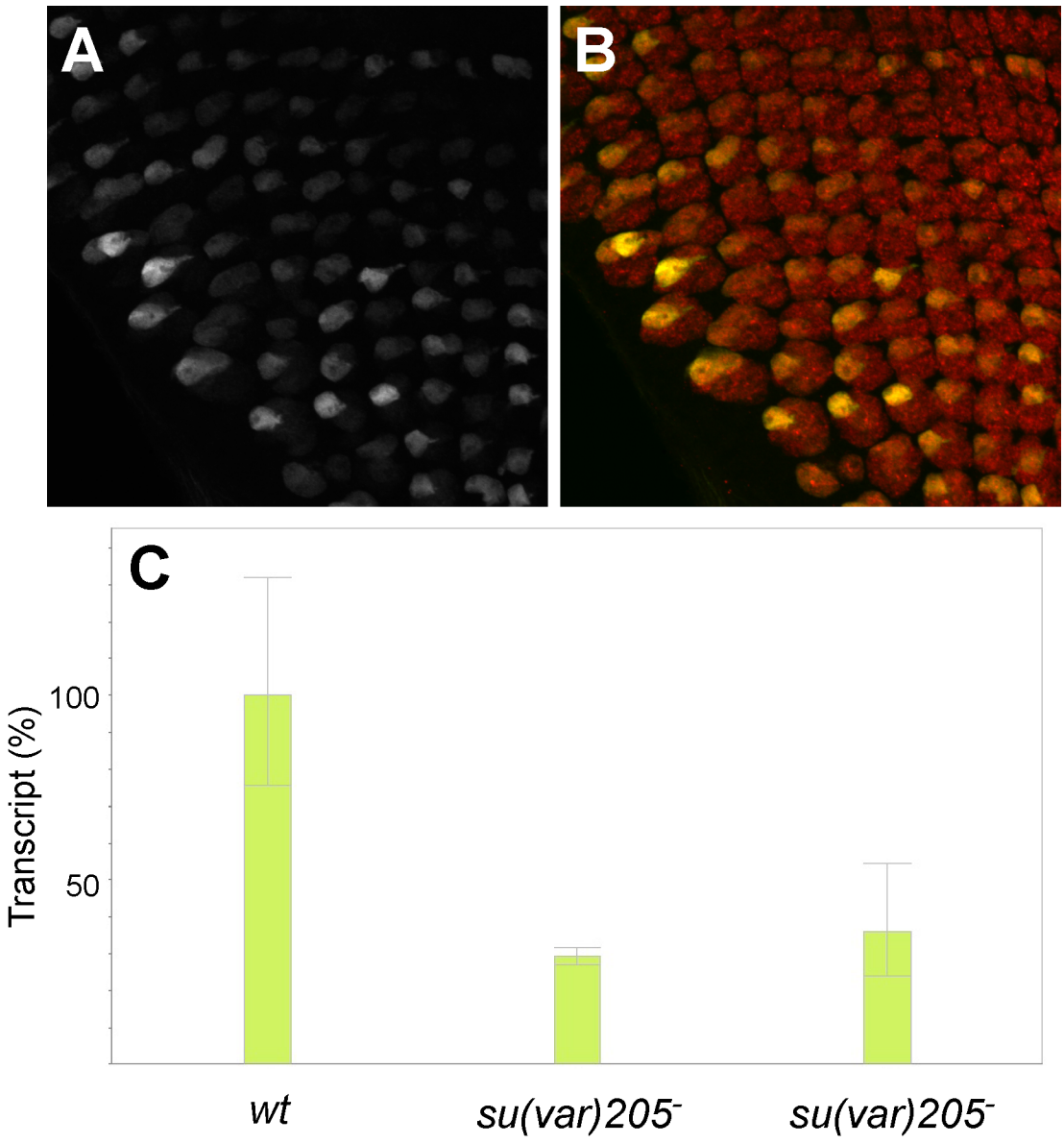

Figure S2 Variegation of GMR action in *trans* is not sensitive to reduction in HP1. (A, B) Max-projected confocal z-stacks showing discs carrying Enhancerless and Promoterless constructs at 53F in a background where *Su(var)205* (encoding HP1) expression is reduced in the eye disc via the expression of a hairpin under the control of *ey-GAL4*. (A) anti-GFP staining only; (B) merged image showing GFP in green and anti-Elav staining in red. Flies carrying the hairpin P[TRiP.HMS00278] targeting *Su(var)205* were obtained from the Bloomington Drosophila Stock Center. (C) Quantitative RT-PCR on cDNA prepared from eye-antennal discs (see Materials and Methods) using primers suvar205_RT_2F (5'-CTGCTGGCGCGTCCTTGAGT-3') and suvar205_RT_2R (5'-CAGCAGTACGAGGCGAGCCG-3') targeting *Su(var)205* exonic sequences. "*wt*", genotype *Enhancerless/Promoterless* (defined as 100%); "*Su(var)205*$^{-}$" genotype *Enhancerless/Promoterless; ey-GAL4/P[TRiP.HMS00278]*. Two independent *Su(var)205*$^{-}$ cDNA preparations derived from separate crosses show a reduction in *Su(var)205* mRNA levels to 29.2% (95% CI, 27.0-31.7%) and 36.1% (95% CI, 23.8-54.6%) relative to *wt*; note that this includes mRNA from the antennal portion of the eye-antennal disc where the hairpin is not expressed, and therefore likely represents an overestimate of transcript abundance in the eye disc. Although this represents a sizeable reduction in *Su(var)205* mRNA, we cannot exclude the possibility that HP1 protein levels are less significantly impacted by expression of the hairpin.

