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2 **The bithorax complex *iab-7* Polycomb Response Element has a novel role in the**  
3 **functioning of the *Fab-7* chromatin boundary.**

4 *Short Title:* PRE facilitates boundary activity

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23 **Abstract**

24 Expression of the three *Bithorax* complex homeotic genes is orchestrated by nine parasegment-specific  
25 regulatory domains. Autonomy of each domain is conferred by boundary elements (insulators). Here, we have  
26 used an *in situ* replacement strategy to reanalyze the sequences required for the functioning of one of the best-  
27 characterized fly boundaries, *Fab-7*. It was initially identified by a deletion, *Fab-7<sup>l</sup>*, that transformed  
28 parasegment (PS) 11 into a duplicate copy of PS12. *Fab-7<sup>l</sup>* deleted four nuclelease hypersensitive sites, HS\*,  
29 HS1, HS2, and HS3, located in between the *iab-6* and *iab-7* regulatory domains. Transgene and *P*-element  
30 excision experiments mapped the boundary to HS\*+HS1+HS2, while HS3 was shown to be the *iab-7* Polycomb  
31 response element (PRE). Recent replacement experiments showed that HS1 is both necessary and sufficient for  
32 boundary activity when HS3 is also presented in the replacement construct. Surprisingly, while HS1+HS3  
33 combination has full boundary activity, we discovered that HS1 alone has only minimal function. Moreover,  
34 when combined with HS3, only the distal half of HS1, dHS1, is needed. A ~1,000 kD multiprotein complex  
35 containing the GAF protein, called the LBC, binds to the dHS1 sequence and we show that mutations in dHS1  
36 that disrupt LBC binding in nuclear extracts eliminate boundary activity and GAF binding *in vivo*. HS3 has  
37 binding sites for GAF and Pho proteins that are required for PRE silencing. In contrast, HS3 boundary activity  
38 only requires the GAF binding sites. LBC binding with HS3 in nuclear extracts, and GAF association *in vivo*  
39 depend upon the HS3 GAF sites, but not the Pho sites. Consistent with a role for the LBC in HS3 boundary  
40 activity, the boundary function of the dHS1+HS3<sup>mPho</sup> combination is lost when the flies are heterozygous for a  
41 mutation in the GAF gene. Taken together, these results reveal a novel function for the *iab-7* PREs in  
42 chromosome architecture.

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44

45 **Author Summary**

46 Polycomb group proteins (PcG) are important epigenetic regulators of developmental genes in all higher  
47 eukaryotes. In *Drosophila*, these proteins are bound to specific regulatory DNA elements called Polycomb  
48 group Response Elements (PREs). PcG support proper patterns of homeotic gene expression throughout  
49 development. *Drosophila* PREs are made up of binding sites for a complex array of DNA binding proteins,  
50 including GAF and Pho. In the regulatory region of the bithorax complex (BX-C), the boundary/insulator  
51 elements organize the autonomous regulatory domains, and their active or repressed states are regulated by  
52 PREs. Here, we studied the domain organization of the *Fab-7* boundary and the neighboring PRE, which  
53 separate the *iab-6* and *iab-7* domains involved in transcription of the *Abd-B* gene. It was previously thought that  
54 PRE recruits PcG proteins that inhibit activation of the *iab-7* enhancers in the inappropriate domains. However,  
55 here we found that PRE contributes to boundary activity and in combination with a key 242 bp *Fab-7* region  
56 (dHS1) can form a completely functional boundary. Late Boundary Complex (LBC) binds not only to dHS1 but  
57 also to PRE and is required for the boundary activity of both elements. At the same time, mutations of Pho  
58 binding sites strongly diminish recruiting of PcG but do not considerably affect boundary function, suggesting  
59 that these activities can be separated in PRE.

60

61

62 **Introduction**

63 Chromosomes in multicellular organisms are subdivided into a series of independent topologically associating  
64 domains (or TADs) [1,2]. The average length of these domains in humans is 180 kb, while they are only on the  
65 order 5-20 kb in flies [3-5]. In mammals, TADs are frequently defined by binding sites for the conserved zinc  
66 finger protein CTCF [6,7]. While a single CTCF is thought to be necessary and sufficient for boundary function  
67 in mammals, this is not true in flies. More than a dozen DNA binding proteins in flies that function as  
68 architectural factors have been identified and it is likely that many more remain to be discovered [8-10].

69 Because of extensive redundancy any one of these individual recognition sequences for these factors might not  
70 be necessary for boundary function.

