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A functionally conserved *Polycomb* response element from mouse HoxD complex responds to heterochromatin factors

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Anterior-posterior body axis in all bilaterians is determined by the Hox gene clusters that are activated in a spatio-temporal order. This expression pattern of *Hox* genes is established and maintained by regulatory mechanisms that involve higher order chromatin structure and *Polycomb* group (PcG) and *trithorax* group (trxG) proteins. We identified earlier a *Polycomb* response element (PRE) in the mouse HoxD complex that is functionally conserved in flies. We analyzed the molecular and genetic interactions of mouse PRE using *Drosophila melanogaster* and vertebrate cell culture as the model systems. We demonstrate that the repressive activity of this PRE depends on PcG/trxG genes as well as the heterochromatin components. Our findings indicate that a wide range of factors interact with the HoxD PRE that can contribute to establishing the expression pattern of homeotic genes in the complex early during development and maintain that pattern at subsequent stages.

The establishment of anterior and posterior (AP) body axis of an animal is determined during early embryonic stages by a set of genes called hox genes[1–3]. Hox genes are known to be evolutionarily conserved and are arranged in the form of clusters[4,5]. One of the most distinguishing features of these genes is their expression pattern, which shows spatio-temporal colinearity of organization in the genome and expression across the AP axis during embryonic development. The genes that are present at the 3′ end are expressed early and in the anterior segments of the body while the 5′ end genes are expressed later during development and in the posterior segments of the body[2,6–8]. This feature of hox genes is conserved in all bilaterians that have been studied[4,6,9]. However, in flies temporal colinearity is absent where all the segments are formed simultaneously during development[10]. The precise expression pattern of these genes is established and maintained by various cis-elements and trans acting factors that function in a highly coordinated manner. The Polycomb group (PcG) and trithorax group (trxG) proteins are the key epigenetic modulators that act throughout the developmental process. Mutations in these genes lead to gross abnormalities along AP axis. Several studies in flies have been reported showing that PcG and trxG group of proteins are required only for the maintenance of the *Hox* expression states[11]. However, in vertebrates, these proteins determine both the establishment and the maintenance of the expression of *Hox* genes along AP axis[12–15]

Generally PcG members are known to maintain the repressive state while trxG members maintain the active state of target genes. Several PcG and trxG proteins have been identified that act in the form of various complexes by interacting with specific cis-regulatory elements called Polycomb/trithorax Response Elements (PRE/TRE) to maintain the segment specific expression pattern of the target genes[16–20]. PREs and TREs, often found to be functionally overlapping, contain multiple motifs for sequence specific DNA binding members of the PcG/trxG proteins[21]. The PREs in fly are known to function from few bases to several kilobases away from the target genes. Some of the sequence specific DNA binding factors of PcG and trxG proteins bind to these PREs, which can mediate recruitment of PcG/trxG complexes and modify the local chromatin into repressed or active form for proper expression of the gene. In order to understand the properties of PREs and their genetic interactions, *Drosophila* model has been used to develop several assays[22–24]. Based on the properties of PREs, several approaches have been used to identify potential PRE throughout the genome, like binding site analysis of PcG factors by ChIP and prediction of PREs using an algorithm based on frequency of small DNA motifs that interact with PcG/trxG proteins[25–30].

In vertebrates, targeting of the PcG to the PRE is poorly understood compared to that in the flies. Three recent studies have identified and characterized novel PREs using a different approach. The first one is mouse HoxD PRE, present in the region responsible for the early repression of HoxD genes, exhibits PcG dependent repressive activity in mouse as well as in flies[31]. The second one is PRE-kr, which is shown to bind PcG proteins in cultured cells and repress reporter genes in flies and mouse embryos in PcG dependent manner[32]. The third one is the PRE present between *Hoxd11* and *Hoxd12*, which is associated with PcG proteins and represses the reporter construct in cell assay system in PcG dependent manner[33,34]. Though these verterbrate PREs (vPREs) have been identified, the mechanism of action of vPREs is not clear yet. It is not yet understood, for example, if vPREs are like fly PREs or they have additional features and complexity. It is of particular interest as the PcG system itself has gone through expansion and diversification in vertebrates[35]. In order to understand this aspect, we analyzed the mouse HoxD PRE element in flies and carried out a systematic genetic approach to dissect out its interacting factors. We report here that the mouse HoxD PRE region not only interacts with PcG/trxG members but also with heterochromatin components for its repressive activity. We show by genetic assays as well as by cell culture assays that a 2 kb fragment from this region is important for the interaction of PcG and Su(var)s to bring about repression. Finally, we demonstrate the direct binding of TRL-GAF and PC proteins to different regions within the HoxD PRE. These results provide a comprehensive characterization of this element from mouse HoxD complex and point to a complex interplay between chromatin-based mechanisms involving multiple factors in its regulation.

Results

Dissection of the mouse HoxD PRE. The repressive activity of the HoxD PRE was earlier identified within a 5 kb DNA located upstream to the *Evx2* gene at the HoxD complex, Figure 1A[31]. In order to map the PRE activity more precisely and to investigate its genetic interactions, we made 3 overlapping constructs (fragments I, II and III) of the full-length repressor element, Figure 1A. We tested all the 3 fragments along with the full length fragment (FL) in a modified pCaSpeR vector with *mini white* reporter gene that encodes for the red eye color and contains *loxp* sites to flip out the test fragment placed at the 5′ end of the reporter gene, using *Cre* expression flies, Figure 1B.

The FL and fragment II show variegated repression of the mini-white reporter in the modified pCaSpeR vector (Supp Fig 1). For better analysis of the repression and variegation, the phenotype has been classified into two different types: lighter eye color and variegating phenotype (Table 1). We show that fragment II behaves like FL fragment having greater percentage of lighter eye color lines as

