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A functionally conserved boundary element from the mouse HoxD locus requires GAGA factor in *Drosophila*

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SUMMARY

Hox genes are necessary for proper morphogenesis and organization of various body structures along the anterior-posterior body axis. These genes exist in clusters and their expression pattern follows spatial and temporal co-linearity with respect to their genomic organization. This colinearity is conserved during evolution and is thought to be constrained by the regulatory mechanisms that involve higher order chromatin structure. Earlier studies, primarily in *Drosophila*, have illustrated the role of chromatin-mediated regulatory processes, which include chromatin domain boundaries that separate the domains of distinct regulatory features. In the mouse HoxD complex, *Evx2* and *Hoxd13* are located ~9 kb apart but have clearly distinguishable temporal and spatial expression patterns. Here, we report the characterization of a chromatin domain boundary element from the *Evx2-Hoxd13* region that functions in *Drosophila* as well as in mammalian cells. We show that the *Evx2-Hoxd13* region has sequences conserved across vertebrate species including a GA repeat motif and that the *Evx2-Hoxd13* boundary activity in *Drosophila* is dependent on GAGA factor that binds to the GA repeat motif. These results show that Hox genes are regulated by chromatin mediated mechanisms and highlight the early origin and functional conservation of such chromatin elements.

KEY WORDS: HoxD, Chromatin domain boundary, GAGA factor, *Drosophila*

INTRODUCTION

The anterior posterior body axis of animals is determined during early embryonic development by a group of genes called the homeotic or Hox genes (Duboule and Dolle, 1989; Krumlauf, 1994; Lewis, 1978; McGinnis et al., 1984a; McGinnis et al., 1984b). These genes are organized as physically linked cluster on the chromosome and encode transcription factors that play a key role in body pattern formation (Kessel and Gruss, 1990; Krumlauf, 1994). A remarkable feature about the organization of Hox genes is that they are positioned on the chromosome in the same order in which they are expressed along the anterior posterior body axis. This phenomenon is called spatial colinearity (Duboule and Dolle, 1989; Featherstone et al., 1988; Gaunt et al., 1989; Krumlauf, 1994; McGinnis and Krumlauf, 1992). Although colinearity was discovered in *Drosophila* (Lewis, 1978), this organization, like the Hox genes themselves, is conserved in all bilaterians (Duboule and Dolle, 1989; Graham et al., 1989). In addition to the spatial colinearity, vertebrates also show temporal colinearity of organization and expression of Hox genes: the anterior genes are expressed earlier than posterior genes during development (Izpisua-Belmonte et al., 1991). Vertebrates have at least four Hox clusters (HoxA, HoxB, HoxC and HoxD) on different chromosomes and these Hox complexes also have the distinction of being more compact compared with the invertebrate clusters (Kessel and Gruss, 1990; McGinnis and Krumlauf, 1992).

It is widely believed that the integrity of the Hox clusters and their peculiar organization and conservation is linked to the tight regulation of the Hox genes, which is achieved by means of higher order chromatin structure (Deschamps et al., 1999; Mihaly et al., 1998; Ringrose and Paro, 2004). Chromatin level regulatory elements, such as chromatin domain boundaries, and cellular memory or Polycomb response elements have been shown to play a key role in the regulation of Hox genes in *Drosophila melanogaster*. The regulatory region of the *Drosophila* BX-C consists of an array of parasegment specific regulatory domains separated by boundary elements (Barges et al., 2000; Hagstrom et al., 1996; Karch et al., 1994; Mihaly et al., 1997). The parasegment specific domains are arranged in a manner that is co-linear to their function along the anterior-posterior body axis. When a boundary is mutated, the domains flanking it start acting together, which leads to the misregulation of homeotic genes seen as homeotic phenotypes (Galloni et al., 1993; Gyurkovics et al., 1990).

Chromatin domain boundary elements functionally subdivide genomic regions to ensure appropriate enhancer-promoter interactions and restrict long-range regulatory elements to specific domains. In vertebrate Hox complexes, distinctly expressed Hox genes are closely spaced. This raises the possibility of boundary elements being present in the intergenic locations to mark the regulatory features of each gene, in a manner similar to *Drosophila* homeotic gene regulation. The *Evx2-Hoxd13* region in the mouse HoxD complex is one such example where two differentially expressed genes are separated by ~9 kb of intervening DNA. Earlier studies used targeted inversion to show the presence of a polar silencer element in the HoxD complex that could selectively prevent one of the two genes from responding to a distally located intestinal hernia enhancer (Kmita et al., 2000a). It was also suggested that the evolutionarily conserved sequences from *Evx2-Hoxd13* region may have a boundary property to facilitate this selective long-range interaction (Kmita et al., 2002; Yamagishi et al., 2007).

With the availability of HoxD sequences from a number of organisms whose genomes have been sequenced, it has become possible to carry out extensive sequence comparison to identify potential regulatory elements. We therefore analyzed the intergenic region between *Evx2* and *Hoxd13* and detected conserved elements

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in addition to the ones that were reported earlier (Kmita et al., 2002; Kmita et al., 2000b). We tested the regions containing these elements for possible boundary function using a transgenic approach in *Drosophila melanogaster*, as well as in human cell culture assays. Our results show that conserved sequences in the *Evx2-Hoxd13* region, which include GA repeats, function as a boundary element in flies as well as in human cells. We also show that GAGA factor (GAF), which is encoded by the *Trl* gene (Farkas et al., 1994) is involved in this boundary function in fly. These data are significant considering the importance of chromatin structure in regulating differential Hox expression as they demonstrate the existence of a functional chromatin domain boundary between two distinctly regulated genes, *Evx2* and *Hoxd13*. Finally, our results show that chromatin features that mediate the regulation of Hox complexes have evolved early on and have been conserved all the way up to vertebrates.

MATERIALS AND METHODS

P-element constructs and transformation

All the overlapping fragments were PCR amplified and cloned in one basic vector-TA or pBlueScript. From here *Bgl*II-*Hin*dIII or *Eco*RI-*Hin*dIII fragment was recovered and inserted between two *loxP* sites of a modified pBlueScript vector, LML. For GAGA deletion construct, I and II fragments were PCR amplified using: FL, 5′-CAAGATCTCTCAGCT-TTCTAAAAAATGTC-3′; FR, 5′-AAAGAACTCAGAATTCTATTTG-TCTAGACA-3′; SL, 5′-CATCTAGATGTAGACTTTTGAAAATGC-TTTC-3′; and SR, 5′-GTAAAAAGCCACTTGTAATTGTATACCT-GCAGCA-3′. These fragments were cloned separately in pGEM-T cloning vector. From fragment I clone and from fragment II clone, *Bgl*II-*Pst*I and *Pst*I-*Nco*I subfragments, respectively, were recovered and ligated into *Bgl*II-*Nco*I-digested pLxbL vector (ED1 fragment in LML vector). From here *Sma*I-*Sma*I fragment was recovered and cloned in similarly digested LML vector.