71 One of the best examples of functional redundancy is the *Fab-7* boundary in the *Drosophila* bithorax  
72 complex (BX-C). BX-C contains three homeotic genes, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and  
73 *Abdominal-B* (*Abd-B*), which are responsible for specifying the parasegments (PS5 to PS13) that make up the  
74 posterior two-thirds of the fly segments [11-14]. Expression of the homeotic genes in the appropriate  
75 parasegment-specific pattern is orchestrated by a series of nine *cis*-regulatory domains, *abx/bx*, *bxp/pbx*, *iab-2*—  
76 *iab-9* (Figure 1A). For example, the *iab-5*, *iab-6*, *iab-7*, and *iab-8* *cis*-regulatory domains direct *Abd-B*  
77 expression in PS10-PS13 [15,16]. BX-C regulation is divided into two phases, initiation and maintenance  
78 [11,17]. During the initiation phase, a combination of gap and pair-rule proteins interact with initiation elements  
79 in each regulatory domain, setting it in the *on* or *off* state. In PS10, for example, initiators in *iab-5* activate the  
80 domain, while *iab-6*, *iab-7*, and *iab-8* are set in the *off* state. In PS11, *iab-6* is activated, while *iab-7* and *iab-8*  
81 are *off*. Once the gap and pair-rule gene proteins disappear during gastrulation, the *on* and *off* states of the  
82 regulatory domains are maintained by Trithorax (Trx) and Polycomb (PcG) group proteins, respectively [18-  
83 21]. These maintenance factors are recruited to the domains by special *cis*-acting elements called Trithorax  
84 Response Elements (TREs) and Polycomb Response Elements (PREs) [22-26]. In addition to elements that  
85 establish and maintain the *on/off* state, each domain has a series of tissue and cell type specific enhancers that  
86 direct the expression of the target homeotic gene in an appropriate pattern [11,16]. For example, the tissue/cell  
87 type enhancers in *iab-6* drive *Abd-B* expression in a pattern that orchestrates the proper differentiation of cells  
88 with a PS11 identity. This pattern of expression is distinct from that in PS12, where *Abd-B* is regulated by  
89 enhancers in *iab-7*.

90 The *Fab-7* boundary, like other boundary elements in BX-C, is required to ensure that the flanking  
91 regulatory domains, *iab-6* and *iab-7*, are able to function autonomously [27-29]. During the initiation phase it  
92 blocks crosstalk between initiators in *iab-6* and *iab-7*, while during the maintenance phase it keeps the domains

93 in *on* or *off* state by preventing interactions between their PREs and TREs. In addition, like most other BX-C  
94 boundaries, *Fab-7* must also facilitate “bypass”, enabling the distal regulatory domains *iab-5* and *iab-6* to  
95 “jump over” and contact *Abd-B* in PS10 and PS11, respectively. Like blocking, bypass activity is essential for  
96 proper *Abd-B* regulation. *Fab-7* was initially defined by a 4 kb X-ray induced deletion that had an unusual  
97 dominant gain-of-function (GOF) phenotype, transforming PS11 into a duplicate copy of PS12 [27]. The *Fab-7<sup>l</sup>*  
98 deletion spanned four nuclease hypersensitive regions, HS\*, HS1, HS2, and HS3 [30]. Subsequent transgene  
99 studies showed that a 1.2 kb fragment spanning HS\*+HS1+HS2 had enhancer blocking activity in embryos and  
100 in adults [31,32]. The fourth hypersensitive region, HS3, had no detectable boundary activity; however, when  
101 included in a *white* transgene, it induced pairing sensitive silencing, which is a characteristic activity of PREs  
102 [33]. This separation of functions was confirmed by Mihaly et al [29] who generated a series of new deletions  
103 that removed either HS\*+HS1+HS2 or HS3. Unlike *Fab-7<sup>l</sup>*, deletions that removed only HS\*+HS1+HS2 have  
104 a mixed GOF and LOF (loss-of-function phenotype) (PS11→PS12 and PS11→P10, respectively). Mutations in  
105 *PcG* genes enhance the GOF phenotypes, while mutations in *Trx* enhance the LOF phenotypes. By contrast,  
106 *PcG* and *Trx* mutations have no effect on the GOF phenotypes of deletions, like *Fab-7<sup>l</sup>*, that remove all four  
107 hypersensitive regions. Finally, flies carrying HS3 deletions are typically wild type as heterozygotes or  
108 homozygotes and only infrequently weak GOF phenotypes are observed in homozygous animals [29,34]. While  
109 the LOF phenotypes in HS\*+HS1+HS2 deletions require silencing of the *iab-6* domain by the *iab-7* PRE, this is  
110 not the only mechanism that can give rise to LOF transformations of PS11 (and PS10). LOF phenotypes are  
111 also observed when *Fab-7* is replaced by heterologous boundaries such *scs*, *su(Hw)*, or the BX-C boundary *Mcp*  
112 [35-37]. Like *Fab-7*, these boundaries prevent crosstalk between *iab-6* and *iab-7*; however, they fail to support  
113 bypass, and instead block the *iab-6* domain from regulating *Abd-B*. Thus far, the only heterologous boundary  
114 that recapitulates both the blocking and bypass activity of *Fab-7* is the neighboring boundary *Fab-8* [36,38].