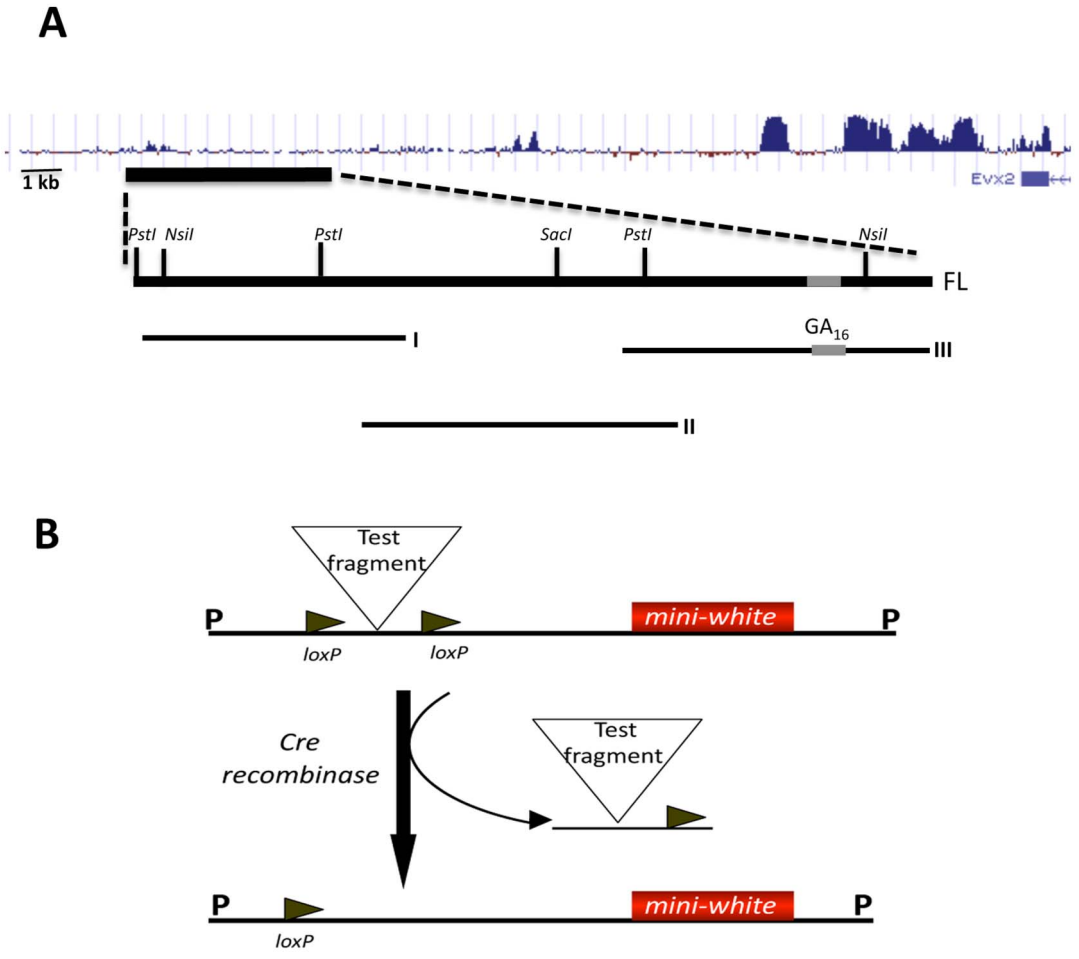

Figure 1 | Location of mouse repressor element in the mouse HoxD complex and the map of the pCaSpeR vector used in the study. (A)Snap shot of UCSC genome browser from *Evx2* gene to the much upstream region. Location of the repressor element is shown in black which shows that there is no conservation when compared to either *Evx2* - *Hoxd13* boundary region or to CR1, 2, 3 of HoxD complex. 5 kb repressor element is divided into three over lapping fragments called as I, II and III. The fragment III is near to the *Evx2* gene. GA repeat is highlighted with gray bar. (B) All the three fragments and full length fragment were cloned in pCaSpeR construct, upstream region of *mini white* reporter gene. All the fragments were flanked by loxp sites. After excision with *Cre recombinase*, vector alone is present with one *loxP* site intact.

Table 1 | Properties of mouse PRE lines

	FL	I	II	III
Number of lines	34	10	21	19
% Lines-variegating	59	0	75	21
% Lines-light eye color	88	40	85	21

well as variegating lines whereas fragments I and III do not show such repression or variegation. To confirm that the repression of the transgenic flies is because of the mouse fragment and not because of the insertion site effect, we crossed the transgenic lines to *Cre* expressing flies to flip out the test fragment[36]. After flipping out, the FL lines show increase in the eye color suggesting that the repression caused is because of the test fragment and not because of the insertion site, Figure 2. None of the three overlapping fragments shows increase in eye color after flip-out. These observations suggest that the FL fragment is functioning like a typical PRE and that fragment II has weak PRE activity similar to the ones that are reported earlier[22]. Since we expected fragment II to show increased eye color upon flipping out, we carried out inverse PCR to map the insertion sites of these lines for further investigation. It turns out that all the fragment II lines were inserted within 500 bp from transcription start site of different genes that are silent in adult eye. It is likely that upon flipping out, the local repressive program continues to repress the *white* gene. Similar weakening of eye color upon flipping out the PRE fragment has been reported earlier where regulatory elements at the site of insertion influence the reporter gene activity[22,37].

Mouse HoxD PRE functions as repressor in mammalian cells. We then examined whether this element can regulate gene expression in vertebrate cells. In order to study this, the putative repressor element and overlapping fragments were cloned in pGL-3 promoter vector upstream of constitutively active SV40 promoter and *luciferase* reporter gene and the activity of the reporter gene was assayed. This reporter plasmid, along with a β-gal expressing plasmid, was transfected in HEK293 and NIH3T3 cells and 30 hrs after transfection the cells were lysed for the reporter assays. Compared to pGL-3 promoter transfected cells, the reporter gene activity was greatly inhibited in cells expressing full length fragment (Figure 3 & Supp Fig 2). In contrast, expression of fragment I resulted in ~2.3 fold activation of the reporter gene, suggesting that this region may behave distinctly as opposed to its role in the context of the full length fragment. Neither fragment II nor fragment III showed repression of reporter gene activity. These observations confirm that as in *Drosophila*, in mammalian cells too the full length fragment encompassing the 5 kb region of the HoxD PRE functions as a repressive element.

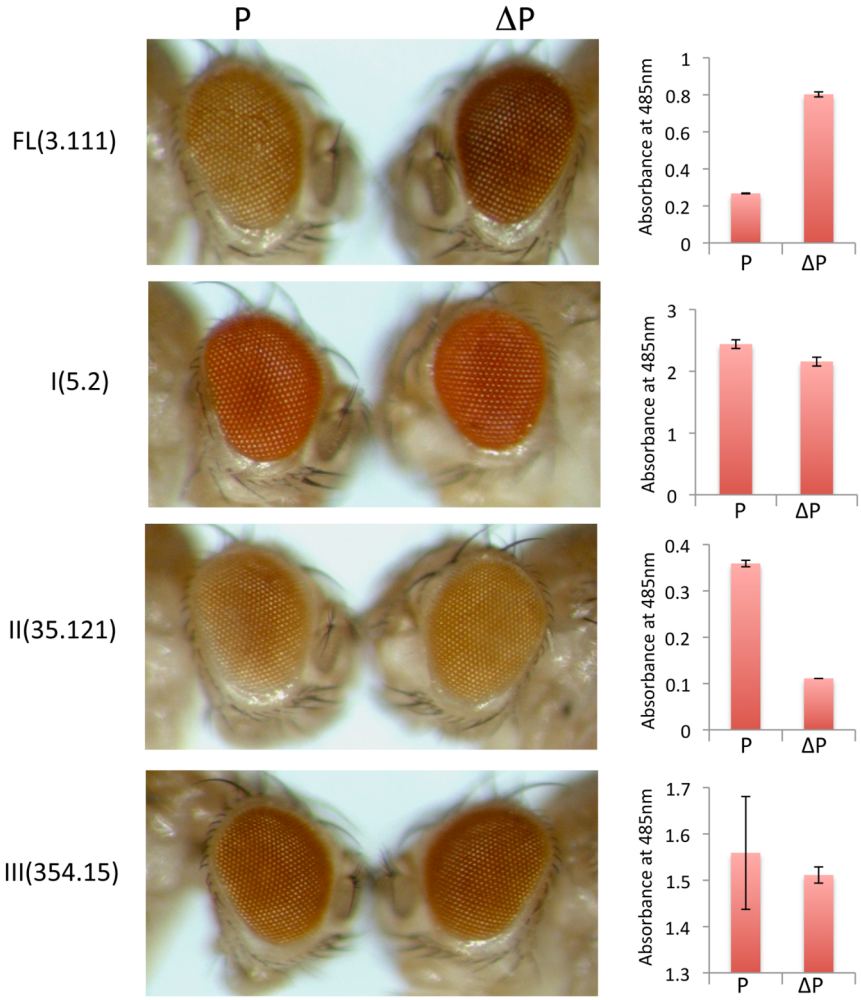

Figure 2 | Repressor activity of mouse repressor element after flip out of the element from the transgenic lines. The eye color of the representative male transgenic lines were shown that were imaged at the same age. The left eye of each box represents the transgene (P) and towards right represents its flip out version of FL, I, II and III fragments (ΔP). The graphs on the right side represents the red pigment value of male flies in homozygous condition of transgenes as well as flip out versions. Error bars represent standard deviation from three independent experiments.