From these constructs, *Xho*I or *Not*I fragments (containing the *loxP* sites and the test DNA) were excised and inserted into the *ftz enhancer:hsp70/lacZ* vector (Hagstrom et al., 1996) between the UPS/NE enhancers of *fushi-tarazu* (*ftz*) and *hsp70* promoter. All fragments were confirmed by sequencing.

For each *P*-element construct 0.5 μg/μl of construct DNA was injected into *yw;P^pKiΔ2-3* embryos. Transformants were identified by presence of the *mini-white* selectable marker and were out-crossed to w^{1118}. Individual flies of each transgenic line were then crossed to marked balancer chromosomes to generate balanced stocks.

Evx2-Hoxd13 boundary lines in *Trl* background

Trl^{R85} mutations used were obtained from Francois Karch's laboratory (University of Geneva, Switzerland). The 'Blue balancer' *TM3, Ubx-lacZ* that was used was obtained from Ruth Lehmann's laboratory (New York University, NY, USA). To examine the boundary activity of *Evx2-Hoxd13* fragment in *Trl* mutant background, genetic crosses were carried out as follows. Homozygous P element stock on second chromosome was crossed to *Pin/CyO*; Trl^{R85}*/TM3,Ubx-lacZ* and males of P/*CyO*;+/*TM3,Ubx-lacZ* were back crossed to *Pin*/*CyO*;Trl^{R85}*/TM3,Ubx-lacZ*. From this cross, P/*CyO*; Trl^{R85}*/TM3,Ubx-lacZ* flies were selfed to obtain P/P; Trl^{R85}*/TM3,Ubx-lacZ* flies. Embryos stained (shown in Fig. 7 and see Fig. S4 in the supplementary material) are from a cross between male P/P (homozygous for the transgene) and virgins from P/P; Trl^{R85}*/TM3,Ubx-lacZ* stock. In the progeny, P/P; Trl^{R85}/+ embryos were identified by the absence of *Ubx-lacZ* staining. All flies were raised at 25°C in standard cornmeal medium.

β-Galactosidase assay

Embryos were collected overnight (0-16 hours) and stained for β-galactosidase activity following published protocol (Grossniklaus et al., 1989) except that the embryos were fixed for 20 minutes with saturated heptane (10 ml heptane was saturated by vigorously mixing with 5 ml PBS and 5 ml 50% glutaraldehyde, phases were then allowed to separate and the top phase of heptane was used). To compare the relative levels of *lacZ* for each transgenic line, staining was preformed simultaneously in a grid. Lines containing lambda DNA insert and vector alone were used as negative controls and lines containing 12 and 5 binding sites for *Suppressor of Hairy wing* [*Su(Hw)*] were used as positive controls (Hagstrom et al., 1996). Staining was repeated at least five times to ensure a consistent pattern.

Excision of insert using *Cre recombinase* and PCR analysis

For excision of the test fragment from both the constructs, we used *Cre recombinase*-expressing fly from Bloomington Stock Center. The cross was performed as explained earlier (Siegal and Hartl, 1996). Excision was confirmed by PCR using following specific primers: *lacZ* is amplified using primers LF 5′-ACTATCCCGACCGCCTTACT-3′ and LR 5′-GATGGCTGGTTTCCATCAGT-3′. For checking ED1a and ED1, primers used were 1F 5′-GAATTCTGAGTTCTTTCTCTC-3′ and 3R 5′-GAATTCCTCTATTTGAGAAGG-3′. For ED1b, primers were BBF 5′-ATTTGAGAATTTGACCCAGCAT-3′ and BBR 5′-GTCCCTTGGG-TAGAGAAGGATT-3′.

Gel mobility shift assay

The SRII fragment of *Evx2-Hoxd13* intergenic sequence was amplified using the following primers: 169 bp fragment 1 was generated using primers 1F 5′-GAATTCTGAGTTCTTTCTCTC-3′ and 1R 5′-CAGCCACGTAGCAAGAAAGC-3′; 177 bp fragment 2 was generated using primers 2F 5′-GCTTTCTTGCTACGTGGCTG-3′ and 2R 5′-GAGAATGCGAGGGTGAGAAGC-3′; and 176 bp fragment 3 was generated using primers 3F 5′-GCTTCTCACCCTCGCATTCTC-3′ and 3R 5′-GAATTCCTCTATTTGAGAAGG-3′. Fragment 1-2 was amplified using 1F and 2R and fragment 2-3 was amplified using 2F and 3R. Fragment RR of 479 bp was amplified using 1F and 3R. The corresponding PCR products were gel purified from 1.5% agarose gel using Qiagen kit for extraction of DNA from agarose gels. The purified DNA was labeled with [γ-^{32}P]ATP using polynucleotide kinase. After labeling, the reaction mixture was ethanol precipitated in presence of sodium acetate and incubated at –20°C for 2 hours and centrifuged at 16,000 ***g*** at room temperature for 20 minutes. the pellet was washed with 1 ml of 70% ethanol and centrifuged at 16,000 ***g*** for 5 minutes. The pellet was dried and resuspended in 1× Tris EDTA buffer. Nuclear extract was prepared from 0 to 16 hour *Drosophila* embryos S2 cells according to published procedures (Mishra et al., 2001).

In a gel mobility shift experiment, 2 μg of *Drosophila* S2 nuclear extract was incubated with a mixture containing end-labeled DNA (~1 ng), 0.5 μg of poly(dI-dC), 1 μg of tRNA (in some experiments 0.5 μg of *E. coli* genomic DNA was also used, as indicated in respective figure legends) in binding buffer [25 mM HEPES (pH 7.6), 100 mM NaCl, 10% glycerol, 0.1 mM PMSF, 0.1 mM EDTA, 1 mM DTT] at room temperature for 15 minutes. Sodium deoxycholate (SDC, 1%) was added to the incubation mix and the mixture was then loaded on 1% agarose gel or 4% acrylamide-bis acrylamide (79:1) gel in 0.5× TBE (Tris-borate EDTA) containing 2.5% glycerol. The gel was run in 0.5× TBE at 40 mA for agarose gels at 16°C or at 10 mA for acrylamide gels at 4°C, dried and exposed to X-ray film.