115 Previous studies indicate that *Fab-7* (HS\*+HS1+HS2) boundary activity is generated by a combination  
116 of ubiquitously expressed factors and stage/cell type specific factors. One ubiquitously expressed factor is the  
117 zinc finger protein Pita which binds to sites in HS2 [37,39]. The known developmentally regulated factors are

118 Elba, Inensitive (Insv), and the LBC [40]. The LBC is a multiprotein complex that contains at least three  
119 distinct DNA binding proteins, the GAGA factor (GAF), Clamp, and Mod(mdg4) [41]. In the case of *Fab-7*,  
120 three contiguous sequences spanning GAGA sites 3, 4, and 5 generate LBC shifts [34,42].

121 While transgene experiments indicated that sequences spanning HS\*+HS1+HS2 are required for  
122 blocking activity [43,44], boundary replacement using an *attP* platform that deletes HS\*+HS1+HS2+HS3  
123 suggested that the requirements for boundary function out of context are more demanding than those in BX-C.  
124 These replacement experiments indicated that HS\* and HS2 are not required for boundary activity, while the  
125 largest hypersensitive region, HS1, is both necessary and sufficient [34]. However, there was a confounding  
126 factor in these experiments: because the HS3 *iab-7* PRE has Polycomb silencing activity and as such is an  
127 important regulatory component of the *iab-7* regulatory domain, it was retained in these replacement  
128 experiments. Here we have asked whether HS1 alone is sufficient in the absence of HS3 *iab-7* PRE.  
129 Surprisingly, it is not. Instead, our studies indicate that HS3 not only has PRE activity, but also that it  
130 contributes to the boundary function of *Fab-7*. Moreover, it appears that like HS1, the LBC is important for the  
131 boundary (and PRE) functions of HS3.

132

## 133 **Results**

### 134 **HS3 rescues boundary activity of HS1**

135 Studies by Wolle et al [34] have shown that HS1 is both necessary and sufficient for boundary function.  
136 However, in these experiments HS3 was also present. For this reason we wondered whether HS1 would be  
137 sufficient in the absence of HS3. Figure 1B shows the *HS1* replacement, and as controls, the starting platform  
138 *Fab-7*<sup>*attP50*</sup>, *HS1+HS3*, and *HS3* alone. The *Fab-7*<sup>*attP50*</sup> platform deletes HS\*+HS1+HS2+HS3, and as observed  
139 for the *Fab-7*<sup>*l*</sup> deletion, the A6 segment is completely transformed into a duplicate copy of A7 (Figure 1C). As  
140 reported previously, we found that flies carrying the *HS1+HS3* replacement are indistinguishable from wild

141 type, while for the *HS3* replacement, there is a strong GOF transformation, and the A6 tergite is greatly reduced  
142 in size or absent, while the sternite is missing (Figures 1C and S1). Unexpectedly, like *HS3*, *HS1* alone also has  
143 a strong GOF phenotype. The sternite is typically missing, while the tergite is typically greatly reduced in size  
144 (Figures 1C and S1).

145 The fact that *HS1* is insufficient for boundary function in the absence of *HS3* prompted us to reassess  
146 the role of *HS3* in *Fab-7* boundary function. To address this question, we examined the functional properties of  
147 different combinations of the *HS\**, *HS1*, and *HS2* regions either in the presence or absence of the *iab-7* PRE,  
148 *HS3*. We first attempted to rebuild the boundary using combinations of *HS\**, *HS1*, and *HS2*. As previously  
149 reported in studies on the Pita sites in *HS2*, *HS1+HS2+HS3*, or *HS1+HS2<sup>4Pita</sup>+HS3* replacements are fully  
150 functional [37]. In contrast, the boundary function of the *HS1+HS2* replacement is tissue-specific (Figure 2). In  
151 *HS1+HS2* flies, the A6 tergite is fully wild type. In contrast, the sternite is absent indicative of a GOF  
152 transformation of PS11→PS12 in the cells that give rise to the ventral cuticle. In this context, the Pita sites in  
153 *HS2* are essential for boundary function in the cells that give rise to the tergite [37].

