The Pleiohomeotic Functions as a Negative Regulator of *Drosophila even-skipped* Gene during Embryogenesis

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Polycomb group (PcG) proteins maintain the spatial expression patterns of genes that are involved in cell-fate specification along the anterior-posterior (A/P) axis. This repression requires *cis*-acting silencers, which are called PcG response elements (PREs). One of the PcG proteins, Pleiohomeotic (Pho), which has a zinc finger DNA binding protein, plays a critical role in recruiting other PcG proteins to bind to PREs. In this study, we characterized the effects of a *pho* mutation on embryonic segmentation. *pho* maternal mutant embryos showed various segmental defects including pair-rule gene mutant patterns. Our results indicated that *engrailed* and *even-skipped* genes were misexpressed in *pho* mutant embryos, which caused embryonic segment defects.

INTRODUCTION

Segment identity along the anterior-posterior (A/P) axis is defined by the spatial expression patterns of homeotic genes in the Antennapedia complex (ANTC) and Bithorax complex (BXC) (McGinnis and Krumlauf, 1992). The spatial expression patterns of these complexes are maintained through interactions between Polycomb group (PcG) and trithorax group proteins (trxG) (Orlando and Paro, 1995; Pirrotta, 1998).

A major role of PcG proteins is to maintain the anterior-boundary of homeotic gene expression by repressing their expression in cells where their transcription has not been initiated (Pirrotta, 1998). Loss of function PcG mutations commonly cause ectopic homeotic expression and consequently a homeotic transformation, which is similar to the gain of function mutations in homeotic genes (Sinclair et al., 1992). However, derepression phenotypes vary widely among PcG mutations. Most PcG genes function maternally as well as zygotically (Breen and Duncan, 1986; Girton and Jeon, 1994).

PcG proteins function as part of Polycomb repressive complexes (PRCs), including PRC1 (Francis et al., 2001), PRC2

(Francis et al., 2001; Saurin et al., 2001), and PhoRC complex. Of the molecularly characterized PcG proteins, only Pleiohomeotic (Pho) and its homolog, Pho-like (Phol), have been found to have DNA binding domains (Brown et al., 1998; 2003). Pho binds directly to PRC1 as well as to the Brahma (BRM) chromatin remodeling complex in *Drosophila* embryonic nuclear extracts (Mohd-Sarip et al., 2002). Pho also directly binds to a PRC2 in the wing imaginal disc (Wang et al., 2004). In addition, Pho interacts with drosophila Scm-related gene containing four mbt(dsfmbt) displaying methyl-lysine-binding activity when forming a Pho-repressive complex (PhoRC) (Klymenko et al., 2006), suggesting that Pho could be the initial recruiter of the PcG complex. The repression of gene expression by the PcG complexes occurs through Polycomb response elements (PREs) (Muller and Kassis, 2006). DNA sequences bound to Pho have been identified in a number of PREs of several genes and these sequences are required for silencing of gene expression (Brown et al., 1998; Fritsch et al., 1999; Mihaly et al., 1998). Moreover GAGA, which is encoded by the gene *Trithorax-like* (*Trl*) and colocalized with several PcG proteins at PREs, seems to facilitate Pho binding to chromatin (Mahmoudi et al., 2003; Strutt et al., 1997).

A large number of key developmental genes including the Hox genes have been shown to be Polycomb (Pc) targets (Kwong et al., 2008). Through whole-genome mapping to identify sites bound by the Pho, the majority of Pc targets were found to also be associated with Pho binding, indicating that Pho plays a widespread role in the maintenance machinery in cell fate determination. So far, over 200 PcG target genes have been identified (Brown et al., 1998; Kwong et al., 2008). These targets contain segmentation genes including *en*, *eve*, *odd-skipped* (*odd*), *Kruppel* (*Kr*), *wingless* (*wg*), suggesting that PcG gene mutation may affect segmentation. Zygotic mutant embryos of *Additional sex combs* (*Asx*) and maternal effect mutant embryos of *Polycomblike* (*Pcl*) showed segment defects (Breen and Duncan, 1986; Sinclair et al., 1992).

Although Pho is molecularly well-characterized and expected

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to play an important role in the gene silencing process, its functions are still not fully understood. Here, we carefully characterized the effects of the *pho* maternal effect mutation on segmentation. Using various approaches to assess the effect of Pho, we argue that maternal Pho functions as a negative regulator of segmentation genes during early embryogenesis.