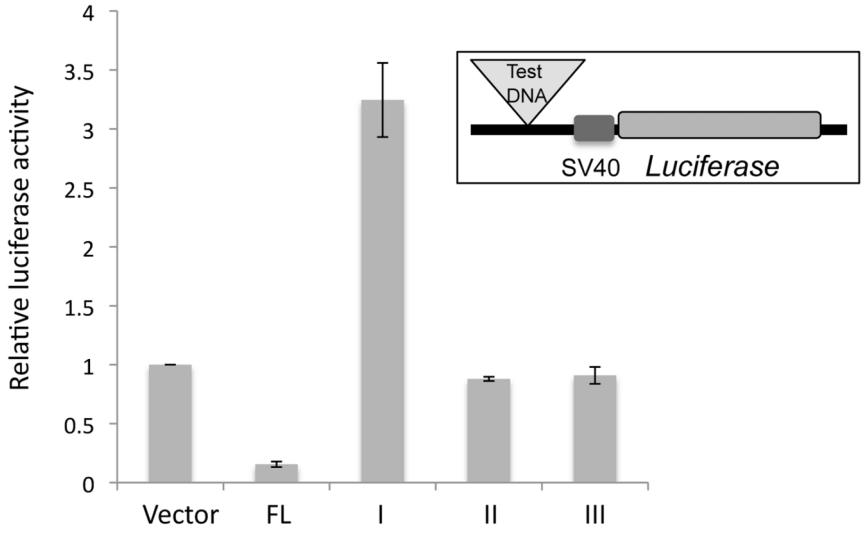

Figure 3 | Mouse repressor element represses luciferase activity in NIH3T3. A) NIH3T3 cells were transected with different constructs that include empty vector and constructs carrying full length fragment and fragment I, II and III. Map of the vector used is shown in the inset. All the transfections were done along with β gal expressing vector. Relative luciferase activity of the mouse fragments is shown on the y axis. Error bars represent standard deviation from three independent experiments.

Mouse HoxD PRE interacts with both *PcG* and *trxG* genes in flies. One of the key features of PREs is their interaction with PcG and trxG genes. We investigated the effect of these mutations on the repressor activity of the mouse element in flies (Table 2). The FL fragment, as reported earlier[31], shows significant derepression in several PcG mutation backgrounds while the control flip out lines do not show effect with these mutations (Supp Fig 3). Like the FL fragment, fragment II also shows derepression of reporter gene with several mutations of PcG members, Figure 4. Fragment I and III, on the other hand, do not respond to PcG mutations, except effect of *Asx* mutation on the fragment III, which shows derepression of the reporter gene (Supp Fig 4). These results suggest that the repressive activity of the full-length fragment lies in the fragment II. The full-length fragment, fragment I and fragment III show repression with some of the trxG mutations like mor^{1} and Trl^{13c} (as well as Trl^{R85}) (Figure 4 and Supp Fig 5). We noticed similar repression with brm^{2} and trx^{1} mutation on fragment I and fragment III while they had no effect on the flipped out lines (Supp Fig 6). The effect of TRL factor is more prominent on these fragments. We used Trl^{13c} allele, which is a hypomorphic allele where some rare escapers of homozygous mutant flies were found. In the homozygous condition of the mutation, full-length, fragment I and III show considerable reduction in eye color (Figure 5). Several other PcG/trxG mutations tested do not show any effect on mouse repressor element transgenic flies (Table 2). Interestingly, *Kismet* mutation, which has been classified as trxG member, shows derepression of *white* gene in FL, II and III fragments. These results indicate while the repressor activity is concentrated at fragment II, the fragments I and III appear to function mainly as TRE. Considering the relatively weaker nature of these elements, however, greater influence of regulatory environment, like presence of promoter, etc., at the site of insertion of at least some lines can not be ruled out.

Studies on PREs suggest that the fate of the target gene, which is under control of the PRE, is maintained by a fine balance between activation factors and repressor factors[38,39]. A decrease in PcG factors weakens the repression as in the case where we demonstrated derepression of the target gene in PcG mutant backgrounds, while decrease in trxG factors compromises the activation of the target gene thereby leading to increased repression of the target gene as seen in our assays in the trxG mutation backgrounds. Our results

Table 2 | Mutations used to test mouse PRE

Mutation	Description	Effect
Pc^{1}	Amorphic allele	Increase
Psc^{1}	Hypomorphic allele	Increase
Asx^{XF53}	Loss of function allele	Increase
Pcl^{T1}	-	Increase
Pcl^{11}	Amorphic allele	Increase
pho^{b}	Amorphic allele	Increase
$Ph\text{-}d^{401}, Ph\text{-}p^{602}$	-	Increase
esc^{2}	Amorphic allele	Increase
$E(z)^{731}$	Amorphic allele	Increase
$Suz2^{1.a1}$	Loss of function allele	Increase
Scr^{w}	Hypomorphic allele	Increase
$Scm^{R5\text{-}13B}$	Hypomorphic allele	Increase
$Su(z)12^{3}$	-	Increase
$Su(z)12^{1}$	Gain of function allele	Increase
grh^{B37}	Loss of function allele	No effect
Trx^{01}	Amorphic allele	Decrease
Trl^{R85}	Amorphic allele	Decrease
Trl^{13c}	Hypomorphic allele	Decrease
Piq^{D91}	-	No effect
kto^{01}	Hypomorphic allele	No effect
brm^{2}	Amorphic allele	No effect
urd^{2}	Hypomorphic allele	No effect
$ash1^{B1}$	Hypomorphic allele	No effect
$ash2^{1}$	Amorphic allele	No effect
mor^{1}	Hypomorphic allele	Decrease
kis^{1}	Loss of function allele	Increase
osa^{2}	Hypomorphic allele	No effect
sls^{1}	-	Increase
skd^{02}	Hypomorphic alele	Increase
$Su(var)3\text{-}9^{01}$	Loss of function allele	Increase
$Su(var)3\text{-}9^{06}$	-	Increase
$Su(var)2\text{-}5^{01}$	-	Increase
$Su(var)2\text{-}5^{5}$	Loss of function allele	Increase
$Su(var)2\text{-}10^{01}$	-	Increase
$Su(var)2\text{-}10^{02}$	-	Increase
$Su(var)3\text{-}4^{02}$	-	No effect
$Su(var)2\text{-}1^{01}$	Antimorphic allile	No effect
P[Su(var)2-5]	Extra copy	Decrease
P[Su(var)3-9]	Extra copy	Decrease