154 We next tested the *HS\*+HS1* combination with or without *HS3*. While the *HS\*+HS1+H3* combination  
155 is fully functional, *HS\*+HS1* retains only limited tissue-specific boundary activity (Figure 2). *HS\*+HS2*  
156 appears to have no boundary activity in the histoblasts giving rise to the ventral cuticle, and in all male flies the  
157 A6 sternite is completely absent. In about 80% of the males, the A6 tergite is greatly reduced in size and has an  
158 irregular shape, as expected for an A6→A7 (or PS11→PS12) transformation in segment identity (Figures 2 and  
159 S2). In the remaining 20%, the boundary appears to be at least partially functional and there is only a slight  
160 reduction in the size of the A6 tergite.

161 The experiments described above indicate that *HS3* complements the blocking defects of boundaries  
162 composed of just *HS1* or *HS1* plus either *HS2* or *HS\**. We wondered whether *HS3* might also contribute to the  
163 bypass activity. To test this possibility, we reinvestigated the effects of inverting the *Fab-7* boundary. In  
164 previous study, we showed that the blocking and bypass activity of *HS1+HS2* is orientation independent [36].

165 However, this experiment was done in the presence of HS3. To determine if HS3 contributes to orientation  
166 independence, we generated forward and reverse  $HS^*+HS1+HS2$  replacements that lacked HS3 (Figure 2). The  
167 properties of the  $HS^*+HS1+HS2$  replacement resemble Class III deletions described by Mihaly et al [29].  
168 While the size of A6 tergite is normal in all but about 5% males, the sternites are typically thinner and slightly  
169 malformed. This phenotype is indicative of a very weak GOF transformation of the A6 sternite (Figures 2 and  
170 S2). A different result was obtained for the inverted  $[HS^*+HS1+HS2]^R$  replacement (Figure 2). All flies had a  
171 fully wild type tergite, while the sternites were weakly malformed, exhibiting weak LOF transformation of A6  
172 into A5. Since the HS3 *iab-7* PRE was absent in these replacements, the weak LOF phenotypes are expected to  
173 arise because bypass activity is partially compromised in cells giving rise to the ventral adult cuticle.

174 **Functional dissection of HS1.**

175 In contrast to HS1 alone, the  $HS1+HS3$  combination has full boundary function. To further probe the  
176 requirements for HS1 boundary activity when combined with HS3, we subdivided HS1 into proximal and distal  
177 parts, pHS1 and dHS1 (Figure 3). Previous experiments have implicated the LBC in the late blocking activity of  
178 dHS1, while an Elba/Insv recognition sequence is likely to contribute to early blocking activity of pHS1 [40].  
179 Figures 3 and S3 show that the  $pHS1+HS3$  combination gives a strong GOF transformation of A6, indicating  
180 that the pHS1 sequence is not able to reconstitute boundary activity. In contrast, the  $dHS1+HS3$  combination  
181 has a fully wild type A6 segment, just like the  $HS1+HS3$  combination.

182 Wolle et al [34] showed that the LBC can bind independently to ~65-80 bp probes spanning the  
183 GAGA3, GAGA4, and GAGA5 sequences in dHS1, and that binding to the 65 bp GAGA3 and GAGA4 probes  
184 requires the GAGAG motif. However, we subsequently found that optimal LBC binding is to larger DNA  
185 probes that span GAGA3-4, GAGA3-5, or even GAGA3-6. The experiments shown in Figure S4 A, B compare  
186 LBC binding to the 65 bp GAGA3 and GAGA4 probes with binding to a larger GAGA3+4 probe. In both  
187 binding and competition experiments we found that there is a 6-12 fold differential in LBC binding to the larger  
188 GAGA3+4 probe. This difference in relative affinity isn't due to just probe length. The competition

189 experiment in Figure S4C shows that a hybrid GAGA3+LacZ probe of the same length as GAGA3+4 is a poor  
190 competitor for LBC binding compared to GAGA3+4. Even larger differences in relative affinity are observed  
191 for probes spanning GAGA3-5 or GAGA3-6.

192 While LBC binding to the 65 bp GAGA3 and GAGA4 probes requires the GAGAG motif, how  
193 mutations in this motif affect LBC binding to larger dHS1 probes hasn't been investigated. For this purpose, we  
194 compared LBC binding to dHS1 probes that are wild type or have mutations in GAGA3-6. Figure 4A shows  
195 that LBC binding to the dHS1 probe is largely abrogated when all four GAGA motifs are mutant (GAGAmut3-  
196 6).