MATERIALS AND METHODS

Fly stocks and culture

The *pho^cv* allele was recovered as a recombinant from the *pho^c* strain of *Drosophila* (Girton and Jeon, 1994). The *pho^cv* allele produced by the insertion of mdg4/gypsy elements (Brown et al., 2003) into the *pho^cv* allele. The mutation *pho^cv* / *pho^cv* is viable. Females produce embryos that die before hatching. Other mutations and balancers are described in Lindsley and Zimm (Lindsley and Zimm, 1992) and flybase (http://flybase.org). Flies were reared in 20 mm-diameter vials containing a standard cornmeal/yeast medium seeded with live yeast. Stocks were maintained at 20°C. Flies were reared at 25°C; eggs were also collected at 25°C.

Immunohistochemical and embryological studies

Immunohistochemical staining was performed with anti-En (4D9 from DSHB), anti-Eve (2B8 from DSHB) and anti-Kr (gift from Dr. Hiromi) primary antibodies and detected using a Vectastain ABC kit (Vectorlabs). RNA *in situ* hybridization on whole-mounted embryos was performed using digoxigenin-labeled antisense RNA probes, in accordance with Tautz and Pfeifle (Tautz and Pfeifle, 1989). For cuticle preparation, eggs were collected at 12 h intervals and further incubated for 24 h at 25°C.

Plasmids, antibodies, and reagents

Drosophila eve promoter DNA fragments containing from -667 to +174 were cloned into a pGL2-basic vector (Promega, USA) to yield pGL2-*eve*-Luc reporter plasmids. *Drosophila* GAGA cDNA and *pho* cDNA fragments were cloned into pPAC-PL to yield cloning pPAC-GAGA and pPAC-Pho, respectively. To prepare recombinant GST-fusion proteins, cDNA fragments encoding GAGA-ZFDBD and Pho-ZFDBD generated by PCR were cloned into pGEX4T3 (Amersham Biosciences, USA). All plasmid constructs were verified by sequencing.

GST fusion protein purification

To prepare bacterial-expressed recombinant fusion proteins, pGEX4T3-GAGA-ZFDBD and pGEX4T3-Pho-ZFDBD plasmids were transformed into *E. coli* (BL21-DE3). After culture and disruption of bacteria, soluble fractions of lysate were incubated with 2 ml GSH-agarose resin (Peptron, Korea) for 2 h at 4°C. After washing, bound proteins were eluted in 2 ml elution buffer (5 mM GSH, 50 mM Tris-HCl, final pH 8.0).

Electrophoretic mobility shift assay (EMSA)

Oligonucleotide probes (500 picomoles each in 83.3 mM Tris-HCl, pH 8.0, 16.7 mM $MgCl_2$, 166.7 mM NaCl) were annealed. 100 picomoles of annealed oligonucleotides for EMSAs were labeled with [α-^{32}P]-ATP and Klenow enzyme (Roche, Germany). [α-^{32}P]-labeled, double-stranded oligonucleotides were purified with SephadexTM G-50 (Amersham Biosciences, Sweden). Sequences of various oligonucleotide probes designed using well-known consensus binding sequences (Fritsch et al., 1999; Mihaly et al., 1998) used in EMSA are as follows (only the top strand is shown).

GAGA #1, 5′-GATCACACACTCTCTGCCG-3′; GAGA #2, 5′-GATCCGGAACTCTCTCTCTCTCCAGCT-3′; GAGA #3, 5′-GATCGCACCGAGAGCACAG-3′; GAGA #4, 5′-GATCATCCACTCTCAGCAC-3′; GAGA #5, 5′-GATCTGGCTGAGAGCAGCA-3′. Pho #1, 5′-GATCGGGCGGCCATTATCT-3′; Pho #2, 5′-GATCAGGTTATGGCTGGAC-3′; Pho #3, 5′-GATCAAGTCATGGCAGCCATGGCAGTGT-3′; Pho #4, 5′-GATCATAATGCCATATCAT-3′; Pho #5, 5′-GATCGGGAGTGCCATGGCCAGCATGGCCAGGAC-3′; Pho #6, 5′-GATCCGGCGGCCATTTGCC-3′.