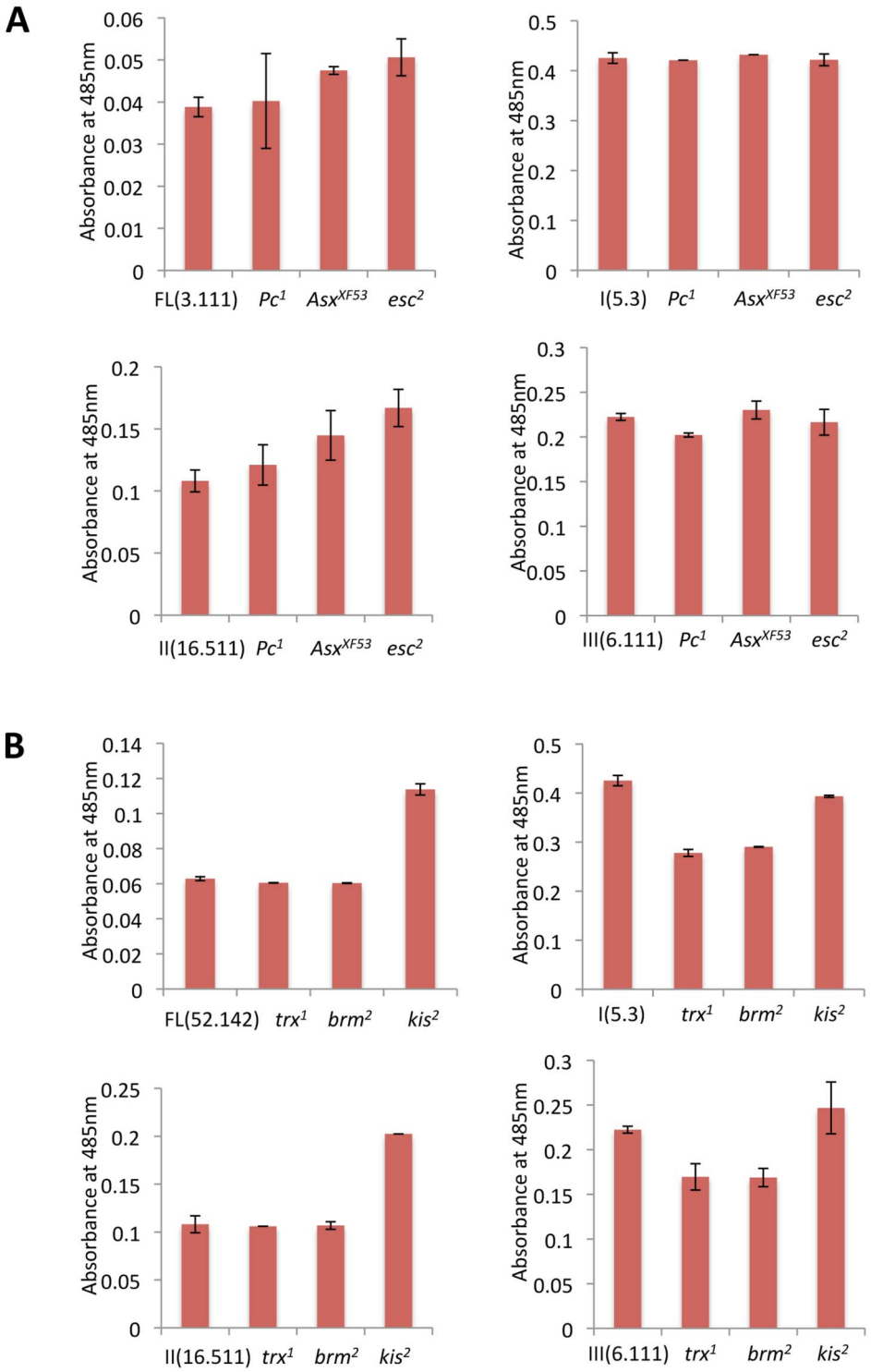

Figure 4 | Effect of PcG/trxG mutations on mouse PRE. (A) The graphs represent the red pigment values of male flies heterozygous for the transgene as well as the PcG mutations. (B) The graphs represent the red pigment values of male flies heterozygous for the transgene as well as the trxG mutations. The error bars represent standard deviation for three independent values.

suggest that, like fly PREs, mouse HoxD PRE has features of both *Polycomb* and *trithorax* response elements, PRE and TRE, delineated in separate regions within 5 kb element.

Repressor activity involves heterochromatin components. We have shown that the silencing activity of mouse HoxD PRE is dependent on PcG system of flies. Many features of PcG mediated silencing resembles heterochromatin silencing and position effect variegation (PEV)[40]. Like PcG silencing, in the heterochromatic silencing also the decision of whether or not to silence a particular gene is made early in development resulting in variegated expression of the target gene. It has also been shown that many Pc binding regions are overlapped with HP1 binding[41]. However, involvement of heterochromatin components has not been shown to affect PRE in any system till date. Here we tested involvement of heterochromatin components on the mouse repressor element. We noticed that *Su(var)2-10*^{2}, *Su(var)3-9*^{1} and *Su(var)2-5*^{1} show derepression in full length fragment and fragment II (Figure 6). The inherent

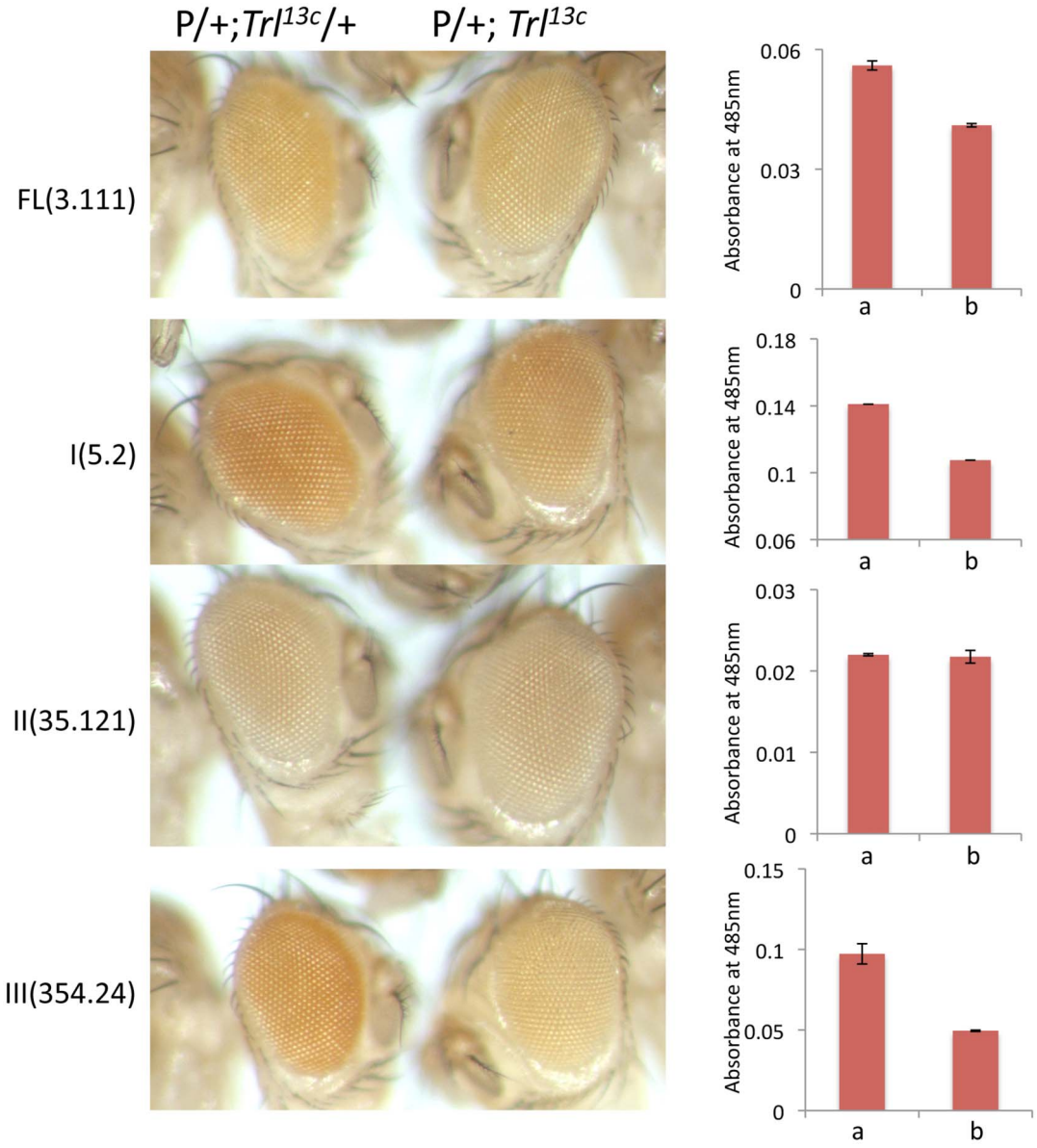

Figure 5 | ***Trl* functions as an activator on mPRE.** Effect of Trl^{13c} on the fragments were shown in heterozygous condition for the transgene and with Trl^{13c} mutation in homozygous background. The graphs on the right side represent the red pigment values of male flies that were heterozygous for the transgene as well as with Trl^{13c} mutation in homozygous background represented as a and b respectively. The error bars represent standard deviation for three independent values.

components of heterochromatin, *Su(var)3-9* and *Suvar)2-5* display haplosuppressor/triploenhancer dosage-dependent effect on PEV[42]. Additional genomic copies of these genes causes strong enhancement of this variegation in w^{m4} lines[42]. We checked for a possible role of extra copies of *Su(var)3-9* and *Su(var)2-5* on the repression mediated by mouse PRE. Interestingly, an extra copy of *Su(var)3-9* and *Su(var)2-5* shows cooperative effect and results in more repression of reporter gene similar to the classical PEV effect (Figure 3). This suggests that the heterochromatin components are involved in the repression mediated by the mouse HoxD PRE.