Super-shift

In super-shift experiment, the labeled probe was incubated with 2 μg of *Drosophila* S2 cell nuclear extract and incubated on ice for 15 minutes. GAGA antibody [from Carl Wu's laboratory (National Cancer Institute, Bethesda, MD, USA);1 μl diluted 1:10 in milliQ water] was added to the reaction mixture and incubated at room temperature for 15 minutes. Antibody against *Drosophila* protein Lolal/Batman (Mishra et al., 2003) was used as negative control. The sample was loaded on 2% agarose gel and run at 16°C.

ImmunoFISH analysis

A detailed protocol is available at http://www.epigenome-noe.net/WWW/researchtools/protocol.php?protid=4. Anti-GAF antibody used in this study was raised in rabbit and the dilution used was 1 in 50. Full-length fragment ED1 was used to make fluorescent in situ hybridization probes using Biotin-Nick translation kit from Roche.

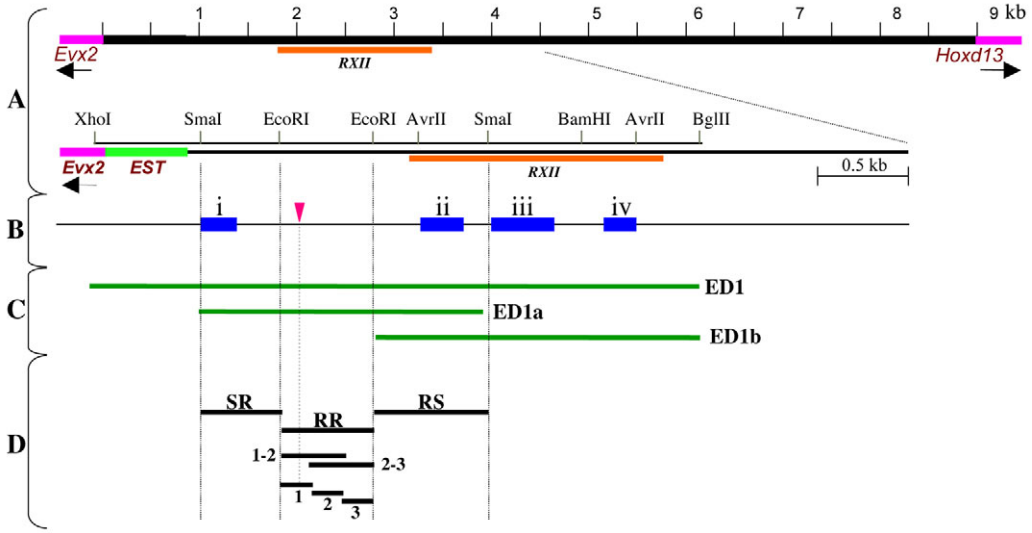

Fig. 1. The *Evx2-Hoxd13* boundary region of mouse HoxD complex. (**A**) Map of *Evx2-Hoxd13* region of mouse HoxD complex. Orange lines indicate the conserved region, RXII, identified in earlier studies. The coding region and EST are shown as pink and green lines, respectively. (**B**) Four blocks, i-iv (blue bars), represent blocks conserved among mammals. The pink triangle between i and ii represents a conserved GA-repeat motif present in all vertebrates. (**C**) ED1 (~3 kb) and two overlapping fragments, ED1a (~1.4 kb) and ED1b (~2 kb), used to make the constructs to test boundary activity are indicated by green lines. (**D**) ED1a subfragments used for DNA-protein interaction studies. SR, *Sma*I-*Eco*RI region; RR, *Eco*RI-*Eco*RI region; RS, *Eco*RI-*Sma*I region. Corresponding restriction endonuclease sites are indicated in A. The RR subfragment was further divided into three parts and various individual and overlapping parts (1, 2, 3, 1-2 and 2-3) were used for gel-shift assays.

Chromatin immunoprecipitation

Third instar larvae containing ED1a or dED1a transgenes were collected, washed well in 1× PBS and homogenized in homogenization buffer (Chopra et al., 2008) supplemented with DTT, PMSF and protease inhibitor cocktail (Roche). Homogenate was filtered through two layers of Mira cloth and centrifuged twice at 100 ***g*** at 4°C for 1 minute to remove debris. To recover cells, the supernatant was centrifuged at 1100 ***g*** for 10 minutes at 4°C. Purified cells were resuspended in cell homogenization buffer, crosslinked with 1% formaldehyde for 10 minutes at room temperature and quenched with 0.125 M glycine. The cells were washed with PBS supplemented with protease inhibitors and collected by centrifugation at 1100 ***g*** for 10 minutes at 4°C.

Chromatin immunoprecipitation (ChIP) was performed using ChIP Assay Kit (Upstate Biotechnology, #17-295) following the manufacturer's protocol. Briefly, purified cells were resuspended in lysis buffer supplemented with PMSF, DTT and protease inhibitors, and the chromatin was sheared to an average size of 200-600 bp by sonication using Biorupter (Diagenode). Pre-cleared chromatin (25 µg) was incubated with anti-GAF polyclonal antibody raised in rabbit at 1:125 dilution (from the IgG stock concentration of 50 µg/µl) or 0.5 µg of non-specific IgG antibody (Calbiochem, #401590) for control. Following elution and purification of DNA, relative abundance of GAF at target transgenes and at control regions was estimated using Power SYBR Green qPCR Master mix (Applied Biosystems) on an ABI7900HT Fast Real-Time PCR System (2 minutes at 50°C; 10 minutes at 95°C; 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 68°C, followed by dissociation curve analysis). Enrichment in the ChIP DNA was determined as percentage Input, where Input DNA represents an aliquot of the same crosslinked and sonicated chromatin used for ChIP and processed in parallel. Enrichment was determined from two independent ChIP assays performed on biological replicates of both the ED1a and dED1a chromatin samples. Statistical significance of the enrichment was calculated using the Wilcoxon paired *t*-test on the raw data (Yuan et al., 2006). Primers were designed against fragment 2 of ED1a (Fig. 1D) that is 28 bp away from the GA sites to assess the enrichment of GAF interacting sites (2F, 5′-GCTTTCTTGCTACGTGGCTG-3′; 2R, 5′-GAGAATGCGAGGGTC-AGAAGC-3′) and against *iab7*-PRE (IF, 5′-GGAATACCG-CACTGTCGTAGG-3′; IR, 5′-GCAGCCATCATGGATGTGAA-3′) and hexokinase (HF, 5′-GGGAAAACACTTGACGTTGG-3′; HR, 5′-GGAGGTGCGAGAACTTATGC-3′) regions as positive and negative controls, respectively.