Each binding reaction was performed in 20 μl binding buffer containing 10 mM HEPES (pH 7.9), 20 mM KCl, 0.8 mM EDTA, 4 mM $MgCl_2$, 5 μM $ZnCl_2$, 1 mM dithiothreitol, 1% BSA, 8% glycerol and purified recombinant GST-GAGAZFDBD (1 μg), GST-PhoZFDBD (1 μg). Protein-DNA complexes were resolved from free probes by 4% non-denaturing poly-acrylamide gel electrophoresis at room temperature in 0.5× TBE buffer (89 mM Tris-Borate, 2 mM EDTA, pH 8.3).

Cell culture and transient transfection

Drosophila SL2 cells were transfected with 1 μg plasmid DNA using Cellfectin reagent (Invitrogen, USA). Cells were then harvested 24 h after transfection and lysed in 100 μl of reporter lysis buffer (Promega, USA). Luciferase activities were measured using the Luciferase Assay reagent (Promega, USA). Luciferase activity was normalized to β-galactosidase activity (Lee et al., 2011).

RESULTS

The *pho* maternal effect mutation affects the expression of *engrailed*

Pho was previously shown to bind to a PRE region of the *en* gene and mutation in *pho* released the pairing sensitive silencing generated by the engrailed fragment (Brown et al., 1998). This result suggested that *pho* might be involved in the regulation of segmentation. Therefore, we examined the expression of *en* in *pho* mutant embryos.

At stage 14 in wild type embryos, *en* was expressed in a series of 14 stripes approximately 1-2 cells wide (Fig. 1A). Mid-laterally, a spur of *en* expression was extended in the anterior direction for 2-3 cells (DiNardo et al., 1985). When *pho* mutant embryos were analyzed on a larger scale, they frequently showed ectopic expression of *en* (41%, n = 39, Figs. 1B, 1C, arrows). This suggests that Pho is involved in maintaining the normal *en* expression pattern like other PcG proteins, such as Pc (Moazed and O'Farrell, 1992). In addition, *en* stripes frequently disappeared, were broken, or fused (Figs. 1D, 1E, Supplementary Table 1). Furthermore, defects in *en* stripes often occurred alternatively along the thoracic and abdominal segments of embryos (Fig. 1F), suggesting that *en* stripe defects may be due to the abnormal expression of upstream segmentation genes caused by *pho* mutations.