197 If the LBC binding to dHS1 is important for boundary function in the dHS1+HS3 combination, then it  
198 should be disrupted when the dHS1<sup>mGAF</sup> fragment is used for the replacement instead of the wild type dHS1  
199 fragment. Figure 5 shows that this is the case. Like the HS3 replacement alone, the dHS1<sup>mGAF</sup>+HS3  
200 combination exhibited a strong GOF phenotype.

201 **The GAGA motifs in HS3 are required for boundary function while the Pho motifs are dispensable**

202 A number of different mechanisms could potentially explain how HS3 contributes to the boundary  
203 functions of the *Fab-7* hypersensitive regions HS\*, HS1, and HS2. One intriguing possibility is that the PcG  
204 dependent silencing activity of the HS3 *iab-7* PRE is needed for the boundary activity of replacements that  
205 contain only HS1/dHS1 (or HS1 plus either HS\* or HS2). The HS3 *iab-7* PRE has two GAF recognition  
206 sequences (GAGAG) and three recognition sequences for the zinc finger protein Pleiohomeotic (Pho) [45].  
207 Like many other *Drosophila* PREs [45,46], these DNA binding motifs are important for the PcG dependent  
208 silencing activity of the *iab-7* PRE. Mutations in either the GAGAG or Pho sequences compromise the  
209 silencing activity of the *iab-7* PRE in *mini-white* transgene assays [47]. Moreover, consistent with a role for the  
210 GAF (Trl) and Pho proteins in silencing, mutations in the *Trl* and *pho* genes suppress the silencing activity of  
211 the HS3 *iab-7* PRE [33,48].

212 If the PcG dependent silencing activity of the *iab-7* PRE is required to complement the boundary defects

213 of HS1, then mutations in either the HS3 GAGA or Pho sequences should abrogate the boundary activity of the

214 dHS1+HS3 combination. To test this prediction, we generated *dHS1+HS3<sup>mGAF</sup>* and *dHS1+HS3<sup>mPho</sup>*

215 replacements. As expected, *dHS1+HS3<sup>mGAF</sup>* lacks boundary activity, and flies carrying this replacement exhibit

216 a strong GOF transformation of A6 (PS11) (Figure 5). However, contrary to our predictions, most

217 *dHS1+HS3<sup>mPho</sup>* flies are fully wild type, and exhibit no evidence of either GOF or LOF transformation. As was

218 reported by Mihaly et al [29] for HS3 deletions, a small percentage (~2-5%) of the male *dHS1+HS3<sup>mPho</sup>* flies

219 have a weak GOF transformation. In these flies the size of the tergite is reduced and/or the sternite is

220 misshapen.

221 **The LBC binds to HS3 and binding depends upon two GAGA motifs**

222 The fact that mutations in the GAGA motifs disrupt the boundary activity of HS3, while those in the Pho

223 sites do not would argue that the PcG dependent silencing activity of HS3 is probably not responsible for its

224 ability to complement HS1. Instead, it would appear that HS3 boundary function is separable from PRE

225 activity. With aim of identifying factors contributing to HS3 boundary activity, we used three overlapping

226 probes spanning HS3 (227 bp) for EMSA experiments with embryonic nuclear extracts. The EMSA experiment

227 in Figure S5 shows that each probe generates several shifts. Though the identity of most of these shifts is

228 unknown, the prominent slowly migrating shift observed with probe 2 resembles the shift generated by the

229 LBC.

230 To determine if this slowly migrating shift corresponds to the LBC, we used a 200 bp fragment

231 containing most of HS3, rather than the shorter probe 2. Like probe 2, the larger probe generates an LBC-like

232 shift (Figure 4B). Two experiments indicate that this HS3 shift corresponds to the LBC. The first is a

233 competition experiment with two different DNA fragments known to bind the LBC, *Fab-7* G3+4 and CES

234 *roX2*. As could be predicted, the HS3 shift is competed by itself and also by excess unlabeled G3+4 and *roX2*.

235 In the second experiment, we used peak LBC fractions from a gel filtration column, plus a control fraction that

236 lacks LBC activity for antibody “supershift” experiments. Previous studies showed that the LBC shift is  
237 sensitive to antibodies direct against Clamp, GAF, and Mod(mdg4). Figure 4D shows that LBC binding to HS3  
238 in the peak gel filtration fractions 45 and 47 is inhibited by Clamp antibody, while GAF and Mod(mdg4)  
239 antibodies generate a supershift.