Construction and transfection of plasmid DNA into K562 cell line and colony assay

Assay vector construct used in this study was made using PJC5-4, a generous gift from Gary Felsenfeld (NIH, Bethesda, MD, USA). The test fragments (ED1, ED1a, DED1a) and β-globin blocker were placed between the LCR and the *neo* gene in the assay vector and used for transfection. Transfection and colony assay in K562 erythroleukemia cells was carried out according to standard procedures (Chung et al., 1993). Relative numbers of surviving colonies in G418 medium were calculated by comparing with the mock-transfected colonies. Statistical significance of the enrichment was calculated using the unpaired *t*-test.

RESULTS

Sequence comparison of *Evx2-Hoxd13* intergenic region

We compared the DNA sequences between the *Evx2* and *Hoxd13* genes from 14 different vertebrates to identify conserved regions that may have potential regulatory functions. We identified several conserved sequences (Fig. 1; see Fig. S1 in the supplementary material) and also observed that the length of the conserved stretches gradually decreases as we move towards the lower vertebrates. This kind of gradual expansion of the conserved region from fish to mammal has been reported earlier (Bejerano et al., 2004; Sabarinadh et al., 2004). We identified four blocks (i, ii, iii and iv) of conserved sequences (Fig. 1B) in addition to the conserved blocks adjacent to the coding regions of *Evx2* and *Hoxd13* (see Fig. S1 in the supplementary material) that probably correspond to the highly conserved promoter region. In an earlier study, a conserved region named RXII was identified by comparing sequences from fewer organisms (Kmita et al., 2002). In our study, blocks ii, iii and iv map to the RXII region as shown in Fig. 1. The conserved block i, present 1 kb upstream of *Evx2*, has not been

previously described. We also identified a GA repeat motif between i and ii (Fig. 1B, pink triangle) that is conserved across vertebrates. In an earlier study, deletion of a 1.2 kb region that included the conserved RXII region, which corresponds to blocks ii-iv identified in our analysis, did not show mis-expression of *Evx2* or *Hoxd13* genes (Kmita et al., 2002). As deletion of a boundary element should lead to misexpression of flanking genes, this deletion perhaps did not remove any significant part of the putative boundary. As this deletion left block i, including the GA repeat, intact, we reasoned that block i and the GA repeat motif may be key components of the putative boundary activity in this region. Taking these observations into account, we tested several subfragments from this region (Fig. 1C; see Fig. S1 in the supplementary material) for the boundary activity in transgenic assays.

Evx2-Hoxd13 region functions as a boundary in *Drosophila*

We tested three overlapping fragments from the *Evx2* and *Hoxd13* regions (ED1, ED1a and ED1b) in enhancer blocking assays for boundary activity in *Drosophila melanogaster* (Fig. 1C). We used an assay vector (Fig. 2A) where the test fragment is placed between *fushi-tarazu* (*ftz*) enhancer and *hsp70* promoter driving *lacZ* gene (Hagstrom et al., 1996). Two *ftz* enhancers, UPS (seven stripes in early embryos) and NE (CNS pattern in late embryos), drive *hsp70-lacZ* gene in the transgenic flies carrying the assay construct. In all constructs, the test fragments were flanked by *loxP* sites to flip them out from the transgenic lines in order to rule out any position effect.

In transgenic lines carrying vector alone or constructs with random DNA insert, both the UPS and NE enhancers of *ftz* appeared to drive a high level of *lacZ* expression (Hagstrom et al., 1996). By contrast, the UPS enhancer driven *lacZ* expression was significantly reduced in all the lines carrying ED1, ED1a and ED1b fragments from the *Evx2-Hoxd13* region (Fig. 2B; see Fig. S2 in the supplementary material). However, we did not see a significant change in the late NE driven *lacZ* expression pattern. ED1 lines showed strong boundary activity while overlapping fragments ED1a and ED1b lines showed decreasing boundary activity in that order (see Fig. S2 in the supplementary material). These results suggest that the boundary function is spread out in almost the entire ED1 fragment.

We flipped out the test fragments from the blocker lines by crossing them to a *Cre* expressing fly and confirmed the excision event by PCR (Siegal and Hartl, 1996). All the excised lines showed clear increase in the expression levels of *lacZ* (Fig. 3), confirming that the blocking activity of *Evx2-Hoxd13* region is entirely due to ED1 regions and not a position effect. As shown in Fig. 1B, we also noticed that the late NE enhancer was not blocked by these elements. These observations suggest that either ED1 is a developmentally regulated early embryonic blocker or neural tissues (CNS) in fly lack trans acting factors needed for this activity. We conclude that ED1 has a strong boundary activity in early embryos that prevents enhancer from acting on a promoter.

GAF binds to *Evx2-Hoxd13* boundary region

In our enhancer blocking assays, ED1a was the smallest of the tested fragments to show prominent enhancer blocking activity. Interestingly, this fragment also contains the conserved GA repeat sequences (Fig. 1B). These sequences recruit GAF, a protein known to be involved in boundaries in the homeotic gene complex of *Drosophila* (Belozerov et al., 2003; Nakayama et al., 2007; Schweinsberg et al., 2004). We decided to check whether this important function of GAF is evolutionarily conserved by testing, as a first step, the interaction of proteins from embryonic nuclear extract of *Drosophila* with ED1a fragment. To achieve this, the *Sma*I-*Sma*I fragment (ED1a), was further divided into three subfragments: *Sma*I-*Eco*RI (SR), *Eco*RI-*Eco*RI (RR) and *Eco*RI-*Sma*I (RS). Electrophoretic mobility shift assay (EMSA) was carried out with all three fragments and all showed specific shifts, indicating that several factors from the nuclear extract associate at many sites on the ED1a DNA (see Fig. S3 in the supplementary material). We focused on the RR fragment for further analysis as it contained GA repeat motif that is known to bind to the GAGA factor GAF. This fragment was further divided into overlapping subfragments RR1, RR2, RR3, RR1-2 and RR2-3 (Fig. 1D) for gel shift experiments, in order to narrow down the region interacting with the nuclear extract. Data presented in Fig. 4A show that the major binding site of RR fragment resides in RR1, as it can compete for the gel shift of RR fragment, whereas RR2, RR3 or RR2-3 could not compete for this shift. Finally, we used mouse liver nuclear extract and Jurkat cell nuclear extract to test whether similar DNA-protein interaction could be seen. Here again, only fragment RR1 showed gel mobility shift which was efficiently competed by excess of cold self-DNA but not by unrelated DNA, suggesting that the binding observed is specific to this region (see Fig. S4A,B in the supplementary material).