The *pho* maternal effect mutation causes segment-defects

We previously reported that *pho* mutant embryos produced from *pho^cv*/*pho^cv* females displayed segment defects, suggesting that *pho* might be involved in segmentation (Breen and Duncan, 1986; Girton and Jeon, 1994; Kwon et al., 2003). To better understand the effects of *pho* on segmentation, we first carefully analyzed denticle defects in embryos from *pho^cv* /*pho^cv* females. Wild type embryos contained three fine thoracic denticle belts and eight abdominal ones (Fig. 2A). Denticle defects occurred in all segments with a high frequency in the second thoracic (T2) and odd-numbered abdominal segments (Figs. 2B-2E and Supplementary Table 2). More than 70% of *pho* mutant embryos showed segment defects in the T2, A1, and
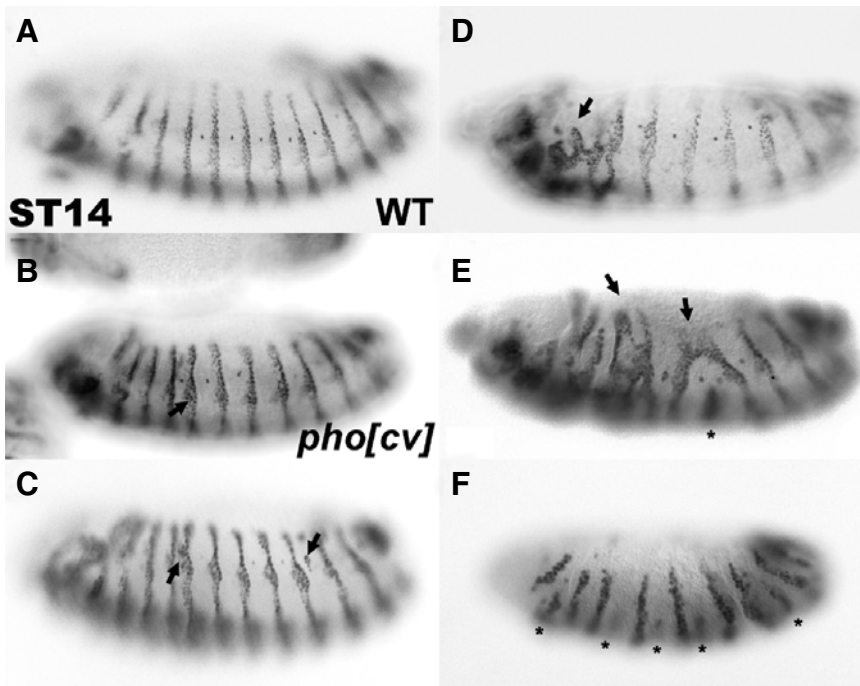
Fig. 1. A *pho* mutation caused abnormal expression of *eve*. (A) At stage 14 in WT embryos, *en* was expressed in a series of 14 stripes approximately 1-2 cells wide and 2-3 cells mid-laterally. (B-E) At stage 14, *pho* mutant embryos displayed different degrees of ectopic En expression (arrows). (D) The number of stripes was reduced, or (E-F) the stripes were reduced in *pho* mutant embryos (asterisk). Also in some *pho* mutants, stripes were broken (D) and fused with each other (E) or reduced like pair-rule mutants (F).
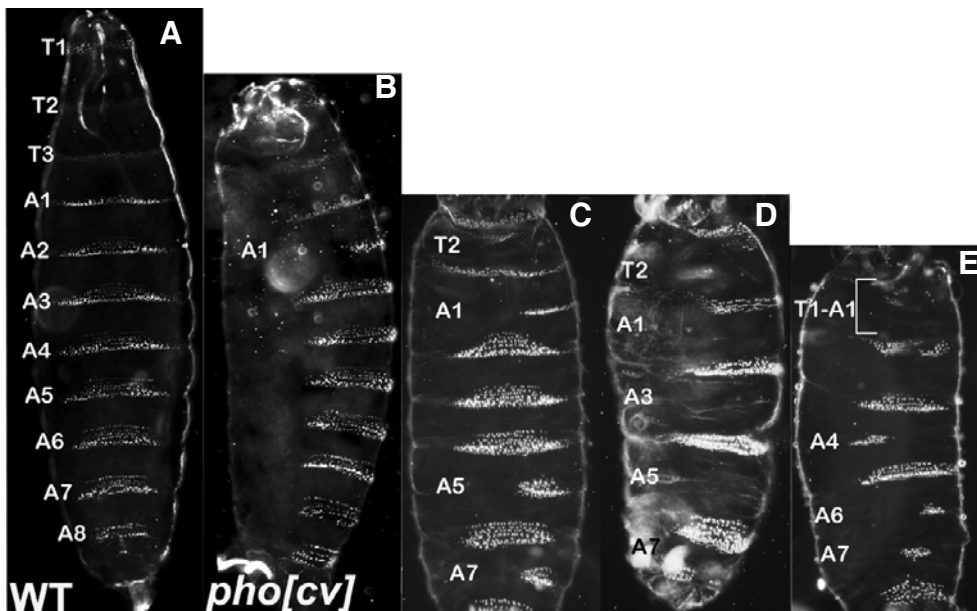

Fig. 2. Embryonic denticle patterns. (A) WT first instar larva. The ventral cuticle pattern was characterized into three thoracic and eight abdominal denticle belts. (B-E) *pho^cv^/pho^cv^* maternal effect embryos. The *pho* mutants contained various segment defects. Labeled segments were partially or completely missing in denticle belts. The odd numbered abdominal segments were mostly affected.

A7 segments, and quite a high percent of mutants (40 to 60%) displayed defects in the A3 and A5 segments. However, less than 10% of pho mutant embryos had defects in the A2, A4 and A8 abdominal segments. Such a high frequency of segment defects in *pho* mutant embryos has never been observed in other PcG mutant embryos.

*pho^cv^/pho^cv^* maternal effect mutation affects the expression of pair-rule genes

Frequent pair-rule pattern of denticle belt defects in *pho* mutant embryos (Supplementary Table 2) and abnormal *en* expression (Fig. 1) suggested that Pho might be involved in the regulation of segmentation genes at an earlier stage of the embryo. Therefore, we examined the expression of pair-rule genes.