240 These experiments indicate that like dHS1, the LBC binds to the full length HS3 sequence *in vitro*. This  
241 finding suggests a plausible mechanistic explanation for why HS3 contributes to *Fab-7* boundary and is able to  
242 reconstitute boundary activity when combined with dHS1. If this explanation were correct, we would expect  
243 that LBC binding to HS3 should require the GAGA motifs but not the Pho binding sequences. This is indeed  
244 the case. Figure 4C shows that mutations in the HS3 GAGA motifs (HS3<sup>mGAF</sup>) abrogate the LBC shift, while  
245 mutations in the Pho binding sequence (HS3<sup>mPho</sup>) have no effect. The requirement for the GAGA motifs, but  
246 not the Pho binding sequences is confirmed by competition experiments (Figure 4B) with mutant HS3 DNAs.  
247 HS3<sup>mPho</sup> competes as well as the wild type HS3 for LBC binding, while HS3<sup>mGAGA</sup> is a poor competitor.

#### 248 **Protein occupancy is altered by mutations that impair function**

249 To extend this analysis, we used chromatin immunoprecipitation (ChIP) experiments to compare GAF  
250 association with wild type and mutant versions of dHS1+HS3 in embryos and pupae. In the wild type  
251 *dHS1+HS3* replacement, GAF is found associated with both dHS1 and HS3 in embryos and pupae (Figure 6).  
252 As would be predicted from the loss of LBC binding to dHS1<sup>mGAF</sup> DNA in nuclear extracts, GAF association  
253 with dHS1 in the *dHS1<sup>mGAF</sup>+HS3* replacement is substantially reduced. Interestingly, GAF association with  
254 HS3 is also reduced in the *dHS1<sup>mGAF</sup>+HS3* compared to the wild type *dHS1+HS3* replacement. This secondary  
255 effect is seen not only in embryos, but also in pupae (Figure 6).

256 Our EMSA experiments predict that GAF association with HS3 will be disrupted by mutations in the  
257 GAGA sequences, but not the Pho sequences. This expectation is correct. GAF association with HS3 is reduced  
258 in the *dHS1+HS3<sup>mGAF</sup>* replacement while mutations in the Pho site (*dHS1+HS3<sup>mPho</sup>*) have no effect (Figures 4C  
259 and 6).

260 We also compared the dHS1/HS3 association of the Polycomb Repressive Complex 1 (PRC1) protein

261 Polyhomeotic (Ph) in the different dHS1+HS3 replacements. As would be predicted from our previous studies  
262 on the silencing activity of HS3 in transgene assays, mutations in either the GAGA or Pho sequences reduce Ph-  
263 HS3 association (Figure 6). Ph also binds to a nearby sequence in the *iab-7* *cis*-regulatory domain and, to a  
264 lesser extent, to HS1. In both cases, this association is reduced by mutations in the HS3 GAGA and Pho  
265 sequences. By contrast, mutations in the dHS1 GAGA sequences (dHS1<sup>mGAF</sup>) have limited effects on Ph  
266 association in HS3 or *iab-7*.

267 Our mutant replacement experiments showed that *dHS1+HS3<sup>mPho</sup>* has nearly full boundary activity,

268 while boundary activity is compromised in *dHS1+HS3<sup>mGAF</sup>*. These findings, together with our *in vitro*  
269 experiments on the LBC, would predict that the boundary activity of *the dHS1+HS3<sup>mPho</sup>* replacement should  
270 depend on the GAF protein. If this is case, boundary activity might be sensitive to the dose of the gene  
271 encoding the GAF protein, *Trl*. To test this possibility we recombined the *dHS1+HS3<sup>mPho</sup>* replacement with a  
272 *Trl* null mutation, *Trl<sup>R85</sup>*. The GOF transformations evident in *dHS1+HS3<sup>mPho</sup>/Trl<sup>R85</sup>* *dHS1+HS3<sup>mPho</sup>* flies  
273 show that the boundary activity of this replacement is compromised by a reduction in the dose of the *Trl* gene  
274 (Figure 5B).

275

276

## 277 Discussion

278 Previous functional studies indicated that the *Fab-7* region of BX-C is subdivided into two seemingly

279 distinct elements [29]. One of these elements spans the distal nuclease hypersensitive site HS3 and corresponds  
280 to a PRE for the *iab-7* regulatory domain. The other element spans the three proximal nuclease hypersensitive  
281 sites, HS\*, HS1, and HS2. This element corresponds to the *Fab-7* boundary. In each case, the functional  
282 assignment was based on a combination of transgene assays and analysis of deletions in the *Fab-7* region

283 generated by excision of a P-element insertion located between HS2 and HS3. This functional analysis has  
284 recently been extended with an *attP* replacement platform in which the entire region has been deleted [34].  
285 This *attP* platform makes it possible to systematically test the functional properties of different *Fab-7* sequences  
286 in their native context. Using this platform, we recently found that hypersensitive site HS1 was both necessary  
287 and sufficient for wild type *Fab-7* boundary activity in a context in which the *iab-7* PRE, HS3, was present.

