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Enhancer-PRE communication contributes to the expansion of gene expression domains in proliferating primordia

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SUMMARY

Trithorax-group and Polycomb-group proteins interact with chromosomal elements, termed PRE/TREs, to ensure stable heritable maintenance of the transcriptional state of nearby genes. Regulatory elements that bind both groups of proteins are termed maintenance elements (MEs). Some of these MEs maintain the initial activated transcriptional state of a nearby reporter gene through several rounds of mitosis during development. Here, we show that expression of *hedgehog* in the posterior compartment of the *Drosophila* wing results from the communication between a previously defined ME and a nearby cis-regulatory element termed the C enhancer. The C enhancer integrates the activities of the Notch and Hedgehog signalling pathways and, from the early wing primordium stage, drives expression to a thin stripe in the posterior compartment that corresponds to the dorsal-ventral compartment boundary. The ME maintains the initial activated transcriptional state conferred by the C enhancer and contributes to the expansion, by growth, of its expression domain throughout the posterior compartment. Communication between the ME and the C enhancer also contributes to repression of gene expression in anterior cells. Most interestingly, we present evidence that enhancers and MEs of different genes are interchangeable modules whose communication is involved in restricting and expanding the domains of gene expression. Our results emphasize the modular role of MEs in regulation of gene expression within growing tissues.

KEY WORDS: *hedgehog*, Epigenetic inheritance, Maintenance element, Notch, Ci, *Drosophila*

INTRODUCTION

Gene expression is generally governed by cis-regulatory elements that integrate cell-type and temporal information to generate accurate and stereotyped patterns of expression in space and time (Levine, 2010). One class of regulatory modules is embryonic enhancers, which drive gene expression as a result of transcription factor binding at the enhancer sequence. Very frequently, these transcription factors are only transiently expressed during development and the gene expression state of their target genes is maintained by the Polycomb group (PcG) and the Trithorax group (TrxG) of proteins. These form the basis of a cellular memory system that maintains the transcriptional state of the target genes heritable during development (for reviews, see Muller and Kassis, 2006; Schwartz and Pirrotta, 2007). The genes controlled by the PcG/TrxG system have PcG and TrxG response elements (PRE/TREs), to which these proteins bind and keep the gene either permanently repressed (PcG) or active (TrxG). In order to reflect the dual function of these regulatory elements that bind both groups of proteins, PRE/TREs are termed maintenance elements (MEs) (Brock and van Lohuizen, 2001). Some of these elements have been shown to maintain the initial activated transcriptional state of a nearby reporter gene through several rounds of mitosis during development (Cavalli and Paro, 1998). This is the case for *Drosophila* Hedgehog (Hh), a signalling molecule expressed in the posterior (P) compartment of all limb primordia and involved in anterior-posterior axis formation (Basler and Struhl, 1994; Tabata et al., 1992). An ME situated upstream of the *hh* transcription start site is able to maintain Gal4-driven *lacZ* reporter gene expression through several rounds of mitosis (Bejarano and Milan, 2009; Maurange and Paro, 2002).

Both genetic and epigenetic mechanisms are involved in regulation of *hh* expression in the *Drosophila* wing (Fig. 1A). The transcription factor Ci is expressed in the anterior (A) compartment, where it is required to repress *hh* expression (Apidianakis et al., 2001; Bejarano et al., 2007; Méthot and Basler, 1999), and the homeodomain proteins Engrailed (En) and Invected are expressed in the posterior (P) compartment, where they are required to repress Ci expression (Eaton and Kornberg, 1990) and relieve Ci-mediated repression of *hh* (Bejarano and Milan, 2009). PcG proteins help to maintain the repression of *hh* expression in A cells whereas TrxG proteins contribute to maintaining the expression of *hh* in P cells (Bejarano and Milan, 2009; Chanas and Maschat, 2005). Notch activity at the dorsal-ventral (DV) compartment boundary has also been reported to participate in the regulation of *hh* expression; however, the mechanistic basis behind this is uncertain (Bejarano and Milan, 2009). Although communication between the *hh*-ME and those cis-enhancers that integrate positional information conferred by the activities of Ci, En and Notch has been proposed as a way to initiate and maintain the transcriptional state of *hh* in P cells, the identity of these enhancers and the proposed communication remain elusive.

Here, we have isolated a 4.3-kb cis-regulatory region that recapitulates *hh* expression in the P compartment of the wing primordium. This fragment includes the previously defined *hh*-ME and a nearby enhancer (termed C enhancer) that responds to En, Ci and Notch and drives gene expression to a thin stripe in the P compartment that corresponds to the DV compartment boundary. We present evidence that the ME maintains the initial transcriptional

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activated state conferred by the C enhancer and contributes to expansion, by growth, of the expression domain throughout the P compartment. Communication between the ME and the C enhancer also contributes to repression of gene expression in A cells. Most importantly, we show that enhancers and MEs of different genes are interchangeable modules whose communication is involved in the expansion and repression of gene expression in the *Drosophila* wing. These results emphasize the modular role of MEs in regulating gene expression within growing tissues.

MATERIALS AND METHODS

Drosophila strains

Df(2R)en[E] deletes both *engrailed* and *invected* (Gustavson et al., 1996); *Su(z)2$^{1b.7}$* (Wu and Howe, 1995); *UAS-Ci^{Cell}* [Ci^{Cell}, a truncation of Ci at amino acid residue 975 that behaves as a constitutive transcriptional repressor (Méthot and Basler, 1999)]; *vg-BE-Gal4* (Williams et al., 1994); *UAS-N^{dsRNA}* (Presente et al., 2002); *ap^{gal4}*, *brm^{2} trx^{E2}*, *gro^{E48}*, *UAS-en*, *UAS-lacZ*, *UAS-GFP*, *UAS-N^{INTRA}* and *hh^{P30}* (*hh-lacZ* in the text) are described in FlyBase.

Antibodies

Rat anti-Ci (Motzny and Holmgren, 1995), rabbit anti-βGal (Cappel), rabbit anti-GFP (Upstate), mouse anti-Ptc [Apa 1 (Capdevila et al., 1994)], mouse anti-En [4D9 (Patel et al., 1989)], mouse anti-Wg [4D4 (Brook and Cohen, 1996)], mouse anti-Notch [C458.2H (Diederich et al., 1994)], mouse anti-Cut (2B10) and rabbit anti-βGal (40-1A) are described in the Developmental Studies Hybridoma Bank.

Genetic mosaics

The following *Drosophila* genotypes were used to generate loss-of-function clones (Xu and Rubin, 1993):

hs-FLP; *FRT42B Df(2R)en^{E} Su(Z)2^{1b-7} / FRT42B Ubi.GFP*; *hh-lacZ/+*;

hs-FLP; *FRT42B Df(2R)en^{E} Su(Z)2^{1b-7} / FRT42B Ubi.GFP; ABC-lacZ/+*.

Clone induction by heat-shock was carried out 2-4 days before wing disc dissection.

Molecular characterization of the Mrt allele of *hh*

A collection of primers were used to amplify and sequence the whole 4.3-kb genomic region upstream of the *hh* transcription start site from wild-type and *hh^{Mrt}* homozygous larvae. Primers (5′-3′) used to amplify the region containing the deletion in the *hh^{Mrt}* allele were:

Hh-3Kb-Top: GGCGTCTCCGTCTGCTTTTAATTTC;

Hh-3Kb-Bot: ATACGAACCATTTTAGCTGCCATTA.

The *hh^{Mrt}* deletion maps to the following BDGP Release 5/dm3 assembly coordinates: chr3R: 18970938-18970961.

Reporter constructs of *hh*

Reporter genes were built from the *hs43-nuc-lacZ* vector, which contains the minimal (TATA box) promoter from the *hsp43* gene (Estella et al., 2008). The A, B and C modules spanning the 4.3-kb genomic region upstream of the *hh-RB* transcription start site (according to FlyBase), and excluding the *hh* promoter, map to the following FlyBase sequence locations:

ABC (from position –4428 to –61 bp upstream of the *hh-RB* transcription start site): BDGP Release 5/dm3 assembly, coordinates: chr3R: 18967689-18972117;

A (–2265 to –105 bp): chr3R:18967689-18969849;

B (–2996 to –2340): chr3R:18969924-18970580;

C (–4265 to –3214 bp): chr3R:18970798-18971949.