As RR1 contains the GA repeat motif known to interact with GAF of fly, which has also been shown to play a role in boundary function at several loci (Belozerov et al., 2003; Ohtsuki and Levine, 1998; Schweinsberg et al., 2004), we directly checked whether GAF interacted with this region by testing fragment RR1 for super-shift with anti-GAF antibody. As shown in Fig. 4B, the RR1 fragment showed super-shift with the GAF antibody and not with the control antibody. Moreover, the shift was lost when excess of cold GA repeat oligo was added to the binding mix as

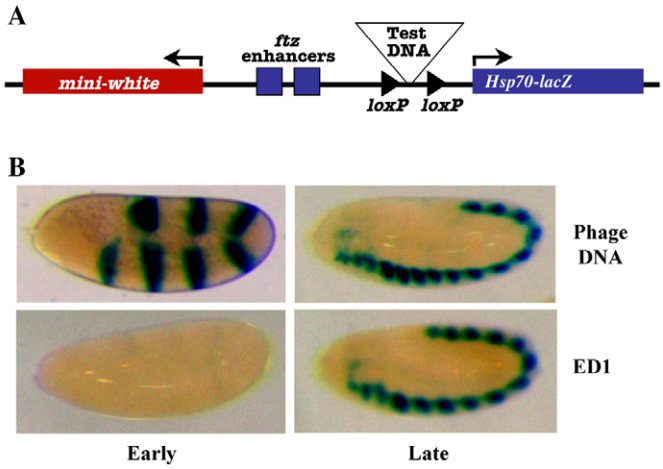

Fig. 2. β-Galactosidase staining of transgenic lines. (**A**) Map of the *ftz* enhancers:hap70/lacZ test construct used for enhancer blocking assay. (**B**) The UPS enhancer-driven seven-stripe staining pattern in germband-extended embryos of stage 10 is shown on the left and the NE enhancer-driven CNS staining pattern in germband-retracted embryos of stage 14 is shown on the right. β-Galactosidase assay was performed on all embryos in a single grid to detect *lacZ* expression so that the expression levels could be directly compared between different lines. Upper row is the control construct where random DNA is inserted in the test site and the lower row is the ED1 fragment.

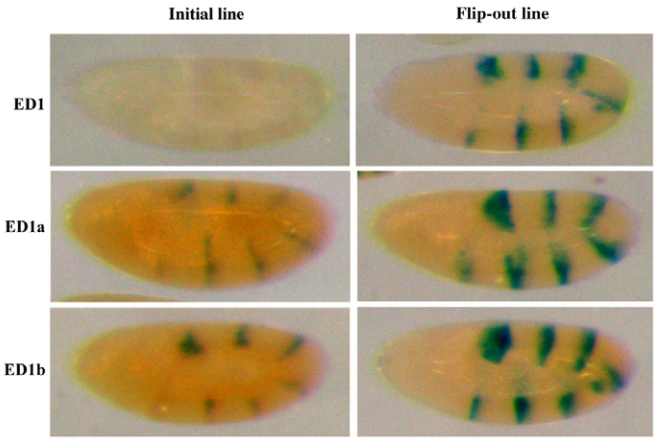

Fig. 3. Boundary activity of the *Evx2-Hoxd13* region. Enhancer-blocking activity of ED1 and two overlapping subfragments, ED1a and ED1b, are shown. Left column is representative staining of initial lines; right column shows staining of the flip-out version of the corresponding lines. All genotypes were stained under identical conditions on a common grid.

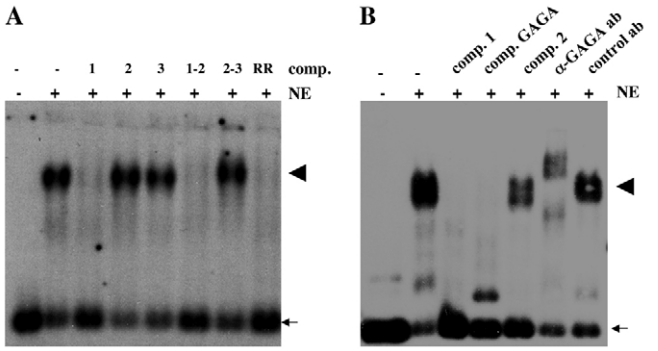

Fig. 4. Interaction of the *Evx2-Hoxd13* region with *Drosophila* nuclear proteins. (**A**) Gel shift of RR fragment using *Drosophila* embryo nuclear extract. –, absence of nuclear extract (NE); +, presence of NE. Different cold competitors, indicated at the top, were used in 100-fold molar excess. Each competitor that contains '1' (1, 1-2 and RR) eliminates the binding of the RR probe. (**B**) Gel mobility shift experiment with 169 bp '1' of RR DNA. 100-fold molar excess of '1' (comp. 1) or double-stranded $(GAGA)_{10}$ oligo (comp. GAGA) eliminate binding, whereas 100-fold molar excess of '2' (comp. 2) fails to compete. In the super-shift experiment, 1:10 diluted GAF antibody was added, which results in a slower moving band while unrelated antibody (control ab) at similar protein concentration does not have any effect. Minor fast-moving bands are non-specific as they are not consistently observed and are competed out with GA-containing DNA. Arrowheads indicate specific bands; arrows indicate the free probe.

competitor, confirming that GA repeat motif is the sole sequence recognition site for interaction with the nuclear extract (Fig. 4B). These observations clearly establish that fly GAF binds in vitro to the RR1 part of ED1a.

GAF binds to *Evx2-Hoxd13* region in vivo and contributes to its enhancer blocking activity

Immuno-FISH assay on polytene chromosomes

To test whether GAF interacts with the *Evx2-Hoxd13* DNA in the *Drosophila* transgenic lines that show enhancer-blocking activity, we carried out immunostaining and DNA fluorescent in situ hybridization (FISH) on polytene chromosomes of third instar larvae. This double staining (immunostaining with the anti-GAF antibody and DNA FISH with probe containing ED1 DNA) to colocalize the protein and DNA was carried out on the initial blocker lines while their flip-out versions were used as controls. We observed a clear colocalization of GAF antibody with the ED1 insertion sites (Fig. 5), whereas no colocalization was seen in case of flip-out lines (see Fig. S5 in the supplementary material).

ChIP assay

As resolution of polytene chromosome colocalization is limited, we used high resolution chromatin immunoprecipitation (ChIP) assay to map the sequence where GAF binds in the *Evx2-Hoxd13* region. We performed ChIP assay using anti-GAF antibody on larvae of ED1 transgenic flies. Considerable enrichment of the GA associated Fragment 2 (28 bp away from the GA sites) was seen in GAF antibody pull-down compared with that of IgG control (Fig. 6A). We used the known GAF-binding region of *iab7-PRE* locus as a positive control and the hexokinase promoter region as a known negative control for GAF interaction (Chopra et al., 2008). The results were found to be statistically significant: P=0.0033 for Fragments 2 (the test fragment), P=0.0002 for the positive control *iab7-PRE* and P=0.0232 for the negative control hexokinase promoter. These results demonstrate that the GA-repeat-rich region of mouse *Evx2-Hoxd13* boundary element binds GAF in the context of transgenic *Drosophila*.