288 Since boundary activity in transgene assays required a sequence spanning HS\*, HS1, and HS2, this  
289 finding suggested that the demands for full activity are considerably less stringent in the native context than in  
290 enhancer blocking assays. To confirm this conclusion, we tested the HS1 by itself. Unexpectedly, it is not  
291 sufficient for wild type boundary activity. Likewise, combinations of HS1 with either HS\* or HS2 have only  
292 partial, tissue-specific boundary activity. In both combinations, the A6 sternite in males is absent, indicative of a  
293 PS11→PS12 GOF transformation. For the HS1+HS2 combination, the A6 tergite is wild type, while, for the  
294 HS\*+HS1 combination, the tergite displays weak to moderate GOF phenotypes. The only combination of these  
295 *Fab-7* sequences that has nearly complete boundary activity in the native context is HS\*+HS1+HS2.

296 Like HS1 alone, the impaired boundary function of HS\*+HS1 and HS1+HS2 can be rescued by the  
297 addition of HS3. These findings argue that HS3 must be able to contribute to *Fab-7* boundary function. Two  
298 different models could potentially explain the boundary activity of HS3. In the first, its boundary function  
299 would depend on the ability of HS3 to recruit P<sub>c</sub>G proteins and induce silencing. In the second, boundary  
300 function would reflect a PRE associated activity that is independent of P<sub>c</sub>G recruitment and silencing. For  
301 example, since P<sub>c</sub>G silencing is facilitated by pairing interactions between PRE containing transgene inserts on  
302 each homolog, the *iab-7* PRE, HS3, might have a chromosome architectural activity just like the classical  
303 boundaries [48-52]. A number of lines of evidence are consistent with this second model.

304 We compared the factors binding to HS1 and HS3 sequences using EMSA experiments with embryonic  
305 nuclear extracts. While probes spanning HS3 gave multiple shifts, the most prominent HS3 shift corresponds to  
306 a HS1 boundary factor, the LBC. This conclusion is supported by several observations. First, the HS3 LBC  
307 shift is competed by two DNA sequences, GAGA3+4 (*Fab-7*) and *roX2*, which are known to bind the LBC.

308 Second, as observed for other LBC recognition sequences, antibodies against GAF, Clamp, and Mod(mdg4)  
309 either generate a supershift or interfere with binding to the HS3 sequence. Third, the peak LBC fractions after  
310 gel filtration of embryonic nuclear extracts have an apparent molecular weight of ~1,000 kD. These peak LBC  
311 fractions generate a shift with the HS3 probe and were used for the antibody “supershift” experiments.

312 LBC binding to the *Fab-7* probes GAGA3, GAGA4, and dHS1 and to three X-linked CES depends upon  
313 GAGAG motifs (or GA rich sequences). This is also true for HS3. Mutations in the two HS3 GAGA motifs  
314 substantially reduce the yield of the HS3 LBC shift. Consistent with this finding, competition experiments  
315 indicate that the HS3<sup>mGAF</sup> is a poor competitor for LBC binding to the wild type HS3 probe. While the HS3  
316 GAGAG motifs are required for LBC binding, the two Pho sites are not.

317 Previous studies on the HS3 *iab-7* PRE indicate that like other fly PREs, it requires the GAF and Pho  
318 proteins for its silencing (and pairing-sensitive) activity [33,47,48]. Mutations in the two GAGAG motifs and  
319 in the two Pho recognition sequences disrupt silencing activity. Pho interacts with Sfmbt and is directly  
320 involved in the recruitment of PRC1 to PREs [53-56]. In vitro, GAF facilitates Pho binding to a chromatinized  
321 template [57].

322 If PcG silencing activity is critical for HS3 boundary activity, then boundary activity should be  
323 eliminated by mutations in either the GAGAG motifs or the Pho binding sites. In contrast, if LBC binding to  
324 HS3 is important, then mutations in the GAGAG motifs should disrupt the boundary function of the dHS1+HS3  
325 replacement, while mutations in the HS3 Pho binding sequences should not. Consistent with the expectations of  
326 the second model, we found that the HS3 GAGAG motifs are important for boundary function, while the Pho  
327 binding sequences are not.