The predicted *hh* promoter maps to the following FlyBase sequence location: chr3R: 18967584-18967675.

For *ABC-lacZ* and *ABC^{Mrt}-lacZ*, a 4.3-kb fragment upstream of the *hh* transcription start site was amplified by PCR from genomic DNA from wild-type and *hh^{Mrt}* homozygous larvae using the following primers (5′-3′):

ModABC-Fwd: ACACGCACACACACACTATCGCCTCGAGTTC;

ModABC-Rev: TAAGTAATCTTGGGAAATATACATAAG.

The corresponding PCR products were cloned into the pTZ57R vector (Fermentas), then excised from this vector using *Xba*I and *Bam*HI and subsequently cloned via the *Spe*I and *Bam*HI sites into the pHs43n-nuc-*lacZ* vector.

For *A-lacZ*, *B-lacZ*, *C-lacZ* and *C^{Mrt}-lacZ*, in order to amplify by PCR the A, B and C modules from genomic DNA of wild-type or *hh^{Mrt}* homozygous larvae, the following primers (5′-3′) were used:

ModA-Fwd: GGAATTCACACGCACACACACACTATC;

ModA-Rev: ATTTGCGGCCGCTTTTAACTCTTTTCTGTATT;

ModB-Fwd: GGAATTCACTATATGATCAACGAAAAG;

ModB-Rev: ATTTGCGGCCGCACATAAAATACTTGCAGCCA;

ModC-Fwd: GGGGTACCAAGTCTTTTGTTTTGGCTGC;

ModC-Rev: TTGCGGCCGCTTAATTTTTTTTTCCATCGA.

PCR fragments containing modules A and B were digested with *Eco*RI and *Not*I, the fragment containing the module C was digested with *Kpn*I and *Not*I, and subsequently cloned into the pHs43n-nuc-*lacZ* vector (Estella et al., 2008).

For *AB-lacZ* and *BC-lacZ*, module AB was amplified by PCR from genomic DNA of wild-type larvae using ModA-Fwd and ModB-Rev primers and module BC was amplified by PCR using ModB-Fwd and ModC-Rev primers. Both fragments were digested with *Eco*RI and *Not*I and cloned into the pHs43n-nuc-*lacZ* vector.

For A*C-lacZ* and *AC^{Mrt}-lacZ*, module C was amplified by PCR from genomic DNA of wild-type and *hh^{Mrt}* homozygous larvae with the following primers (5′-3′):

ModAC-Fwd: ATTTGCGGCCGCAAGTCTTTTGTTTTGGCTGC;

ModAC-Rev: TTGGATCCTTAATTTTTTTTTCCATCGA.

The fragment was digested with *Not*I and *Bam*HI and cloned downstream of ModA in the pHs43n-nuc-*lacZ* vector.

For *AλC-lacZ*, a 650-bp fragment from Lambda (λ) Phage was amplified by PCR with the following primers (5′-3′):

Lambda-Fwd:
ATAAGACTAGCGGCCGCATGAAATAAGAGTAGCCT;

Lambda-Rev: ATAAGAATGCGGCCGCTAGCAACTGGAAATCATT.

The fragment was digested with *Not*I and cloned in the *Not*I site of ModAC in the pHs43n-nuc-*lacZ* vector.

For *ACB-lacZ*, module AC was amplified by PCR from *AC-lacZ* with the following primers (5′-3′):

ModA-Fwd: GGAATTCACACGCACACACACACTATC;

ModC-ERI-Rev: TTGGATCCTTAATTTTTTTTTCCATCGA.

The PCR product was digested with *Eco*RI and cloned upstream of ModB in the pHs43n*lacZ* vector.

For *A-vgBE-lacZ*, module A was digested from the *ModA-lacZ* with *Eco*RI and *Not*I; vgBE was digested from *vgBE-lacZ* with *Not*I and B*am*HI. Both fragments were ligated into the pHs43n*lacZ* vector digested *Eco*RI and *Bam*HI.

For *iab-7-BC-lacZ*, the iab-7 PRE region (chr3R:12725498-12726604) regulating *Abd-B* was amplified by PCR with the following primers (5′-3′):

iab-7-Fwd: GATGCTATCGCGTTGATTGT;

iab-7-Rev: CGAGTTTCGGTCGCTGACGTC.

The 1120-bp PCR fragment was cloned in the pCR-XL-TOPO vector (Invitrogen), then excised from this vector with *Eco*RI and cloned upstream of module BC in the pHs43n-nuc-*lacZ* vector.

FlyBase coordinates of PCR primers are described in Table S1B in the supplementary material.

Reporter constructs of *vg*

Reporter genes were built from the *hs43-nuc-lacZ* vector, which contains the minimal (TATA box) promoter from the *hsp43* gene (Estella et al., 2008). The *vgBE-lacZ* reporter was generated by amplifying the vestigial BE region (Kim et al., 1995) by PCR from genomic DNA (chr2R:8776380- 8777133) from wild-type larvae, using the following primers (5′-3′):

vgBE Fwd GGGCAACGCGGCCGCGAATTCCGCAACTCAAT-GTTG G;

vgBE Rev CTTTGGGATCCGAATTCAGCTCCCTGGTTTATCTGC.

The vgBE PCR product was digested with *Not*I/*Bam*HI and cloned into the pHs43n-*lacZ* vector.

The *vgPRE-lacZ* reporter was made by amplifying the predicted PRE region of *vestigial* (chr2R:8793522- 8790996) by PCR using the following primers (5′-3′):

vgPRE Fwd ATTTGGATCCCTATGTCGATTAAAATGTTTGTAATGG;
vgPRE Rev TCATCAGGATCCGAACCTTGCAACCTTATGCCC.

The vgPRE PCR product was digested with *Bam*HI and introduced into the pHs43n-*lacZ* vector.

The *vgBE-PRE2-lacZ* reporter was generated by introducing the vgPRE PCR product (digested with *Bam*HI) into the *Bam*HI site of the pHs43n-vgBE-*lacZ* construct.

The vgBE (Kim et al., 1995) and vgPRE (Schuettengruber et al., 2007) regions map to the following FlyBase sequence locations:

vgBE: chr2R: 8776380-8777132;
vgPRE: chr2R: 8790966-8793542.

FlyBase coordinates of PCR primers are described in Table S1B in the supplementary material.

Bioinformatic identification of Ci and Su(H) motifs

With the predictive models published in the literature for Su(H) and Ci/Gli consensus binding sites (Bailey and Posakony, 1995; Kinzler and Vogelstein, 1990; Lecourtois and Schweisguth, 1995), we used the MatScan program (Blanco et al., 2006) to obtain a list of putative Ci and Su(H) binding sites (up to two changes in the consensus) on the C module of *hh* (BDGP Release 5/dm3 assembly, coordinates: chr3R:18970798-18971949). We filtered out the predictions that were not conserved in at least five species (including *Drosophila pseudoobscura* or more distant species) in the multiple alignments of Drosophilids (UCSC Conservation track) (Fujita et al., 2011).