Effect of temperature. It is known that increase in temperature or stress suppresses heterochromatin spread[41,43,44]. To test how the mouse HoxD PRE, that responds to both PcG silencing as well as heterochromatin silencing mechanisms, responds to temperature we collected 24 hr old embryos of the transgenic flies and exposed them to heat shock at 37C for 1 hr followed by culture at 25C. We observe that heat shock partially, but significantly increased *white* gene silencing on the mouse full length fragment (Supp Fig 7) while there is no effect on the flipped out line. Like the FL fragment, fragment II also responds to temperature though to a lesser extent. These results suggest that the involvement of Su(var)s in repression in mouse HoxD PRE is secondary to the PcG mediated silencing mechanism.

Combined effect of *PcG* and *Su(var)* mutations on mouse PRE. PcG proteins forms different types of complexes to perform their functions, and thus there would be interactions between different PcG genes. We asked whether such interactions have any consequence on the repression phenotype of mouse HoxD PRE lines. For this we made stocks of mouse PRE with Pc^1, esc^2, $Su(var)3\text{-}9^6$ and $Su(var)2\text{-}5^1$ mutations. As shown in Table 3, cumulative increase in eye color was noticed in case of Pc^1 and Psc^1, Pc^1 and Asx^{XF53}, Pc^1 and $Su(z)12^3$ combinations whereas many combinations do not show this increase in eye color (Pc^1 and $Su(z)2^{1.a1}$, Pc^1 and $E(z)^{731}$, Pc^1 and $Scm^{R5\text{-}13B}$, Pc^1 and esc^2) etc

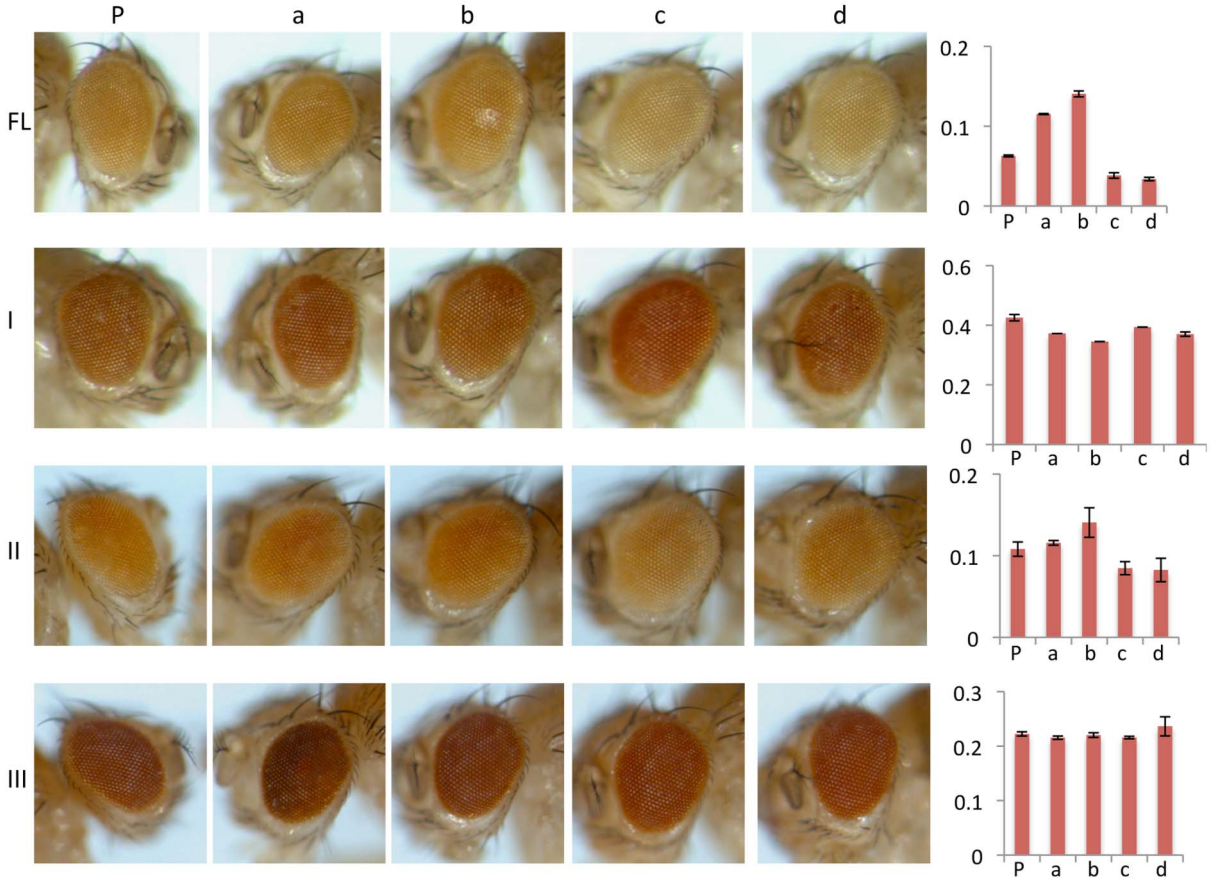

Figure 6 | Effect of heterochromatin components on mouse PRE. The male eye color of FL, I, II and III fragments in heterozygous condition was compared with *Su(var)2-5*^{1}, *Su(var)3-9*^{6}, P[Su(var)2-5] and P[Su(var)3-9] that are denoted as a, b, c and d respectively. The representative flies were imaged at the same age. The graphs on the right side represent the red pigment values of male flies heterozygous for the transgene as well as the mutations. The error bars represent standard deviation for three independent values.

(Figure 7 & Table 3). Also, in all the cases the degree of derepression is dependent on the mutation that is coming from the maternal side. Similarly, we tested different *PcG* and *Su(var)* mutations on mouse PRE. The derepression caused by *Su(var)3-9*^{6} and *Su(var)2-5*^{1} on mouse PRE is not additive (Figure 7). There is derepression of the eye color when the *PcG* mutation is coming from the maternal side while there is no effect of the *Su(var)* mutation coming from the paternal side (Figure 7). This suggests that the PcG machinery is important in establishing repression mediated by mouse PRE while heterochromatin components are involved in stabilizing repression at later stages.