Evx2-Hoxd13 blocker activity depends on Trl

We tested whether the mutation in *Trithorax like* (*Trl*), the gene encoding GAF, has any effect on the boundary activity. For this purpose we used Trl^{R85} null allele in the lines carrying ED1 fragment. As shown in Fig. 6B, in Trl^{R85} background the enhancer blocking activity was clearly compromised. Interestingly, this effect was seen only when we brought the mutation from the female side. This might be because the normal maternal contribution of GAF when the mutation is brought from male is able to sustain boundary activity in the embryos that were heterozygous for *Trl*. The increase in the staining when mutation is brought from maternal side is clear but mild. As these embryos contain half of the maternal contribution, because they are heterozygous and can therefore make a half dose of the protein, the incomplete loss of boundary activity may be due to this partial reduction in protein level. Another possibility could be that the early blocking activity may be dependent on many factors, with GAF being one of the key contributors. This would also explain the incomplete loss of boundary activity in the mutant context. As controls, we tested the flip-out lines for the effect of *Trl* and, as expected, we did not observe any effect of the mutation in these lines (Fig. 6B).

We also tested lines carrying the overlapping fragments ED1a and ED1b for the effect of *Trl* mutation on their blocking activity. Whereas ED1a responded to *Trl* mutation in a manner that was similar to the ED1 lines, there was no effect seen in ED1b lines. Flip-out lines in both these cases had no effect of *Trl* mutation (see Fig. S6 in the supplementary material). The GA repeat motif is present in ED1a and not in ED1b (Fig. 1B). The effect of *Trl* mutation seen in these experiments, therefore, is due to the GAF sites in test fragments and not an indirect effect. These results show that GAF is one of the important factors for the early enhancer blocking activity of *Evx2-Hoxd13* boundary in transgenic flies. In

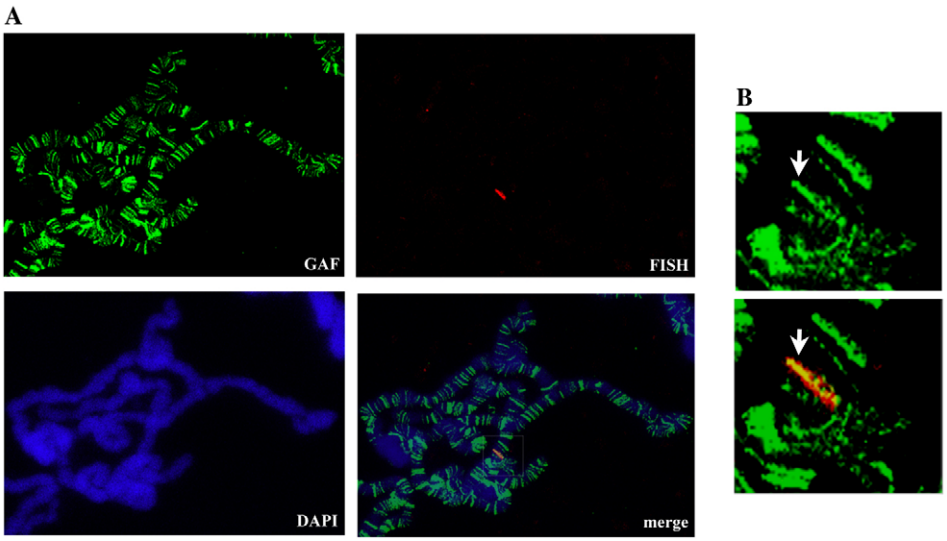

Fig. 5. The *Trl*-GAGA factor of *Drosophila* binds to the *Evx2-Hoxd13* region in vivo. (**A**) Polytene chromosome immuno-FISH was performed on transgenic line containing ED1 fragment. A GAF antibody (green), FISH (red), DAPI for DNA (blue) and the merge of the three are shown. (**B**) Enlarged version of the area indicated in the merged panel in A. The FISH signal colocalizes with one of the *Trl*-GAGA factor bands and is indicated by arrows.

addition, as ED1b does have boundary activity, albeit a bit weak, we conclude that boundary activity of ED1 is spread over much of the fragment and involves multiple factors.

GA deleted fragment shows reduction in boundary activity and loss of GAF binding

The final proof of functional relevance of GA repeats in this region comes from the construct in which we introduced deletion mutation of the GA sites of ED1a. We tested the enhancer-blocking activity of this mutated version, DED1a, in transgenic flies. As seen in Fig. 7A, the boundary activity of the mutated fragment DED1a is considerably weakened when compared with that of the wild-type fragment, ED1a, confirming that the GA repeat motif indeed contributes to the boundary function of the region. Flip-out lines of DED1a, however, still showed increase in the *lacZ* staining, suggesting that part of the boundary activity is still present in the mutated fragment. We also tested the effect of *Trl* mutation on the DED1a lines and its flip-out version. As expected in these lines, there is no effect of *Trl* mutation on the residual enhancer blocking activity (Fig. 7A). These results further establish that the GA motif is the site of action for GAF on this boundary, and that the effect of *Trl* seen in the wild-type fragment is not an indirect one.

We also tested whether the interaction of GAF is eliminated in the DED1a lines by performing ChIP with anti-GAF antibody (Fig. 7B). As the target GA repeats have been deleted in DED1a, we chose to use a common region, Fragment 2, which is just 28 bp away from the GA site, for binding of GAF to this region in both wild-type and mutated lines. Although positive and negative controls behaved in the same way, as in the case of the ChIP experiments with the wild-type construct (Fig. 6A), DED1a lines did not show any enrichment of fragment 2 (Fig. 7B). These observations confirm that enrichment of fragment 2 is due to GAF binding to the GA repeat site and that the interaction of GAF with ED1a is via the GA-repeat motif in this region. We, therefore, conclude that GAF interacts with the *Evx2-Hoxd13* fragment in transgenic flies and that this interaction is exclusively due to GA repeats present in this DNA.