RESULTS

ABC, a 4.3-kb region that recapitulates the expression of *hh* in *Drosophila* tissues

We used a transgenic reporter gene assay to search for *hh* cis-regulatory elements that are active in the wing disc. A 4.3-kb region from position –4428 to –61 bp upstream of the *hh* transcription initiation site, which we named ABC (Fig. 1B), drove *lacZ* reporter expression in a pattern that reproduced the *hh* expression pattern in the wing imaginal disc and in the embryonic ectoderm [Fig. 1C; data not shown; number of independent lines analysed (*n*)=16]. We observed that *lacZ* expression was more robust in the distal portion of the wing disc, the presumptive wing pouch (encircled region in Fig. 1C). We next tested whether the ABC region integrates the genetic and epigenetic mechanisms involved in the regulation of endogenous *hh* (Fig. 1A) (Bejarano and Milan, 2009). En and Invected are expressed in the P compartment where they are required to repress Ci expression (Eaton and Kornberg, 1990) and relieve Ci-mediated repression of endogenous *hh* (Bejarano and Milan, 2009). Consistent with that, expression of a truncated form of Ci that behaves as a constitutive transcriptional repressor (Ci^{Cell}) (Méthot and Basler, 1999) reduced ABC expression in P cells (compare Fig. 1D and 1E). PcG genes are involved in repression of *hh* in the A compartment (Randsholt et al., 2000) and, as shown in Fig. 1F, clones of cells located in the A compartment and mutant for the PcG gene *Suppressor 2 of zeste* [*Su(z)2*] showed ectopic expression of *hh*. In these clones, expression of the ABC reporter was also induced

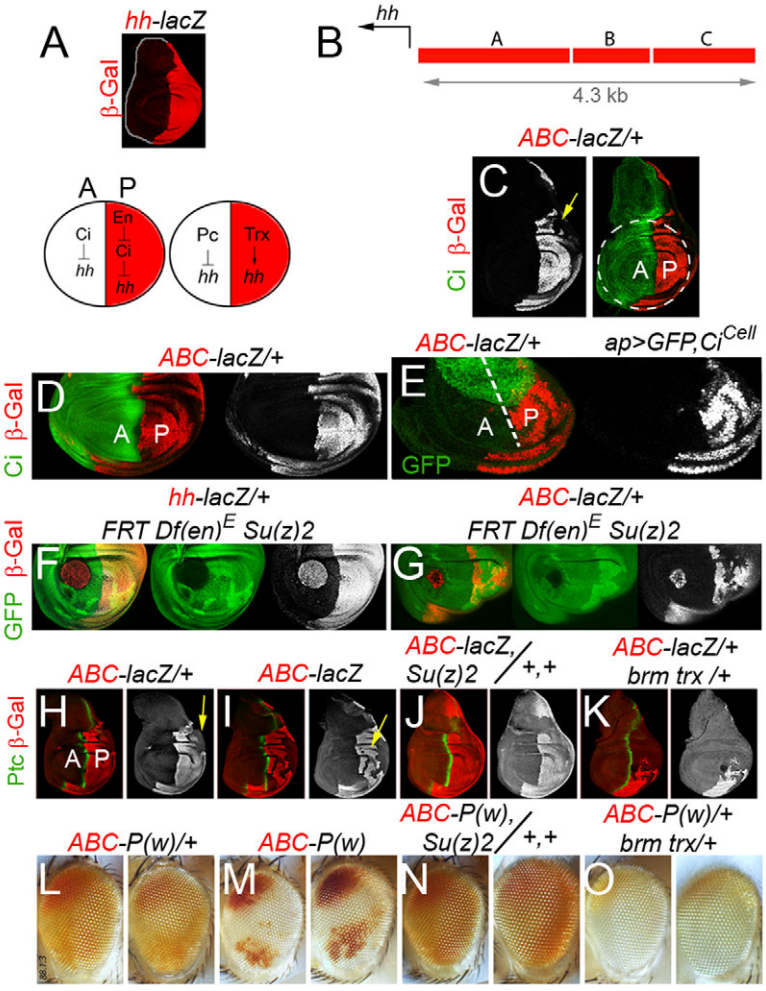

Fig. 1. ABC recapitulates the expression pattern of *hh* in the wing disc and behaves as a PcG response element (PRE) in *Drosophila* wing discs and adult eyes. (**A**) Top: Wing discs carrying the *hh-lacZ* enhancer trap labelled to visualize β-gal protein expression. Bottom: Schematic of the genetic and epigenetic mechanisms involved in the regulation of *hh* expression in the wing primordium. White, anterior compartment; red, posterior compartment. (**B**) Genomic organization of the *hh* locus where the 4.3-kb-long genomic region (ABC) upstream of the *hh* transcription start site is depicted in red. (**C-G**) Wing discs carrying the *ABC-lacZ* reporter construct (C-E,G) or the *hh-lacZ* enhancer trap (F) and labelled to visualize in green Ci (C,D) or GFP (E,F,G) and in red or white β-gal protein expression. In E, Ci^{cell} and GFP were expressed under the control of the *ap-gal4* driver and the anterior-posterior (AP) compartment boundary is depicted by a dashed line. In F and G, clones of cells mutant for $Df(en)^E$ and the *PcG* gene *Su(z)2*, and labelled by the absence of GFP are shown. In C, the yellow arrow points to a group of cells not expressing *lacZ* in the posterior compartment and the wing pouch is encircled. (**H-O**) Transgenic reporter assays for PRE function and expression in the wing disc. Wing discs (H-K) or adult eyes (L-O) of transgenic flies carrying a *lacZ* and a *mini-white* reporter construct containing the ABC region of *hh* [*ABC-lacZ* or *ABC-P(w)*] show expression of *lacZ* in the posterior (P) compartment of the wing (H-K), variegation (H,L, in adult eyes), pairing-sensitive silencing (I,M, in adult eyes), loss of silencing in the *PcG* mutant background *Su(z)2*/+ (J,N) and reduced activation in the *trxG* mutant background *brm trx*/+ (K,O). Wing discs shown in H-K were labelled to visualize Ptc (green) and β-gal (red and white) protein expression. Yellow arrows in H,I point to groups of cells not expressing *lacZ* in the posterior compartment of wing discs. A, anterior; P, posterior.

(Fig. 1G). Note that these clones were also mutant for a deficiency covering both *en* and *invected* [*Df(en)*^{E}], as these two genes are de-repressed in anterior *Su(z)2* mutant clones [data not shown, see also Busturia and Morata (Busturia and Morata, 1988)] and are able to induce de novo expression of *hh* when ectopically expressed in A cells (Tabata et al., 1992).

The ABC cis-regulatory element contains the previously described *hh*-ME (Maurange and Paro, 2002). Consistent with this, *ABC-lacZ* expression in the P compartment was frequently repressed or variegated, with some groups of cells losing *lacZ* expression (Fig. 1C,H, yellow arrows). The *mini-white* reporter gene used to identify transgenic animals also showed a large degree of variegation in adult eyes (Fig. 1L; see Table S1 in the supplementary material). The ABC element showed pairing-sensitive silencing in the expression of the *lacZ* reporter in wing cells (Fig. 1I, yellow arrows) and of *mini-white* in adult eyes (Fig. 1M; see Table S1 in the supplementary material). Thus, *lacZ* expression or eye colour of individuals homozygous for the transgene was usually more variegated than in heterozygous animals. Both types of variegation were also sensitive to a reduction in the doses of the PcG gene *Su(z)2* and the TrxG genes *brahma* and *trithorax*. Expression of the corresponding reporters was less variegated in wing discs and adult eyes of *Su(z)2/+* animals (Fig. 1J,N), and was largely reduced in *brahma trithorax* double heterozygous animals (Fig. 1K,O). Together, these results indicate that the ABC region integrates the genetic and epigenetic mechanisms involved in the regulation of *hh*. Other cis-regulatory regions might also be involved in the control of endogenous expression of this gene, as the *ABC* fragment drove variegated expression of *lacZ* in the P compartment and this expression was sensitive to changes in the doses of PcG and TrxG genes whereas a *hh-lacZ* enhancer trap drove robust expression in all P cells and this expression was not sensitive to changes in the doses of PcG and TrxG genes (data not shown).