The odd-numbered segment defects in *pho* mutant embryos was similar to the mutant phenotypes of the pair-rule gene, *fushi tarazu* (*ftz*) (Weiner et al., 1984). So, we first examined the expression of *ftz* in *pho* mutant embryos. At the cellular blastoderm stage of wild type embryos, *ftz* is expressed in a seven-stripe pattern (Fig. 3A). In many *pho* mutant embryos, *ftz* stripes were partially missing (Figs. 3B and 3C, arrows), which might suggest that Pho could be a positive regulator of the *ftz* gene. However, this is in contrast to the general role of Pho as a negative regulator of genes. Furthermore, the *ftz* promoter of 1
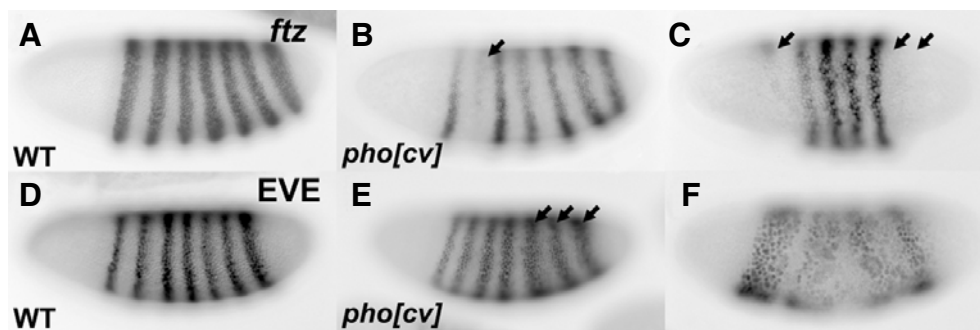
Fig. 3. A pho maternal effect mutation did not maintain the expression of eve. At the cellular blastoderm stage in wild type embryos, *ftz* (A) and *eve* (D) were periodically expressed in even- and odd-numbered segments. Each stripe was 3-4 cells wide. In many pho maternal effect mutant embryos, *ftz* stripes were broken (B) or missing (C). In *pho* mutants, the expression of *eve* was extended (E) or the stripes were fused (F).

kb upstream from the transcription start site has just a presumptive Pho binding site in a reverse direction. Thus, we speculated that the abnormal expression of the *eve* gene, a primary pair-rule gene, might affect the expression of *ftz*, a secondary pair-rule gene. The *eve* promoter has the presumptive nine Pho binding sites within 1 kb upstream from the transcription start site (Fritsch et al., 1999; Mihaly et al., 1998). According to a previous study, ectopic *eve* expression caused a loss of *ftz* expression (Manoukian and Krause, 1992).

Eve is expressed in a seven-stripe pattern in wild type embryos (Fig. 3D), but in *pho* mutant embryos, their expressions were partially extended or even fused within each other's stripe (Figs. 3E and 3F), suggesting that Pho negatively regulates *eve* expression. Although these phenotypes were quite variable, the expansion of *eve* stripes was mainly observed in the posterior region of the embryo (Fig. 3E). The interaction between Pho and *eve* was further verified in the ChIP assay in which *eve* was shown to have the predicted PREs (Kwong et al., 2008). In addition to the ChIP assay, *eve* was also shown to contain many PREs on its 5′ and 3′ locus and one of them was located near the promoter region, which might be involved in its maintenance during early embryogenesis (Fujioka et al., 2008). On the other hand, the PREs played no role in regulating the expression of *ftz*.

Pho acts as a negative regulator of *eve* interacting with GAGA

To further characterize *eve*-PREs, we first searched for predicted binding sites of Pho and GAGA in the 1 kb region of the promoter, which contained the TATA box, based on their well-conserved binding sequence, and found six and five potential binding sites of Pho and GAGA, respectively (Fig. 4A). One of the proteins involved in recruiting PcG proteins to the DNA may be a GAGA factor, which is a protein involved in nucleosome remodeling (Tsukiyama et al., 1994).

EMSA was performed to identify molecular events among Pho, GAGA, and their putative binding regulatory elements (Fig. 4). We also tried to determine which Pho binding sites were more important. Recombinant GST-PhoZFDBD bound to all potential binding sites with varying degrees of affinity (Fig. 4B). Particularly, Pho I, III and VI elements were strongly bound relatively to the others and were all supershifted when incubated with anti-GST after reacting with the recombinant protein (Fig. 4C). In contrast, GST-GAGAZFDBD bound to only one of its five predicted binding sites, the GAGA II element (GII), which were also supershifted under the same condition as the other Pho elements (Fig. 4D), suggesting that GII is a very important element in transcription repression by GAGA.