Table 3 | Cumulative effect of multiple mutations on mouse PRE lines

Female	Male	Observation
3.111;Pc^{1}*/Tb*	*Psc*^{1}*/Pin*	Cumulative Increase
3.111;Pc^{1}*/Tb*	*Su(z)12*^{3}*/TM2*	Cumulative Increase
3.111;Pc^{1}*/Tb*	*Su(z)2*$^{1.a1}$*/Cyo*	No effect
3.111;Pc^{1}*/Tb*	*esc*^{2}*/Cyo*	No change
Asx^{XF53}*/Cyo*	*3.111;Pc*^{1}*/Tb*	Cumulative Increase
Su(var)3-9^{6}*/TM2*	*3.111;Pc*^{1}*/Tb*	No change
Ph-d^{401}*,Ph-p*^{602}*/FM7*	*3.111;Pc*^{1}*/Tb*	No change
Su(var)2-5^{1}*/Cyo*	*3.111;Pc*^{1}*/Tb*	No change
Scm$^{R5\text{-}13B}$*/TM3*	*3.111;Pc*^{1}*/Tb*	No change
E(z)^{703}*/TM3*	*3.111;Pc*^{1}*/Tb*	No change
Su(var)2-5^{1}*/Cyo*	*3.111;Su(var)3-9*^{6}*/Tb*	No change
esc^{2}*/Cyo*	*3.111;Su(var)3-9*^{6}*/Tb*	Increase
Pc^{1}*/Tb*	*3.111;Su(var)3-9*^{6}*/Tb*	Increase
3.111;Su(var)3-9^{6}*/Tb*	*Pc*^{1}*/Tb*	No change
3.111;Su(var)3-9^{6}*/Tb*	*Su(var)2-5*^{1}*/Cyo*	No change

Mouse HoxD PRE is associated with PC and GAF proteins. Next we validated whether the observed genetic interactions on mouse PRE are effected by direct binding events on the PRE region or represent indirect interactions. We used transgenic flies carrying the FL fragment for ChIP with antibodies against the TRL factor (GAF) and PC protein. We found that GAF is specifically bound to the region corresponds to fragment I and fragment III but not at middle fragment (region corresponds to fragment II) on the full length fragment carrying transgenic flies (Figure 8). This binding pattern is expected as fragment I and III show clear genetic interaction with *Trl* mutation. PC, on the other hand was enriched at the region corresponding to fragment II and also at fragment I. These results were found to be statistically significant, $P < 0.05$ in all cases. This binding of PC to the region corresponding to fragment I suggest that the binding of PC is spread over upto fragment I, however fragment I alone cannot mediate PcG dependent repression. Such cases have been reported earlier wherein, on Ubx region PcG protein complexes PhoRC, PRC1 and PRC2 and the Trx protein are all constitutively bound to PRE irrespective of activation status of *Ubx* gene[45]. We also checked for binding pattern of GAF and PC on flip out transgenic lines that serve as a control for this binding efficiencies. While there is no binding of PC in the region next to the insertion site where the primer pair is coming for the 5′ P foot region, GAF occupancy is noticed (Supp Fig 8). Our observations are consistent with a highly localized binding pattern of the PC and GAF on mouse PRE transgenic flies, which could be directly

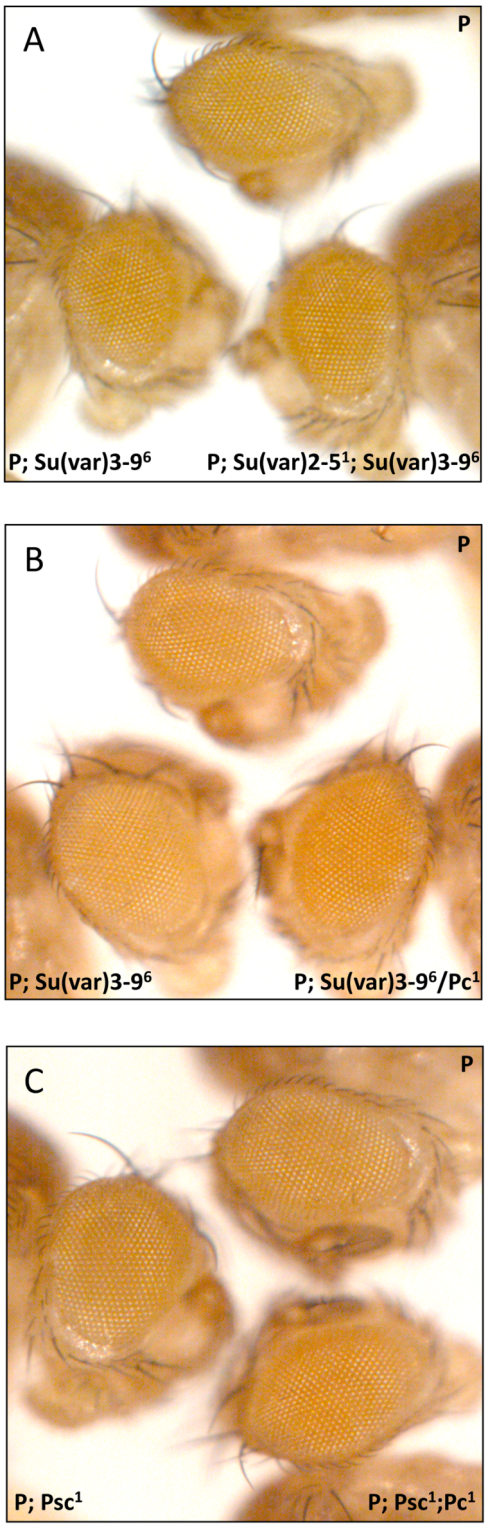

Figure 7 | Cumulative effect of multiple mutations on mPRE. A) Heterozygous line of FL fragment represented as (P) is compared with the progeny of the cross (F) *Su(var)2-5*^{1}/Cyo with P3.111/Cyo; *Su(var)3-9*^{6}/TM2 males. Line 3.111, containing full length fragment doesn't show cumulative increase in eye color upon *Su(var)2-5*^{1} and *Su(var)3-9*^{6} mutations. The eye color remains same. B) Maternal component of *Pc* is important for setting up of repression on mPRE. Line 3.111/Cyo;*Su(var)3-9*^{6}/Tb males were crossed to virgins of *Pc*^{1}/Tb. P/+ *Su(var)3-9*^{6}/*Pc*^{1} shows strong increase in eye color compared to P/+;*Su(var)3-9*^{6}/+ or P/+ line. C) Representative image to show cumulative increase in eye color upon two *PcG* mutations. 3.111/Cyo;*Pc*^{1}/Tb virgins were crossed to males of *Psc*^{1}/Cyo, P/*Psc*^{1};*Pc*^{1}/+ eye color is greater than P/*Psc*^{1} which in turn is greater than P/+ line.

involved in mediating the regulatory effects associated with this element.

Epigenetic modifications associated with mouse HoxD PRE. Based on the association of PC and GAF on the mouse PRE, we also analysed the epigenetic signatures known to be catalyzed by PcG, and Su(var) machinery on the mouse PRE. Real time ChIP-qPCR was performed on the transgenic larvae containing FL fragment using H3K27me3 antibody as read out of *Polycomb* repressor complex (PRC) association, H3K4me2 antibody as an activation mark and also with H3K9me3, H3K9me2 as markers of heterochromatin components (Figure 8). We observed enrichment of both H3K27me3 and H3K9me3 at the region corresponding to fragment II. Interestingly, we also found lower levels of enrichment for H3K9me2 and H3K9me3 marks at fragment I and III (bell shape graph) indicating the spread of these marks across the full-length fragment. In contrast, the activation mark, H3K4me2, showed the opposite trend and was found to be enriched at fragment I and III. Similar enrichments were noticed in cells that were transfected with full length construct (Supp Fig 10). Accumulation of repressive marks at fragment II and activation marks at fragment I and III indicates that the fragment II is more likely to be involved in the repressor activity of the full length fragment while I and III are mainly associated with activation of the target genes. Similar results were obtained when we performed ChIP on mouse ES cells with GAF homolog-ThPOK-antibody. We noticed that THPOK binding is enriched at region corresponding to fragment I and III (Supp Fig 9). These results suggest that apart from the association of PcG/trxG complexes on the mouse repressor element, heterochromatin components also take part in the repression by modifying the local chromatin environment and highlight the role of multiple epigenetic mechanisms associated with the functioning of this complex regulatory element.