Distinct functional modules within the ABC cis-regulatory element

On the basis of sequence conservation with other *Drosophila* species (Vista Genome Browser, data not shown), we subdivided the 4.3-kb region upstream of the *hh* transcription start site in three fragments (A, B and C) (Fig. 1B) and generated reporter constructs carrying them. The A fragment drove some expression of *lacZ* in a patchy manner in wing discs, thereby indicating that it contains some wing enhancers (Fig. 2A, *n*=8). Most interestingly, A drove expression of *lacZ* and *mini-white* in a variegated manner in wing discs and adult eyes (Fig. 2A,E; see Table S1 in the supplementary

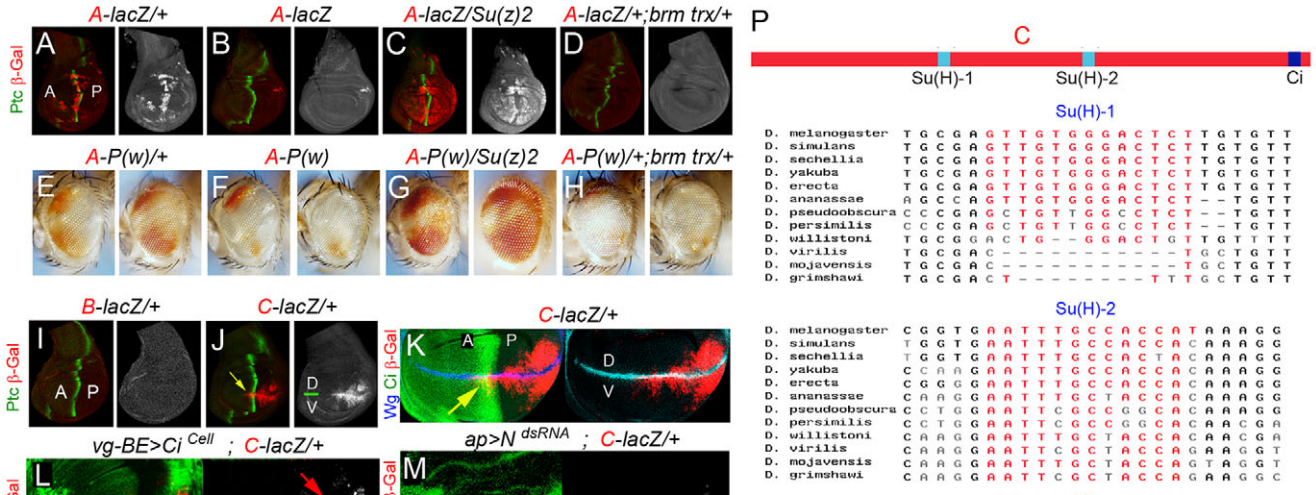

Fig. 2. Dissection of ABC. (**A-H**) Transgenic reporter assays for PcG response element (PRE) function and expression in the wing disc. Wing discs (A-D) or adult eyes (E-H) of transgenic flies carrying a *lacZ* and a *mini-white* reporter construct containing the A region of *hh* [*A-lacZ* or *A-P(w)*] show variegation (A,E), pairing-sensitive silencing (B,F), loss of silencing in a *PcG* mutant background [*Su(z)2*, C,G] and reduced activation in a *trxG* mutant background (*brm trx*, D,H). Wing discs shown in A-D were labelled to visualize Ptc (green) and β-gal (red or white) protein expression. (**I-O**) Wing discs carrying the *B-lacZ* (I) or the *C-lacZ* (J-O) reporter constructs were labelled to visualize in green Ptc (I,J), Ci (K,L,N), Notch (M), or Wg (O) protein expression, in red or white β-gal protein expression and in blue Wg (K). Yellow arrows in J and K indicate *lacZ* expression in anterior cells. In L and N, *Ci*^{Cell} was expressed under the control of the *vg-BE-gal4* (L) or *ap-gal4* (N) drivers. Note loss of *C-lacZ* expression (red or white) in those cells expressing Ci^{Cell} (green). Red arrow in L points to the loss of C-*lacZ* expression in those cells expressing high levels of Ci^{Cell} in the posterior compartment. In M and O, *N*^{dsRNA} (M) or *N*^{INTRA} (O) were expressed under the control of the *ap-gal4* driver. Note that *N*^{dsRNA}-induced loss of Notch protein (green) and C-*lacZ* expression in dorsal (D) cells. Expression of *N*^{INTRA} in the D compartment induced expression of Wg (green) in the anterior (A) and posterior (P) compartments and expression of C-*lacZ* (red or white) mainly in the P compartment. (**P**) Conserved consensus binding sites (red letters) of Su(H) and Ci transcription factors are found in the C module of the *hh* gene. Coordinates of the sites (BDGP Release 5/dm3 assembly) in the *Drosophila melanogaster* genome are: Su(H)-1: chr3R: 18971144-18971156; Su(H)-2: chr3R: 18971421-18971433; Ci: chr3R: 18971833-18971941.

material). Expression of both reporters showed pairing-sensitive silencing (Fig. 2B,F; see Table S1 in the supplementary material) and was modulated by changes in the doses of PcG (Fig. 2C,G) and TrxG genes (Fig. 2D,H). These results indicate that the 2.1-kb long A fragment behaves as a PRE. Consistent with these data, chromatin immunoprecipitation (ChIP) on chip assays to map the chromosomal distribution of Gaf (Trl – FlyBase), Pho, PhoL (Phol – FlyBase) and Dsp1, four DNA-binding proteins thought to be involved in PcG recruitment, identified this region as a potential PRE (Schuettengruber et al., 2009).

The reporter constructs carrying the B or C fragments did not show variegation in adult eyes (see Table S1 in the supplementary material) and expression of *mini-white* was unaffected by changes in the doses of PcG or TrxG genes (data not shown). In wing discs, the B fragment did not drive expression of *lacZ* (Fig. 2I, n=8), whereas the C fragment drove expression in a posterior wedge straddling the DV compartment boundary (Fig. 2J,K, n=5). Some expression in anterior cells abutting the AP and DV compartment boundaries was observed in *C-lacZ* wing discs (Fig. 2J,K, yellow arrows). As the Notch signalling pathway is activated at the DV boundary (Irvine and Vogt, 1997), we examined whether *C-lacZ* expression depends on the activity of Notch. For this purpose, we analysed the effects on reporter expression after blocking or activating this pathway. Expression of a dsRNA form of Notch (N^{dsRNA}) in dorsal cells induced a cell-autonomous loss of *C-lacZ* expression (Fig. 2M), and expression of a dominant active form of Notch (N^{intra}) in the same cells led to an expansion of *C-lacZ* expression throughout the D compartment (Fig. 2O). We analysed next the role of Ci in regulating the expression of *C-lacZ*. A repressor form of Ci (Ci^{Cell}) induced a cell-autonomous loss of *C-lacZ* expression (Fig. 2L,N). Ectopic expression of Engrailed in anterior cells is known to repress *ci* expression (Eaton and