Evx2-Hoxd13 boundary functions in human cells

Having established the boundary activity of *Evx2-Hoxd13* fragment in flies, it was of interest to check whether this boundary activity reflects an evolutionarily conserved feature of this region. To achieve this, we tested the enhancer blocking activity of ED1 fragment in the human erythroleukemia K562 cell line using a colony number assay (Chung et al., 1993). This assay relies on a vector in which the LCR enhancer driven γ-*neo* expression

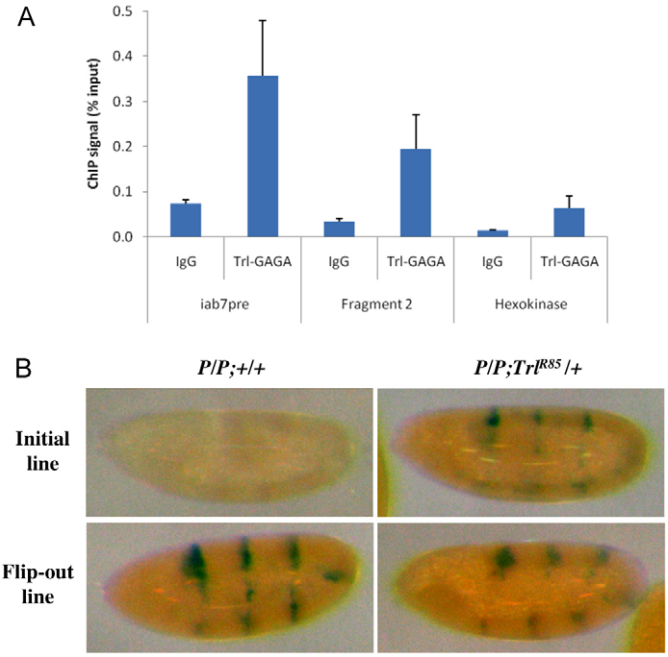

Fig. 6. The *Trl* GAGA factor binds and shows a genetic interaction with the ED1 fragment. (**A**) Binding of *Trl*-GAGA to the transgene was assessed by ChIP-qPCR assays on larvae carrying the transgene. Target region (fragment 2, which is 28 bp from the GA repeat site) is enriched above background levels of the non-specific IgG control in flies carrying the ED1 fragment. Enrichment was also checked at the *iab-7* PRE and hexokinase loci as positive and negative controls, respectively. Results shown are the mean±s.e.m. from two independent ChIP-qPCR experiments. (**B**) ED1 line and its flip-out version is stained in wild-type and *Trl* mutant backgrounds. All genotypes were stained under identical conditions on a common grid. Increase in staining of the initial line in the *Trl* context indicates the requirement of this factor for the boundary activity of ED1 in fly. Lack of such an increase in the case of the flip-out line indicates that the effect seen is mediated through the ED1 DNA.

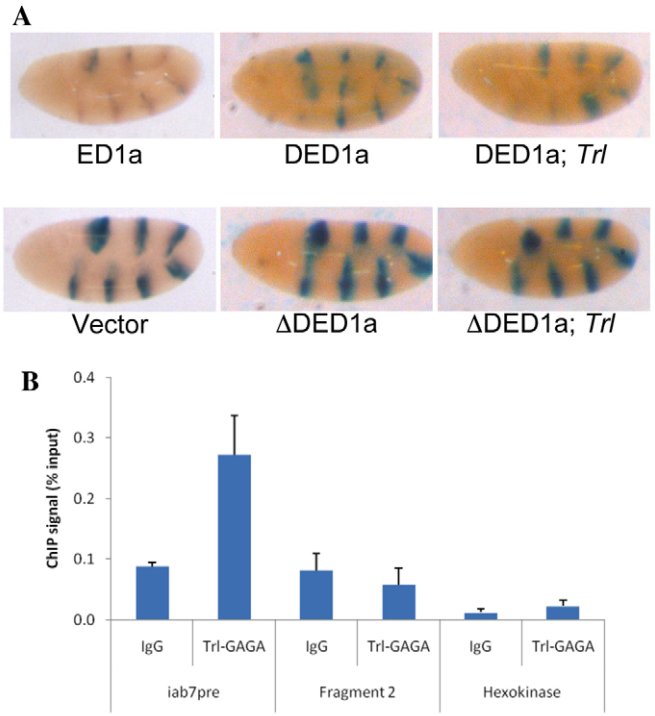

Fig. 7. Role of the *Trl* GAGA factor on DED1a fragment. (**A**) Top row shows the β-galactosidase staining of ED1a, DED1a (a GA-repeat-deleted version of ED1a) and DED1a in the *Trl* mutant background. Increase in staining is observed in case of the DED1a fragment compared with the ED1a fragment, and there is no change in staining of the DED1a line in *Trl* mutant background. The flip-out version of DED1a is shown in the bottom row, which shows the increase in staining compared with DED1a. In the *Trl* mutant background, the staining is unaltered. The vector line serves as a negative control. Staining was performed simultaneously in one grid for direct comparison. (**B**) Binding of *Trl*-GAGA was assessed by ChIP-qPCR assays on larvae carrying the DED1a transgene. Enrichment level of Fragment 2 is decreased below the background level of the non-specific IgG control in flies carrying DED1a fragment. Enrichment was also checked at the *iab-7* PRE and hexokinase loci as positive and negative controls, respectively. Results shown are the mean±s.e.m. from two independent ChIP-qPCR experiments.

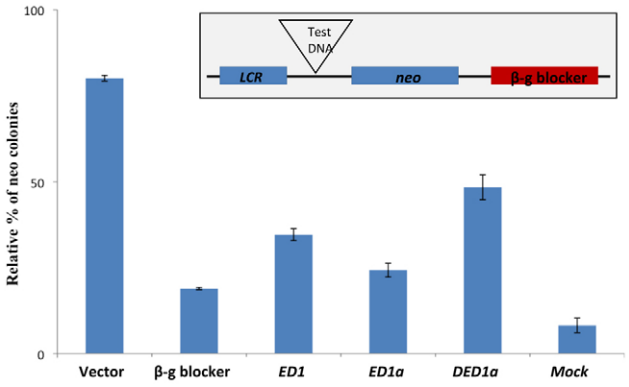

Fig. 8. Boundary activity of the *Evx2-Hoxd13* region in human cells. The human erythroleukemic cell line K562 was stably transfected with different constructs that included empty vector and constructs carrying β-globin boundary, ED1, ED1a and DED1a fragments. Map of the construct used for the boundary assay is shown in the inset. Mock is a transfection control without any DNA. Cells were grown in semi-solid agar medium with and without G418. The colonies from both G418-plus and G418-minus plates were counted after 2 weeks. Ratio (%) of average number of colonies for G418-minus to G418-plus colonies for each construct is shown on the *y*-axis.