Discussion

PcG and trxG proteins are required to maintain the correct expression pattern of *Hox* genes in repressed and active states, respectively, using mechanism that involves higher order chromatin structures. The target genes of PcG/trxG carry cis regulatory elements called PREs that enable to bind and to maintain the status of transcriptional activity of the gene over many cell generations PREs and determine the activation status of the target gene[20,46]. The DNA binding factors of the PcG members target PREs for recruitment of PRC2 complex that puts the repressive H3K27me3 mark. This mark is recognized by PRC1 members followed by H2AK119ub mark that brings about stable silencing of the target genes.

Multicellular eukaryotes also employ a second silencing mechanism, known as position effect variegation (PEV), where genes in close proximity of heterochromatin are repressed by clonal spreading of heterochromatin covering the affected genes. This silencing requires a group of proteins called Su(var)s. These are involved in either modifying nucleosome structure by deacetylation (HDAC/RPD3) or methylation of H3K9 or constitute the building blocks of repressive chromatin structure. Both the mechanisms involve distinct sets of proteins, which are known generally not to overlap functionally[47,48]. There are, however, examples where they do show overlap. It has been shown earlier that Polycomb mediated heterochromatin formation is important from developmentally regulated DNA elimination in *Tetrahymena thermophilia*[47,49]. Added to this, on *kcnq1* imprinting locus, *kcnq1ot1* recruits PRC2 complex and G9a histone methyltransferase in cis to cause transcriptional gene silencing by H3K27me3 and H3K9me9 marks, respectively[50]. Similar to PEV, there is Telomeric position effect (TPE) that shows similarity to heterochromatin silencing which involves some novel factors[51]. We find that at the mouse PRE, factors involved in repression are from multiple silencing mechanisms, PcG/trxG as well as the heterochromatin factors

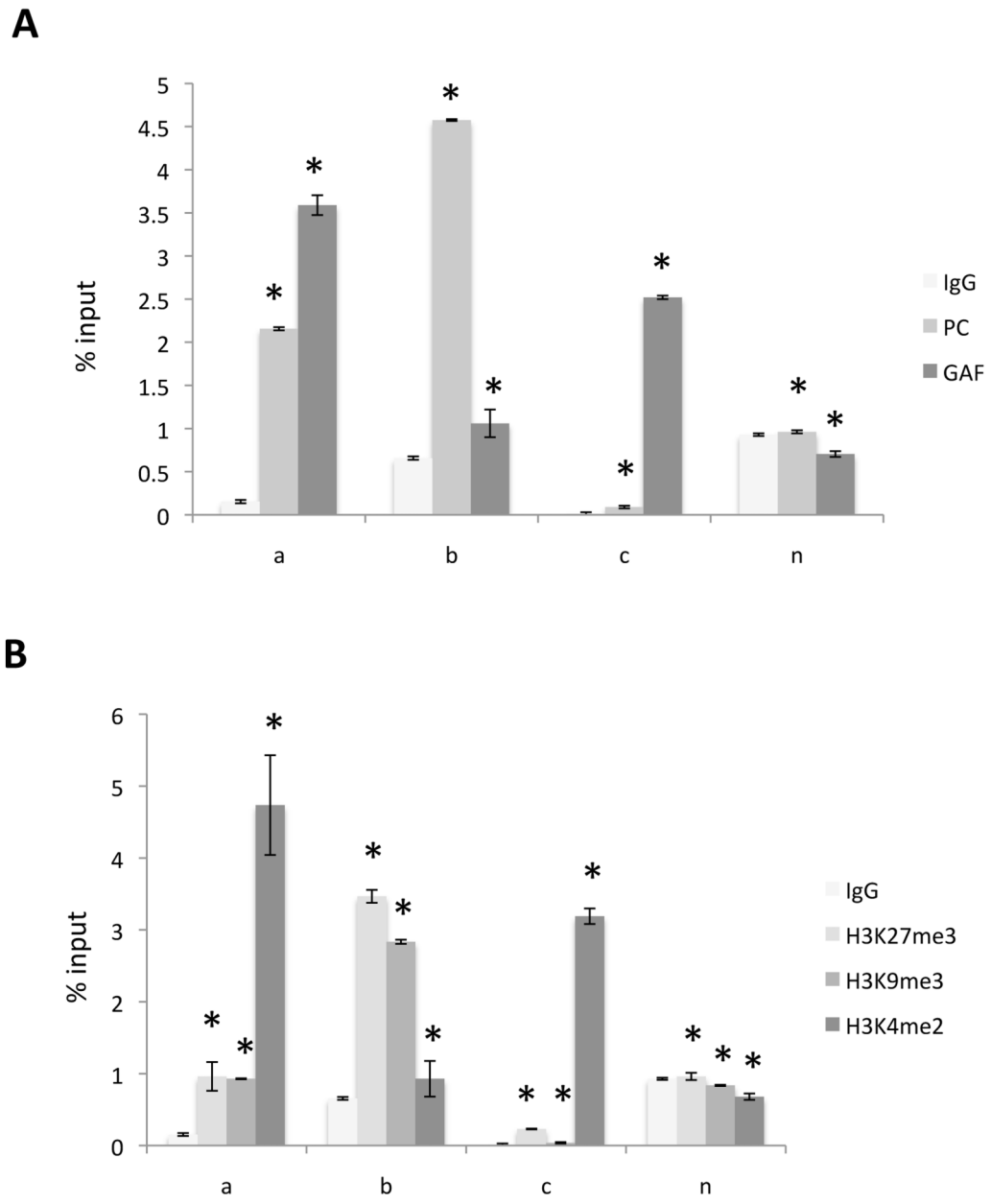

Figure 8 | Binding profiles of PC and GAF and chromatin modifications at transgenic mouse PRE locus. (A) Binding profiles of PC and GAF are shown as percent input. All the primer pairs are from the region itself. Primer pair a lies in the fragment I, b lies in fragment II and c lies in fragment III. Negative control primer pair (n) is from the endogenous *white* gene locus. light gray and dark gray and white bars represent GAF and PC occupancy respectively. IgG is indicated as white bar. (B) ChIP experiment with anti H3K27me3, anti H3K9me3 and anti H3K4me2 were shown. White, light gray, dark gray and black bars represent binding of IgG, H3K27me3, H3K9me3 and H3K4me2, respectively. Data are presented as the mean ± s.e.m. and derived from two independent experiments.*$P < 0.05$ (two-tailed Student's t-test).

involved in PEV and TPE. This suggests that silencing of the Hox locus in mammals involves several components of different silencing mechanisms and that this process is far more complex as compared to fly PREs. Mouse HoxD PRE also has several other features distinct from other PREs. For example, role of *Trl* GAGA as an activator unlike fly PRE where it acts like repressor, lack of pairing sensitive silencing, presence of variegation in large number of transgenic lines and, finally, mild but cumulative effect of PcG mutations distinguish mouse PRE from fly PRE. These observations indicate that while retaining key features during evolution, mouse PREs have acquired novelties, presumably, to render more regulatory functions. It remains to be seen, however, if this is unique feature of this particular PRE or vertebrate PREs are generally of this nature.

Interactions with PcG, trxG and heterochromatin components with mouse repressor element suggest its rather complex nature. In order to precisely delineate the region associated with different factors we analyzed three overlapping fragments of the full length fragment. Our results while in general suggest that the fragment II has most of the repressive activity and fragment I and III the activator activity, the results do not add up to the full-length fragment properties. We ascribe it to multilayered and complex regulatory attributes to the HoxD PRE. The entire length of the PRE, including all the fragments, is necessary for the repressive activity and since properties of the three fragments don't add up to that of the full-length fragment, it is likely that the mouse PRE is of complex nature and multiple motifs spread over the entire length function in a concerted manner.