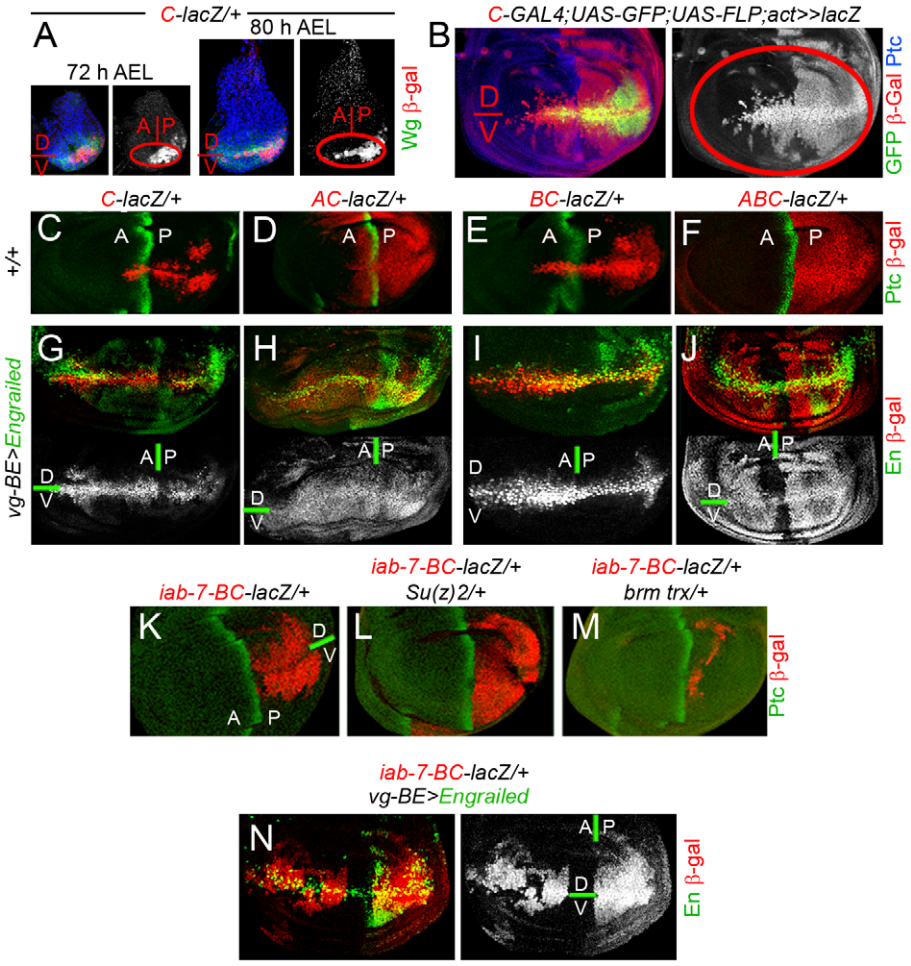

Fig. 3. Epigenetic maintenance of gene expression. (**A**) Early (left) and mid (right) third instar wing discs carrying the *C-lacZ* reporter construct and labelled to visualize Wingless (Wg, green) and β-gal (red or white) protein expression. (**B**) Late third instar wing disc in which all the cells that have ever activated the C-enhancer are labelled by the expression of β-gal (red or white, see text for details), and expression of the C-enhancer is visualized by the expression of GFP (green). The anterior-posterior (AP) boundary is labelled by the expression of Ptc protein (blue) and the dorsal-ventral (DV) boundary is labelled. The presumptive wing pouch is encircled in A and B. (**C-J**) Wing discs carrying the following reporter constructs: *C-lacZ* (C,G), *AC-lacZ* (D,H), *BC-lacZ* (E,I) and *ABC-lacZ* (F,J) were labelled to visualize in green Patched (Ptc, C-F) or Engrailed (En, G-J) and β-gal (red or white) protein expression. In G-J, Engrailed was expressed under the control of the *vg(BE)-gal4* driver. (**K-N**) Wing discs carrying a reporter construct containing the *iab-7* PcG response element (PRE) and the B and C elements of *hh* (*iab-7-BC-lacZ*) were labelled to visualize in green Patched (Ptc, K-M) or Engrailed (En, N) and β-gal (red or white) protein expression. Wing discs in L and M were heterozygous for the *PcG* mutant *Su(z)2* (L) or for the *trxG* mutants *trx* and *brm* (M). In N, Engrailed was expressed under the control of the *vg(BE)-gal4* driver. The anterior (A), posterior (P), dorsal (D) and ventral (V) compartments are labelled in most figure panels. Note that in G-J and N, as previously reported (Crickmore et al., 2009; Garaulet et al., 2008; Tabata et al., 1995), exogenous En was able to induce a reduction in the endogenous levels of En expression in P cells.

Kornberg, 1990) and we showed that it induced the expression of *C-lacZ* along the anterior DV boundary (Fig. 3G). Together, these results indicate that the C fragment integrates the activities of the Notch pathway and the Ci^{rep}. Interestingly, canonical binding sites for Ci and the transcriptional factor Su(H) are found in this fragment (Fig. 2P) and chromatin-binding assays identified this region as a binding domain for Ci^{rep}, Su(H) and Notch (Biehs et al., 2010) (S. Bray, personal communication).

Enhancer-PRE communication mediates epigenetic inheritance of gene expression

The results above indicate that C contains the enhancers that drive expression to a posterior wedge straddling the DV compartment boundary whereas A behaves as a PRE. This PRE has been reported to be able to maintain Gal4-driven *lacZ* reporter gene expression through several rounds of mitosis (Bejarano and Milan, 2009; Maurange and Paro, 2002). It is known that most wing pouch cells are progenies of the cells determined at the DV boundary at early larval stages (Klein, 2001). Thus, we hypothesized that transcription of *hh* is activated, through the C enhancer, in boundary cells during early larval development. The active transcriptional state should then be inherited, by the activity of the *hh*-PRE, to daughter cells after mitosis. This should result in expression of the gene in all wing pouch cells. We undertook the following experimental approaches to evaluate this hypothesis. First, we examined the expression induced by C during wing development. In early wing primordia, C drove *lacZ* expression to a posterior wedge straddling the DV boundary; this posterior wedge occupies a large fraction of the wing pouch and this fraction was reduced as the wing disc grew in size (Fig. 3A). This comparison suggests that cells lose *lacZ* expression as they are displaced out of the C domain by growth of the disc and that most of the wing pouch cells in the posterior compartment are born in the C domain. To verify that this is indeed the case, we lineage-tagged cells born in the C-expressing region using C-Gal4 to direct expression of FLP recombinase. In larvae carrying C-Gal4, UAS-GFP, UAS-FLP and act5c>sto*P*>*lacZ*, FLP recombinase is expressed in cells expressing C-Gal4 and mediates excision of the flip-out 'stop' cassette from the inactive reporter construct to generate an active act5c>*lacZ* transgene (Struhl and Basler, 1993). After excision of the cassette, reporter gene expression is regulated by the actin promoter and is clonally inherited in all the progeny of *C-Gal4*-expressing cells in which the recombination event took place. In *C-Gal4*, *UAS-GFP*, *UAS-FLP*, *act5c>stoP>lacZ* wing discs, expression of *lacZ* was expanded throughout most of the posterior wing pouch (Fig. 3B, compare with the activity of the C-enhancer in mature wing primordia labelled by the expression GFP). These results indicate that most of the wing pouch cells in the posterior compartment are born in the C domain.

We next analysed the capacity of A, as an ME, to mediate epigenetic inheritance of C-induced expression and to expand, by growth, the expression domain of C throughout the posterior wing pouch. The combination of A and C was able to expand the expression domain of C throughout the most distal part of the wing disc (compare Fig. 3C and 3D, *n*=4), and in the presence of the B fragment this expansion took place only in P cells (compare Fig. 3E and 3F, see below, *n*=16). Interestingly, *iab-7*, a well characterized ME of *Abdominal B* (*Abd-B*) (Hagstrom et al., 1997; Mishra et al., 2001), in combination with B and C (in *iab-7-BC-lacZ* wing discs), also expanded the expression domain throughout the most distal part of the P compartment (Fig. 3K, *n*=3) and this expansion depended on the doses of PcG and TrxG genes (Fig. 3L,M). We also tested the capacity of A and *iab-7* to mediate epigenetic inheritance of C-induced expression upon ectopic expression of En in the anterior compartment. Ectopic expression of En along the DV compartment boundary (with the *vg-BE-gal4* driver) induced expression of *C-lacZ* or *BC-lacZ* only in those anterior cells with high levels of En protein (Fig. 3G,I). By contrast, this expression was maintained throughout the wing pouch in the absence of detectable levels of En when the constructs also carried the A fragment or the *iab-7* PRE (e.g. *AC-lacZ*, *ABC-lacZ* or *iab-7-BC-lacZ*, Fig. 3H,J,N). Note, as shown in Fig. 6C, that most of the wing pouch cells were born in the *vg-BE* domain. Together, these results indicate that communication between a PRE (A) and the enhancers located in the C module drive sustained expression throughout the posterior wing pouch.

Enhancer-PRE communication mediates repression of gene expression

Whereas the ABC domain of *hh* drove restricted and sustained expression of *lacZ* in the posterior compartment of the developing wing (Fig. 1), the absence of the B fragment (in *AC-lacZ* reporter constructs) failed to repress *lacZ* expression in anterior cells close to the AP boundary (Fig. 4A, white arrow). In order to address the role of B in mediating repression in the anterior compartment, we combined it with other modules and analysed the resulting expression patterns. B in combination with A was indistinguishable