To investigate whether Pho represses the expression of *eve*

through this regulatory region containing their potential binding sites *in vitro*, a luciferase activity assay in *SL 2* cell was performed (Fig. 4E). In these experiments, Pho was shown to repress the transcription of the luciferase gene, which is a reporter gene, by 50% and GAGA repressed transcription by 65%. In addition, when Pho and GAGA were co-transfected, the reporter gene was almost completely repressed.

In summary, these results imply that Pho and GAGA repress the transcription of the *eve* gene by directly binding to the *eve* PRE element on its promoter, which may be important in maintaining the expression of *eve* during early embryonic segmentation.

DISCUSSION

Apart from homeotic transformations, one of the striking phenotypes produced by the *pho^cv* mutation is the appearance of embryonic segment defects. Segment defects were also observed in the mutant embryos of other PcG genes including *Asx* (Sinclair et al., 1992), *Pcl* (Breen and Duncan, 1986), and *Sex comb extra* (*Sce*) and *Posterior sex comb* (*Psc*) (Adler et al., 1991). These results imply that PcG proteins might be involved in regulating the expression of segmentation genes. However, the molecular mechanisms by which PcG proteins regulate segmentation genes have not been characterized yet.

It was shown that *pho* zygotic mutation caused a slight derepression of *en* (Moazed and O'Farrell, 1992). In fact, Pho was identified as a protein that binds to the PREs of the *en* gene and mediates pairing-sensitive silencing, suggesting that *pho* is involved in the regulation of *en* expression (Brown et al., 1998). Here, we showed that *pho* maternal mutation leads to relatively strong misexpression of *en*, indicating that maternal *pho* functions in maintaining the expression of *en*.

In addition to the ectopic expression of *en*, *pho* mutant embryos showed abnormal *en* stripe patterns including stripe fusion and stripe defects, mainly in even numbered stripes. These results are coincident with the predominant segment defects in the mesothorax and in the odd numbered abdominal segments. These findings strongly imply that these phenotypes are caused by alternations in the expression of the *ftz* gene, which might appear to be indirectly suppressed by ectopic expression of *eve* in *pho* mutation. In addition, Pho was shown to bind to the regulatory sites of *eve* in the EMSA and luciferase activity assay, which suggests that *eve* expression is regulated by maternal Pho. However, since some of the *ftz* stripes were missing, which was not exactly coincident with ectopic expression of *eve* stripes, segment defects may be caused by misregulation of other segmentation genes as well.