We noticed unusual effect of trxG mutations on the full length and different fragments. *Asx* is the only factor among all the PcG members tested has repressive effect on the III fragment. Earlier ASX has been shown to be an active member in PR DUB complex, which has a role in H2A deubiquitination[52]. *Pc* target genes especially at *hox* genes requires H2A ubiquitinase activity as well as deubiquitinase activity resulting in rapid loss of *hox* gene repression there by leading to the maintenance of activation[52,53]. Together, with the effect of *Asx* and *kis* mutations on the III fragment strongly suggests that the fragment III mainly functions as mediator of the PRE and TRE features. Among the trxG genes, *brm* and *trx* mutations have no effect on the full length fragment while fragment I and fragment III show repression, supporting their activation input on these elements. We think that in the full length context, the PcG machinery dominates, at least in flies and that when separated out, fragments I and III become free to interact with trxG members. Since I, II and III activities do not add up to the FL activity, we conclude that mouse PRE, although having major repressive activity around the fragment II, is not separable in independent modules and that multiple features across the entire length of the PRE may be interacting with one another.

Finally our study indicates that the region upstream of mouse HoxD complex that is shown to be important for colinear expression of *Hox* genes, functions as a repressor and involves multiple chromatin level mechanisms including PcG/trxG complexes as well as the heterochromatin components. While the complexity of the mouse element is assayable in *Drosophila*, as demonstrated here, the complexity of the factors involved in repression are several fold higher in vertebrate system. For example, there are multiple homologs of *Polycomb* with distinct additional motifs in higher animals[35]. It is likely that vertebrate PREs will be fully and appropriately interpreted for their full regulatory finesse in equivalent host system. Nevertheless, studies from the fly system, do yield useful clues about the regulatory potential and added complexity over the conserved theme of the epigenetic regulatory mechanisms. Such studies also are likely to be helpful in further exploration of these regulatory mechanisms in vertebrate systems.

Methods

Mouse repressor element constructs and micro injections. Sub-fragments of mouse PRE were PCR amplified using overlapping primer pairs. These amplicons (full-length, fragment I, fragment II and fragment III) were cloned into pMOS plasmid (Amersham Pharmacia Biotech). Further, these fragments were released using appropriate restriction sites and sub-cloned in LML vector. XhoI fragments from the LML construct were ligated into XhoI digested pCasperX construct (incorporated XhoI site upstream to *mini-white* gene). The clones were sequenced to check the orientation of the insert with respect to the *mini-white* reporter gene.

For each *P*-element construct 0.5 μg/μl of construct DNA was injected into *yw;PpKiΔ2-3* embryos. Transformants were identified by the presence of the *mini-white* selectable marker and were crossed with w^{1118}. Individual flies of each transgenic line were then crossed to marked balancer chromosomes to generate balanced stocks. All the crosses were done at 25°C unless specified.

To determine the insertion sites of transposons, genomic DNA was isolated from flies for each transgenic line according to the BDGP protocol. The genomic DNA was digested with Sau3A1 and HinP1I enzymes, ligated and used for inverse PCR with primers according to the protocol sited at –http://www.fruitfly.org/about/methods/inverse.pcr.html, EY.3.F-CCTTTCACTCGCACTTATTG, EY.3.R-GTGAGACAGC GATATGATTGT and Plac1- CACCCAAGGCTCTGCTCCCACAAT, Pwht1-GTA ACGCTAATCACTCCGAACAGGTCACA for 3′ and 5′ P element respectively. The sequence flanking the insertion site was determined using the following primers: EY.3.F-CCT TTC ACT CGC ACT TAT TG, 5.SUP.seq1- TAT CGC TGT CTC ACT CAG.

Red pigment assay. 3 day old flies were collected and flash frozen in liquid nitrogen then decapitated manually under microscope collected separately male and female heads in microfuge tubes. Ten heads were placed in tubes and 125 μl of 0.1% ammonium hydroxide and 125 μl of chloroform was added to it. The heads were homogenized manually with Teflon pestle. Solid debris was removed by centrifugation and the absorbance of aqueous phase was measured in spectrophotometer set at 485 nm. Minimum three independent readings were taken to plot the graphs.

Repressor activity assay in cell cultures. All the mouse fragments were cloned in pGL3 -Promoter luciferase vector (Promega) upstream of SV40 promoter and the luciferase reporter gene at Xho1 site. Mouse fibroblast cell line NIH3T3 and human embryonic kidney cell line HEK293T were grown in DMEM supplemented with 10% FBS in a humidified incubator with 5% CO2 at 37°C. The transfections were performed using Lipofectamine Plus™ reagent (Invitrogen, San Diego, CA, USA), according to the manufacturer's instructions. All the plasmids for transfection were prepared using Qiagen columns. Cells grown in 24-well plates were transfected with 100 or 200 ng of the required pGL3-prmoter construct along with 50 ng of pCMV.SPORT-β-gal (Invitrogen). Lysates were prepared about 30 hours post-transfection to assay luciferase and β-gal activities. Preparation of lysates and luciferase assays were carried out as per the instruction of manufacturer (Promega). Relative luciferase activities were calculated after normalizing with β-galactosidase enzyme activities.

Chromatin immunoprecipitation (ChIP). Third instar larvae containing full length transgene was collected, washed well in 1XPBS 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. 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 (rabbit), PC (Santacruz-sc25762), H3K9Me3 (abcam-ab8898), H3K9Me2 (abcam-ab1220), H3K27Me3 (abcam-ab6002), H3K4Me2 (abcam-7766) along with with non-specific IgG antibody (Calbiochem, #401590) as control. For mouse ES cells, Chromatin immunoprecipitation (ChIP) was carried out as per the Upstate kit (catalog 17–295) protocol. In brief, about 10^6 cross-linked cells were resuspended in 2 ml SDS lysis buffer containing protease inhibitors. After 10 min of incubation on ice, 200 μl aliquots were sonicated using Bioruptor (Diagenode). Pre cleared chromatin was incubated with ThPOK antibody (rabbit abcam-ab20985) and with rabbit non-specific IgG antibody (Calbiochem, #401590) for control. Following elution and purification of DNA, relative abundance of each antibody at target transgene 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 was determined from two independent ChIP assays performed on biological replicates. Primer pairs used to analyze the results are as follows:

aF-TTCCATGCTCAGAAATTCCA, aR-GGTTTTGAAAAACTAAGTCTACAG C, bF-GGCGAACTAACCATCTAGGTTTT, bR-CACTCAGGTGGAAGCTCAGA, cF-GGTCCTTGGCATGTCCATTA, cR-TTCCAGGCATGGGTTAAGAA, n-ACT GCGATTGCAACATCAAA, n-TAGCGAGCACAGCTACCAGA, CCP3F- GCTT GCAATAAGTGCGAGTG, CCP3R- CAGCCAAGCTTTGCGTACT, iab7PREF- G GAATACCGCACTGTCGTAGG, iab7PRER- GCAGCCATCATGGATGTGA, Evx2-d13-F GCTTTCTTGCTACGTGGCTG, R GAGAATGCGAGGGTCAGAAGC.

Statistical significance of the enrichment was calculated using the Wilcoxon paired t-test on raw data[54]. We calculated the percent input for each individual replicate and derived standard deviation between the percent input values of the replicates. The standard deviation is divided by square root of n (where n = 2) and plotted in the graph. For calculation of P value significance, we took the raw data of ct values and used the online available tool (http://www.graphpad.com/quickcalcs/ttest1.cfm) to calculate the P value significance.

Approval for animal experiments. Animal (*Drosophila*) experiments were performed in our fly facility that has been approved by the 'Institutional Animal Ethics Committee' and 'Institutional Bio-Safety Committee'.

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Author contributions

V.D. performed the fly experiments. A.N. performed cell culture assays. N.K.M. performed chip assays. V.D. and R.K.M. interpreted the results and wrote the manuscript.

Additional information

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