provides antibiotic resistance for the cells to grow in presence of neomycin drug G418. Presence of a boundary blocks the LCR enhancer from driving the expression of γ-*neo* and makes these cells sensitive to the drug. It has earlier been shown that the presence of lambda insert between LCR and γ-*neo* does not have any effect on neo expression ruling out the distance effect of LCR on γ-*neo* gene (Chung et al., 1993). We used vector alone as negative control and chicken β-globin boundary as the positive control in this assay and compared them with the boundary activity of *Evx2-Hoxd13* fragments. We observed a significant decrease in the number of colonies in the G418-containing medium in cases of ED1 (P=0.0001) and ED1a (P=0.008) that was comparable with the decrease in colony number seen in case of the chicken β-globin blocker. At the same time, no change was seen in the number of colonies for vector transfected cells (Fig. 8). These results clearly show that the mouse *Evx2-Hoxd13* fragment blocks the LCR enhancer from acting on γ-neo reporter gene, confirming that the sequence acts as an enhancer blocker in the human cell line. We also assessed the contribution of the GA repeat present in this region towards the boundary activity in these cells by using the DED1a fragment in which the GA sites have been deleted. DED1a construct clearly showed more neo resistant colonies when compared with the ED1a fragment (P=0.0001), indicating that the boundary activity was compromised in the GA mutated fragment. The enhancer blocking activity in DED1a fragment, however, was only partially lost and did not reach as low as the vector alone. These results bear a remarkable similarity to our observations in *Drosophila*: that multiple factors contribute to this boundary activity and at least one of these is mediated by the GA-repeat motif.

DISCUSSION

The role of chromatin organization in developmental gene regulation has been well established. In particular, chromatin organization that involves domain boundary elements has been shown to be a key feature of the regulation of homeotic genes in *Drosophila* (Gyurkovics et al., 1990; Mihaly et al., 1998). As the organization of Hox genes is well conserved among bilatarians, it is reasonable to speculate that the constraint that led to this conservation of organization is due to chromatin elements that regulate Hox genes. In general, when differentially expressed genes are in close proximity, as is often the case in Hox complexes, boundary elements are likely to be present between the genes to establish and maintain their distinct expression states. In the mouse HoxD complex, *Evx2* and *Hoxd13* are ~9 kb apart and they are expressed in distinct regions in the developing embryo. This suggests the presence of a boundary within this 9 kb region that prevents the crosstalk between regulatory elements of the two flanking genes.

In order to identify this putative boundary, we carried out sequence comparison of the *Evx2-Hoxd13* region from different vertebrates and identified a cluster of conserved sites along with a GA repeat motif in all the species checked, from fish to mammals. The ~3 kb fragment that included the GA repeats showed enhancer-

blocking activity in *Drosophila* embryos, as well as in a human cell line, indicating the presence of a complex evolutionarily conserved boundary between *Evx2* and *Hoxd13* genes. The boundary activity was shown by both overlapping fragments, ED1a and ED1b, suggesting that the *Evx2-Hoxd13* boundary is spread over several kilobases, unlike *Drosophila* boundaries that tend to be smaller, often less than 1 kb. Spread out boundary function in this region has also been suggested by an earlier study (Yamagishi et al., 2007). The complex nature of the *Evx2-Hoxd13* boundary is also indicated by our observation that only early enhancers of *ftz* are effectively blocked, whereas late enhancers are able to drive expression of the *lacZ* reporter gene even in the presence of this boundary. We have also looked at this boundary activity in the adult eye using a *white* gene enhancer and promoter interaction assay (see Fig. S7 in the supplementary material) and the results clearly showed no enhancer blocking activity in this tissue. These observations indicate that *Evx2-Hoxd13* is a developmentally regulated boundary that functions in early embryos but not in late embryonic CNS and adult eye.

We also find that the boundary activity shown by the fragment containing GA-repeat motif is dependent on GAF in *Drosophila*. This indicates that the conserved GA sites are functionally relevant in *Drosophila*. *Evx2* is the homolog of the *even skipped* (*eve*) gene of *Drosophila*, and both are thought to have evolved from (Bushey et al., 2009; Negre et al., 2010) a common ancestral gene *Evx*. In vertebrates, *Evx* is located near Hox clusters: *Evx1* near HoxA and *Evx2* near HoxD. In *Drosophila, eve* has moved away from the Hox cluster (Garcia-Fernandez, 2005). Our finding that a GAF-dependent boundary is present in the *Evx2-Hoxd13* region is of particular interest in the light of a previous study showing that the *eve* gene in fly is also associated with a GAF-dependent boundary (Ohtsuki and Levine, 1998). These observations suggest that the boundary function evolved early on near the ancestral *Evx* gene and that the same combination has been conserved during evolution even in the organisms where the linkage between *eve* to Hox complex has been lost.

Although several boundary-interacting factors are known in *Drosophila* (Iqbal and Mishra, 2007; Mishra and Karch, 1999), in vertebrates, CTCF is the only protein that has been well studied for its role in boundary function. A CTCF homolog is also present in *Drosophila* and is known to play a role in the *Fab-8* boundary function in the BX-C (Moon et al., 2005). Interestingly, however, the *Fab-7* boundary of the BX-C does not involve CTCF, and instead GAF plays an important role in its function and regulation (Schweinsberg et al., 2004; Schweinsberg and Schedl, 2004). In the case of the *Evx2-Hoxd13* boundary, and in agreement with earlier studies, we have not found CTCF-binding sites (Yamagishi et al., 2007). As in *Fab-7*, this boundary appears to be dependent on GAF. These observations suggest that although several factors act together to establish a boundary, some of them may be mutually exclusive (Bushey et al., 2009; Negre et al., 2010). Further studies in this direction will help in understanding the function and regulation of boundaries during development.

The results presented here strongly indicate the presence of GAGA-binding protein in vertebrates with functional similarity to that of *Drosophila* GAF. Earlier studies have also indicated that transcription of *st-3* gene in *Xenopus* is regulated by GAGA sequences and GAGA factor (Li et al., 1998), but the identity of vertebrate GAF has been elusive (Ringrose and Paro, 2004). In a separate study, we were able to identify c-krox/Th-POK as the vertebrate homolog of GAF and showed that it binds to *Evx2-Hoxd13* region in vertebrates (Matharu et al., 2010). These findings suggest that *eve/Evx2* dependence on GAF is a feature acquired early in evolution and that even after *eve* separated from the Hox context, it retained this association and the functional features as seen in *Drosophila*. Our work indicates that, in vertebrates, the ancient organization (as well as the GAF-dependent regulation) has been maintained at least at one of the Hox complexes. Finally, we suggest that using this approach, other evolutionarily conserved cis elements and trans-acting factors involved in genomic organization and developmental gene regulation can be explored

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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