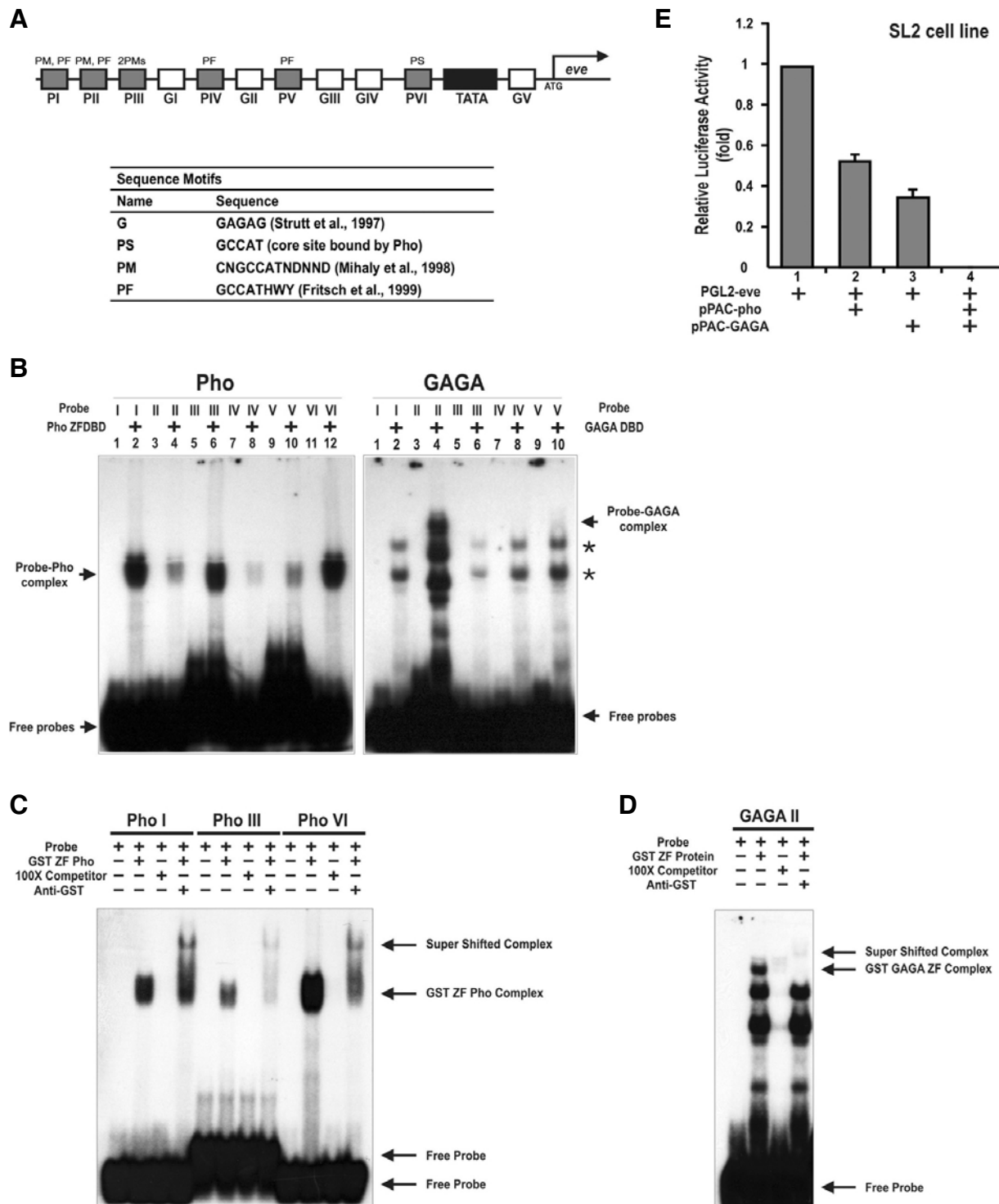Maternal E(Z), which is a PcG, also acts to repress a gap

Fig. 4. Pho and GAGA bind to regulatory elements of the *eve* gene and potently repress its transcription. (A) Structure of the 1 kb regulatory region of the *eve* gene. Putative binding sites are indicated by the filled and open boxes. Open box, GAGA; filled gray box, Pho; filled black box, TATA box. Arrow, *eve* transcription starts. G is the GAGA consensus sequence (Strutt et al., 1997). P is the Pho consensus sequence (Fritsch et al., 1999; Mihaly et al., 1998). (B-D) DNA-Pho or GAGA interaction assays by EMSA. Pho binds to all six putative binding elements with varying degrees of affinity. The Pho-DNA complexes were supershifted by an antibody specific to the GST polypeptide. GAGA binds to only one site (GII) out of five putative sites tested, which was also supershifted by the antibody. Asterisks appear to be non-specific binding. (PhoI: -644 bp, PhoIII: -539 bp, GAGAII: -236 bp, PhoVI: +22 bp) (E) Transient transfection assays in *Drospophila* SL2 cells. Pho and GAGA strongly repressed the expression of a reporter *luc* gene by 50% and 65%, respectively. Co-transfection of Pho and GAGA expression vectors completely repressed *luc* transcription.

gene, *hb* (Pelegri and Lehmann, 1994). In this study, we also found that some *pho* mutant embryos showed an expansion in the expression territory of *Kr* and *kni* to a more anterior domain (Supplementary Figs. S1 and S2), which was shown to contain Pho binding sites in the ChIP assay (Kwong et al., 2008). These findings suggest that misexpressed *Kr* and *kni* in *pho* mutation might repress the expression of pair-rule genes. The ChIP assay also showed numerous other binding sites of Pho to segmentation genes including *giant, odd-skipped, paired* and *wingless*, etc (Kwong et al., 2008), which demonstrates that Pho may regulate other segmentation genes as well.

Taken together, various segment defects were due to derepression of multiple segmentation genes in *pho* mutant embryos, suggesting that Pho is involved in maintaining the expression pattern of segmentation genes as well as homeotic genes.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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