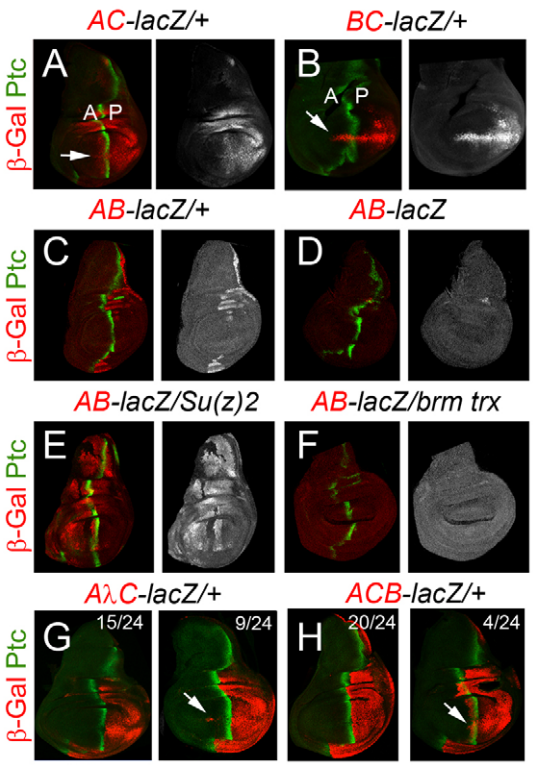

Fig. 4. Repression in the anterior compartment. (**A-H**) Wing discs carrying the following reporter constructs: *AC-lacZ* (A), *BC-lacZ* (B), *AB-lacZ* (C-F), *AλC-lacZ* (G), *ACB-lacZ* (H) were labelled to visualize in green Patched (Ptc) and in red or white β-gal protein expression. *AB* showed variegated *lacZ* expression (C), pairing-sensitive silencing (D), loss of silencing in a *PcG* mutant background (E) and reduced activation in a *trxG* mutant background (F). In A and B, the anterior (A) and posterior (P) compartments are labelled and white arrows point to the expression of β-gal in the anterior compartment. In G and H, the ratio of wing discs with or without ectopic expression of *lacZ* in the anterior compartment (white arrows) is shown.

from A alone (compare Fig. 2A-D and Fig. 4C-F) and the resulting construct, AB, drove expression of *lacZ* and *mini-white* in a variegated manner in wing discs and adult eyes (Fig. 4C, *n*=2; data not shown; see Table S1 in the supplementary material). Reporter expression driven by AB showed pairing-sensitive silencing in wing and eye tissues (Fig. 4D and data not shown; see Table S1 in the supplementary material) and this expression was modulated by changes in the doses of PcG and TrxG genes (Fig. 4E,F; data not shown). Thus, the features of A as a PRE were not visibly affected by the presence of B. Similarly, B in combination with C was indistinguishable from C alone (compare Fig. 4B and Fig. 2J) and the resulting construct, BC, drove expression in a posterior wedge straddling the DV compartment boundary (*n*=5). Some anterior cells expressed *lacZ*, as observed with the C alone (Fig. 4B, white arrow). All together, these results indicate that B participates in mediating repression of *lacZ* in anterior cells receiving the Hh signal only in the presence of both A and C fragments (Fig. 1C). Interestingly, an ACB fragment, where the order of the A, B and C fragments was altered, drove restricted and sustained expression of *lacZ* in the posterior compartment (Fig. 4H, *n*=20/24), suggesting that B contains those enhancers that respond, in the presence of A and C, to the mechanisms involved in the repression of anterior *hh* expression. However, a chimeric A-λ-C fragment, in which the B element was substituted by an heterologous DNA fragment (the λ phage) of the same size (600 bp), also gave similar results in terms of restricted and sustained expression of *lacZ* in all P cells (Fig. 4G, *n*=15/24), suggesting that B also serves as a scaffold in mediating communication between the A and C modules. Thus, B plays a dual and redundant role in the repression of *hh* expression in A cells. Interestingly, we noticed that some wing discs carrying the *ACB-lacZ* or *AλC-lacZ* reporters showed some *lacZ* expression in the anterior compartment (Fig. 4G, *n*=9/24, and Fig. 4H, *n*=4/24, white arrows), suggesting that the dual role of the B fragment confers robustness to the repression of gene expression in anterior cells.

It has been reported that the *hh* gain-of-function allele *Moonrat* (hh^{Mrt}) results in de-repression of *hh* in anterior cells located at the DV boundary (Fig. 5E,F) and causes duplication of anterior structures (Fig. 5A,B) (de Celis and Ruiz-Gomez, 1995; Felsenfeld and Kennison, 1995). The adult wing phenotype is enhanced when halving the doses of *ci* (Fig. 5C) or *groucho* (Fig. 5D), a transcriptional co-repressor involved in repression of *hh* in anterior cells (Apidianakis et al., 2001; Bejarano et al., 2007). We sequenced the ABC domain of hh^{Mrt} homozygous larvae and a small deletion of 24 bp, a highly conserved region among *Drosophila* species (Fig. 5H), was identified in C (Fig. 5G). Interestingly, the ABC domain containing the Mrt deletion (ABC^{Mrt}) drove expression of *lacZ* to some anterior cells located at the DV boundary (Fig. 5H, *n*=16), and this anterior expression was largely increased when halving the doses of *groucho* (Fig. 5I). Intriguingly, the C^{Mrt} mutant module drove expression of *lacZ* to a similar domain as the wild-type C module (Fig. 5J, *n*=3). Although the role of this fragment in mediating anterior repression of gene expression remains to be further characterized, these results indicate that the deletion in C contributes to the Mrt phenotype and reinforce the proposal that communication between A and C mediate repression of *hh* in anterior cells.

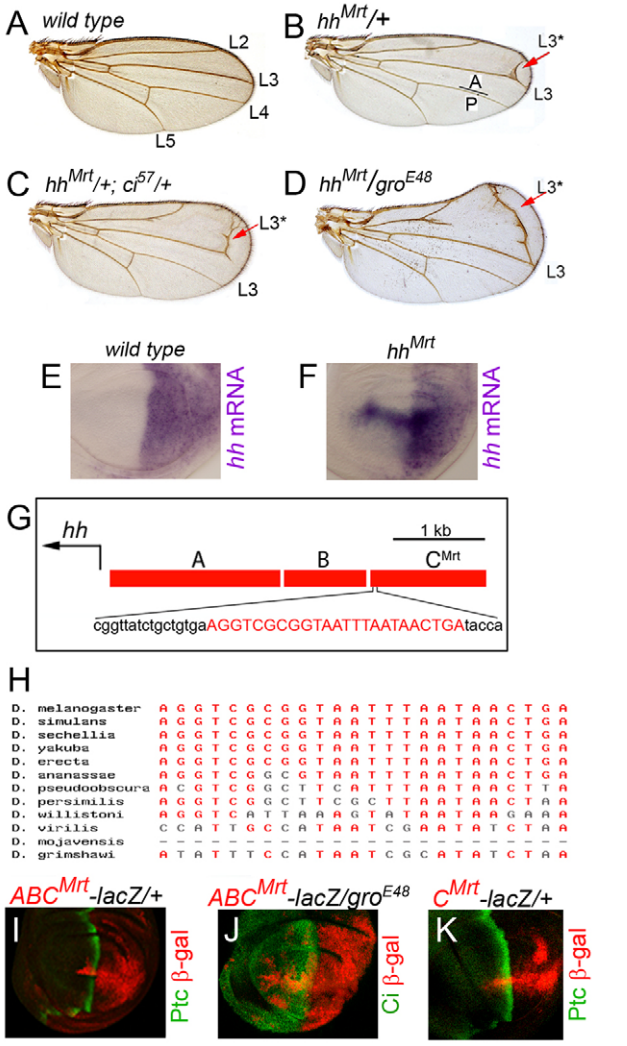

Fig. 5. Molecular characterization of the *Moonrat* allele of *hh*. (**A-D**) Cuticle preparation of wild-type (A), hh^{Mrt}/+ (B), hh^{Mrt}/+; ci^{57}/+ (C) and hh^{Mrt} /gro^{E48} (D) adult wings. Longitudinal veins L1-L5 are shown and ectopic veins L3 (L3*) are indicated by a red arrow. (**E**,**F**) Wild-type (E) and hh^{Mrt} (F) wing discs labelled to visualize expression of *hh* mRNA in purple. (**G**) Genomic organization of the *hh* locus with the deleted nucleotides found in hh^{Mrt} homozygous larvae shown as capital red letters. (**H**) Conservation among *Drosophila* species of the 24 bp deletion found in hh^{Mrt} flies. (**I-K**) Wing discs carrying the following reporter constructs: ABC^{Mrt}-*lacZ* (I,J) and C^{Mrt}-*lacZ* (K) were labelled to visualize in green Patched (Ptc, I,K) or Ci (J) and in red β-gal protein expression. The wing disc shown in J is also heterozygous for gro^{E48}.