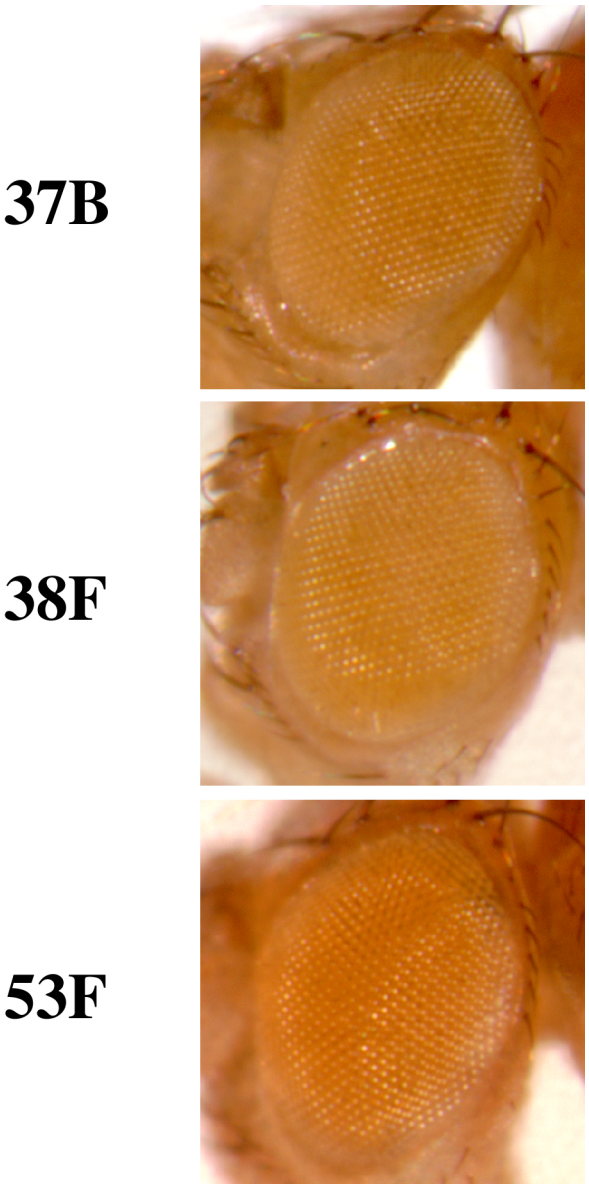

Figure S3 Lack of variegation of *mini-white* expression from RMCE targets at 53F, 37B, or 38F. Each image represents a fly carrying a single copy of the P[attP.w+.attP] target cassette, which is marked with *mini-white*. No variegation is evident.

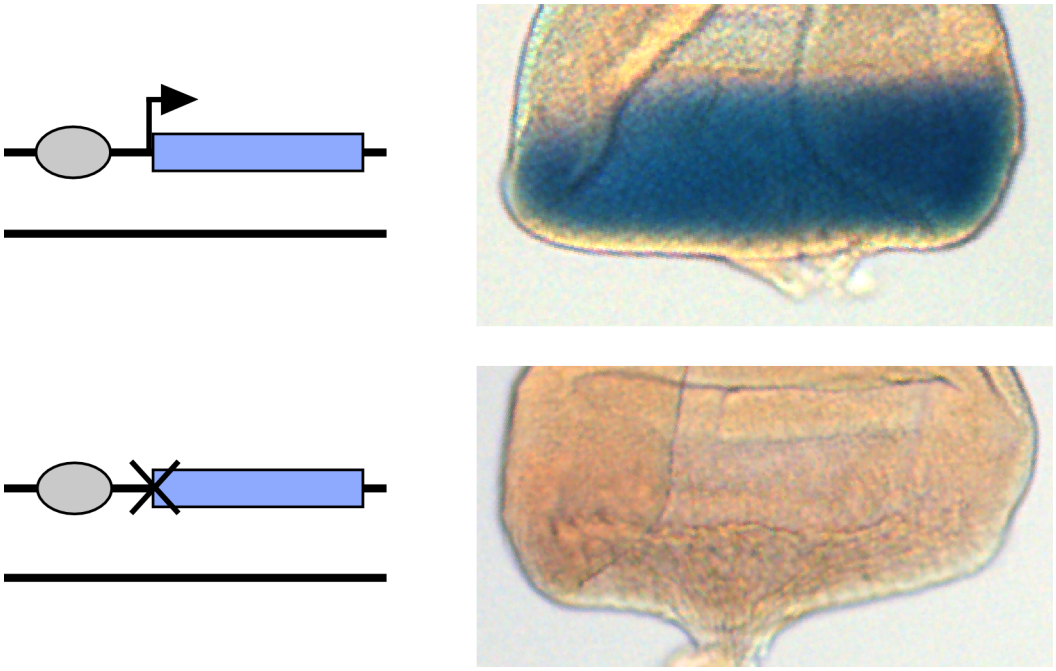

Figure S4 Expression of *lacZ* in *cis*. X-gal stained discs carrying constructs Complete^{lacZ} (top) or Promoterless (bottom) alone at 53F. In the top disc, the staining reaction was stopped after 20 minutes, whereas for the bottom disc, staining was carried out overnight. Quantitative RT-PCR indicates that expression from the Promoterless construct is <1% that of Complete^{lacZ} (data not shown).