Enhancer-PRE communication mediates epigenetic inheritance of gene expression: is this a general mechanism?

Our results indicate that communication between a PRE (A) and the enhancers located in the C fragment that respond to Ci^{rep} and Notch drive sustained expression of *hh* throughout the posterior wing pouch. We then questioned how generally this mechanism is used in development. *vestigial* (*vg*), a gene required for growth and cell survival of wing cells, is expressed in the wing pouch in a graded manner at both sides of the DV boundary (Fig. 6A) (Kim et al., 1996). *vg* expression is regulated by the combined activities of the Notch pathway and the Wingless signalling molecule. The so-called boundary enhancer (vg-BE, located in the second intron of *vg*, Fig. 6K) drives expression to the DV boundary (Fig. 6B, *n*=8) and responds to the activity of Notch (Kim et al., 1996), whereas the quadrant enhancer (vg-QE, located in the fourth intron, Fig. 6K) drives expression in non-boundary cells and responds to the activity of Wingless, a signalling molecule expressed at the DV boundary.

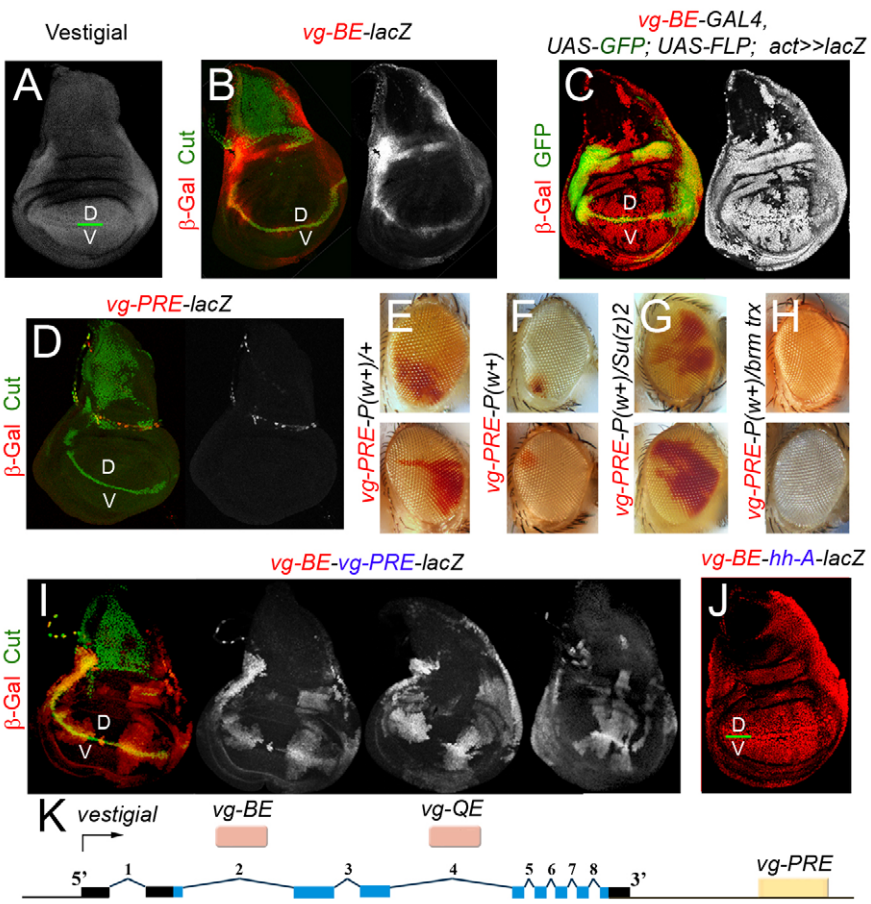

Fig. 6. Enhancer-PcG response element (PRE) interaction: a general mechanism? (**A**,**B**,**D**,**I**,**J**) Wing discs labelled to visualize Vestigial protein expression in white (A) or carrying the following reporter constructs: *vg-BE-lacZ* (B), *vg-PRE-lacZ* (D), *vg-BE-vg-PRE-lacZ* (I), *vg-BE-hh-A-lacZ* (J), were labelled to visualize in green Cut (B,D,I) and β-gal (red or white) protein expression. (**C**) Late third instar wing disc in which all the cells that have ever activated the vg-BE enhancer are labelled by the expression of β-gal (red or white, see text for details) and expression of the vg-BE enhancer is visualized by the expression of GFP (green). Dorsal (D) and ventral (V) compartments are labelled in all wing discs. (**E-H**) Transgenic reporter assays for PRE function in adult eyes. Adult eyes of transgenic flies carrying a *mini-white* reporter construct containing the PRE region of *vg* [*vg-PRE-P(w+)*] show variegation (E), pairing-sensitive silencing (F), loss of silencing in a *PcG* mutant background [*Su(z)2*, G] and reduced activation in a *trxG* mutant background (*brm trx*, H). (**K**) Genomic organization of the *vg* locus where the PRE, boundary enhancer (BE) and quadrant enhancer (QE) are depicted. Blue and black boxes represent coding and non-coding exons, respectively.

Intriguingly, *vg* can be expressed in the wing pouch in the absence of Wingless protein at the DV boundary or in cells lacking the Wingless receptor (Piddini and Vincent, 2009). These surprising results suggest that *vg* expression in the wing pouch is not only regulated by Wingless but also by other redundant mechanisms. Interestingly, genome wide bioinformatic prediction of PRE/TREs identified one potential PRE in the *vg* locus that was confirmed by ChIP as a PcG binding site in S2 cells (Ringrose et al., 2003). In transgenic assays, this PRE caused variegation and pairing sensitive silencing of a *mini-white* gene in adult eyes (Fig. 6E,F; see Table S1 in the supplementary material) (see also Lee et al., 2005). We then addressed whether communication between this PRE and the vg-BE contributes to expansion of the expression domain of *vg* at both sides of the DV boundary in a similar manner to *hh*. Again, we undertook the following experimental approaches to evaluate this hypothesis. First, we lineage-tagged cells born in the vg-BE-expressing region using vg-BE-Gal4 to direct expression of FLP in larvae carrying the act5c>sto*P*>*lacZ* cassette (Struhl and Basler, 1993). In these wing discs, expression of *lacZ* was expanded throughout most of the wing disc (Fig. 6C) (see also Vegh and Basler, 2003), indicating that most of the wing disc cells are born in the vg-BE domain. Next we analysed the capacity of the potential vg-PRE to mediate epigenetic inheritance of vg-BE-induced expression and to expand, by growth, the expression domain of vg-BE throughout the wing disc. vg-PRE did not drive expression of *lacZ* in wing disc cells (Fig. 6D, *n*=10) and induced, as previously reported (Lee et al., 2005), variegation of the *mini-white* gene in adult eyes (Fig. 6E; see Table S1 in the supplementary material), showed pairing-sensitive silencing (Fig. 6F; see Table S1 in the supplementary material) and was modulated by changes in the doses of PcG (Fig. 6G) and TrxG genes (Fig. 6H). Interestingly, the combination in cis of vg-PRE with vg-BE drove expression of *lacZ* reporter not only in DV boundary cells but also at both sides of the DV boundary in a variegated manner (Fig. 6I, *n*=4), indicating that the presence of the vg-PRE was able to induce expansion of the expression domain of vg-BE at both sides of the DV boundary. A second PRE located in the *vg* promoter region was identified by ChIP on chip assays as an enrichment site in PcG proteins (Schuettengruber et al., 2007). However, this PRE was not able to induce expansion of the expression domain of vg-BE at both sides of the DV boundary (data not shown).

Finally, we analysed whether enhancers and PREs from different genes were interchangeable and worked in a modular manner. For this purpose, we combined in cis vg-BE with the hh-PRE (the A module) and analysed the resulting expression pattern. Interestingly, the expression domain of vg-BE was expanded throughout the wing pouch in the presence of the A module of *hh* (Fig. 6J, *n*=5). This observation together with the fact that the PRE of Abd-B (*iab-7*) was able to expand the expression domain of the C enhancer of *hh* when located in cis (Fig. 3K,L) strongly suggest that enhancers and PREs are interchangeable modules whose communication contributes to the expansion of gene expression in growing tissues.

DISCUSSION

Bithorax (*BX-C*) and *Antennapedia* homeotic gene complexes determine the segmental identity of the fly along the anterior-posterior axis and functional analysis of the >300-kb cis-regulatory

region of the *BX-C* complex has been very illustrative with regard to understanding the modular role of enhancers and MEs in the initiation and maintenance of expression of the three BX-C homeotic genes *Ubx*, *abd-A* and *Abd-B* along the anterior-posterior axis of the fly embryo (for a review, see Maeda and Karch, 2006). Here, we provide evidence that communication between enhancers and MEs are also involved in the initiation and maintenance of gene expression within the growing primordium of the fly wing. Functional analysis of the 4.3-kb cis-regulatory region upstream of *hh* has been very illustrative in this regard (Fig. 7). We have identified a cis-regulatory element (termed C) that contains the enhancers that respond to the activity of the repressor form of Ci (Ci^{rep}) (see also Biehs et al., 2010) and the Su(H) transcription factors, and initiates gene expression, from the early wing primordium stage, in a posterior wedge that corresponds to the DV compartment boundary (Fig. 7A). Transcriptional activation is maintained by the activity of a previously identified ME in cis and is expanded, by tissue growth, throughout the posterior compartment (Fig. 7B). This expansion depends on the activity of PcG and TrxG genes, as changes in the doses of these genes modulate the capacity of the ME to maintain the expression in posterior cells. We underscored a dual role of a fragment of 600 bp located between the C enhancer and the ME in mediating repression of gene expression in anterior cells.

Interestingly, repression and expansion of gene expression can also be mediated by the presence of the ME of *Abd-B* in cis with the C element. This observation thus reinforces the proposal that enhancer-ME communication mediates repression and epigenetic inheritance of gene expression (Muller and Kassis, 2006) and that MEs are interchangeable modules (Americo et al., 2002; Chiang et al., 1995; Kozma et al., 2008). It is interesting to note in this context that enhancer-PRE communication appears to contribute to the expansion of the expression domains of other developmental genes, such as *vg*, and that these enhancers also behave as interchangeable modules. Thus, communication between the vg-BE enhancer, which responds to the activity of Su(H) and is expressed in cells at the DV boundary, and a vg-PRE is able to expand, to some extent, the expression to wing pouch cells. This expansion can be also mediated by the presence of the *hh*-ME when in cis with vg-BE. Hence, on the basis of our findings we propose that enhancer-ME communication is a general mechanism at work in highly proliferative tissues that contributes to the expansion of the expression domains of developmental genes.

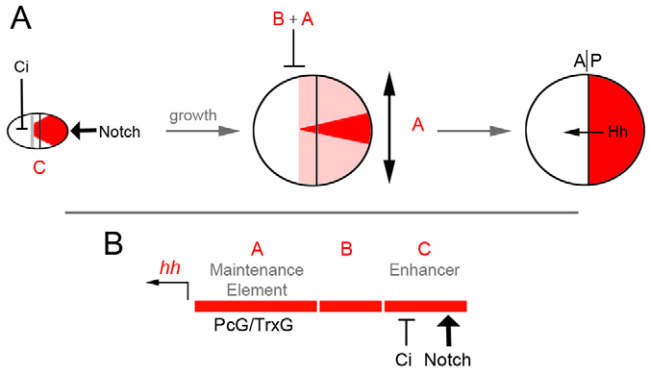

Fig. 7. Enhancer-PcG response element (PRE) communication contributes to *hh* expression in the *Drosophila* wing. (**A**) The combined activities of Ci in anterior cells and Notch in cells along the dorsal-ventral (DV) compartment boundary act through the hh-C Enhancer and result in the initial induction of gene expression to a posterior wedge straddling the DV boundary. Note expression in anterior cells abutting the anterior-posterior (AP) compartment boundary (A/P). Communication between the hh-C Enhancer and the hh-A maintenance element contributes to the expansion, through growth, of gene expression in the posterior compartment and to the repression of gene expression in the anterior compartment. The hh-B element plays a dual role in the repression of gene expression in anterior cells. As a consequence, expression of *hh* (red) is restricted to the posterior compartment. (**B**) Genomic organization of the *hh* locus in which the ABC upstream region of the *hh* transcription start site is depicted in red and the different molecules acting on the hh-A maintenance element (PcG and TrxG protein) and the hh-C Enhancer (Ci and Notch) are shown.

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Competing interests statement
The authors declare no competing financial interests.

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Table S1A. Variegation and pairing sensitive silencing

Reporter construct	Number of variegated lines/ number of lines analyzed	Number of lines with pairing sensitive silencing) number of variegated lines
ABC-lacZ-P(w+)	15/24	6/15
ABC –P(w+)-Casper	8/8	–
ABC^{Mrt}-lacZ-P(w+)	10/30	8/10
ABC^{Mrt} –P(w+)-Casper	23/24	–
A-lacZ-P(w+)	4/16	3/4
B-lacZ-P(w+)	0/15	–
C-lacZ-P(w+)	0/12	–
C^{Mrt}-lacZ-P(w+)	0/11	–
AB-lacZ-P(w+)	7/17	6/7
BC-lacZ-P(w+)	0/15	–
BC^{Mrt}-lacZ-P(w+)	0/11	–
AC-lacZ-P(w+)	4/8	3/4
AC^{Mrt}-lacZ-P(w+)	3/8	3/3
iab-7-lacZ-P(w+)	4/7	2/4
iab-7-BC-lacZ-P(w+)	7/13	5/7
vg-BE-P(w+)	0/23	–
vg-PRE-P(w+)	9/17	4/9
vg-PRE-vg-BE-P(w+)	14/16	7/14
A-vg-BE-P(w+)	4/9	–

Table S1B. Sequence and FlyBase coordinates of PCR primers

Oligonucleotide name	Oligonucleotide sequence (5′-3′)	FlyBase coordinates
Hh-3Kb-Top	GGCGTCTCCGTCTGCTTTTAATTTC	chr3R:18970175-18970199
Hh-3Kb-Bot	ATACGAACCATTTTAGCTGCCATTA	chr3R:18971010-18971034
ModABC-Fwd	ACACGCACACACACACTATCGCCTCGAGTTC	chr3R:18967689-18967719
ModABC-Rev	TAAGTAATCTTGGGAAATATACATAAG	chr3R:18972091-18972117
ModA-Fwd	GGAATTCACACGCACACACACACTATC	chr3R:18967689-18967709
ModA-Rev	ATTTGCGGCCGCTTTTAACTCTTTTCTGTATT	chr3R:18969631-18969650
ModB-Fwd	GGAATTCACTATATGATCAACGAAAAG	chr3R:18969924-18969943
ModB-Rev	ATTTGCGGCCGCACATAAAATACTTGCAGCCA	chr3R:18970560-18970579
ModC-Fwd	GGGGTACCAAGTCTTTTGTTTTGGCTGC	chr3R:18970798-18970817
ModC-Rev	TTGCGGCCGCTTAATTTTTTTTTCCATCGA	chr3R:18971929-18971949
ModAC-Fwd	ATTTGCGGCCGCAAGTCTTTTGTTTTGGCTGC	chr3R:18970798-18970817
ModAC-Rev	TTGGATCCTTAATTTTTTTTTCCATCGA	chr3R:18971929-18971949
iab7-Fwd	GATGCTATCGCGTTCGATTGT	chr2R:12726584-12726604
iab7-Rev	CGAGTTTCGGTCGCTGACGTC	chr2R:12725498-12725518
vgBE-Fwd	GGGCAACGCGGCCGCGAATTCCGCAACTCAATGTTGG	chr2R:8776380-8776401
vgBE-Rev	CTTTGGGATCCGAATTCAGCTCCCTGGTTTATCTGC	chr2R:877109-8777133
vgPRE-Fwd	ATTTGGATCCCTATGTCGATTAAAATGTTTGTAATGG	chr2R:8790970-8790996
vgPRE-Rev	TCATCAGGATCCGAACCTTGCAACCTTATGCCC	chr2R:8793522-8793542
Lambda-Fwd	ATAAGACTAGCGGCCGCATGAAATAAGAGTAGCC	
Lambda-Rev	ATAAGAATGCGGCCGCTAGCAACTGGAAATCATT	