328 This distinction is also reflected in the protein occupancy of wild type and mutant HS1+HS3  
329 replacements. In the wild type replacement, both GAF and Pho are associated with HS3. As would be predicted  
330 from the effects of GAGAG mutations on the PcG silencing activity of HS3 in transgene assays, the levels of  
331 both GAF and the Polycomb protein Ph are reduced in the HS1+HS3<sup>mGAF</sup> replacement. In contrast, mutations

332 in the HS3 Pho sites have no effect on GAF occupancy, while they reduce Ph occupancy. A prediction that  
333 follows from these findings is that the GAF protein is important for the boundary activity of dHS1+HS3<sup>mPho</sup>  
334 replacement. Consistent with this prediction, dHS1+HS3<sup>mPho</sup> boundary function is compromised when the flies  
335 are heterozygous for a mutation in *Trl*.

336 While the findings reported here support the idea that the boundary activity of both dHS1 and HS3 is  
337 mediated, at least in part, by the LBC, many questions remain. For example, why does HS3 *iab-7* PRE have  
338 P<sub>c</sub>G silencing activity while HS1 doesn't? Likewise, why are the X-chromosome CES able to recruit the M<sub>sl</sub>  
339 dosage compensation complexes? One idea is that the silencing activities of the HS3 *iab-7* PRE and the dosage  
340 compensation functions of the CES depend upon the association of functionally specialized ancillary factors  
341 with a platform that is provided by LBC binding. This idea would be consistent with our findings. Both the  
342 silencing and boundary activities of the HS3 *iab-7* PRE depend upon GAF, while only the silencing activity  
343 depends upon Pho (which in other PREs is thought to function in the recruitment of PRC1). That functionally  
344 specialized factors might associate with different LBC recognition elements would also fit with our gel filtration  
345 experiments. We found that the LBC shifts in nuclear extracts (with *Fab-7* and several CES probes) migrate  
346 more slowly than the shifts observed after gel filtration. This difference in mobility suggests that there are  
347 factors that associate with the LBC:DNA complex in nuclear extracts that are not integral components of the  
348 LBC, and thus don't co-fractionate with the LBC during gel filtration. These factors could contribute not only  
349 to the P<sub>c</sub>G silencing or M<sub>sl</sub> recruitment activities of individual LBC recognition elements, but also to the  
350 boundary activity of elements like *Fab-7* dHS1.

351

## 352 **Materials and Methods**

### 353 **Generation of the *Fab-7*<sup>attP50</sup> replacement lines**

354 The strategy of the creation of the *Fab-7*<sup>attP50</sup> landing platform and generation of the *Fab-7* replacement lines is  
355 described in detail in [34,36]. DNA fragments used for the replacement experiments were generated by PCR

356 amplification and verified by sequencing. The sequences of the used fragments are shown in the Supporting  
357 Table S1.

358 **Cuticle preparations**

359 Adult abdominal cuticles of homozygous eclosed 3-4 day old flies were prepared essentially as described in  
360 [36] and mounted in Hoyer's solution. Photographs in the bright or dark field were taken on the Nikon SMZ18  
361 stereomicroscope using Nikon DS-Ri2 digital camera, processed with ImageJ 1.50c4 and Fiji bundle 2.0.0-rc-  
362 46, and assembled using Impress of LibreOffice 5.3.7.2.

363 **Nuclear extracts**

364 Nuclear extracts from 6- to 18-h embryos were prepared as described previously (Aoki et al., 2008) with small  
365 modifications. Embryos from Oregon R were collected from apple juice plates and aged 10 h at room  
366 temperature. The extraction was completed with the final concentration of KCl at 360 mM. Fractionation of the  
367 nuclear extracts derived from 6- to 22-h embryos was performed by size exclusion chromatography using  
368 Superose 6 10/330 GL column (GE Healthcare). Molecular mass markers ranging from 1,350 to 670,000 Da  
369 (Bio-Rad) were used as gel filtration standards.

370 **Electrophoretic mobility shift assay (EMSA)**

371 Electrophoretic mobility shift assays were performed using  $\gamma$ -<sup>32</sup>P-labeled DNA probes under conditions  
372 described previously (Wolle et al., 2015). Probes for EMSA were obtained by PCR, purified on agarose-  
373 1XTris-acetate-EDTA (TAE) gel followed by phenol/chloroform extraction. Probe sequences are listed in  
374 Supporting Table S1. Purified DNA probes (1 picomole) were 5' end labeled with [  $\gamma$ -<sup>32</sup>P]ATP (MP  
375 Biomedicals/ Perkin Elmer) using T4 polynucleotide kinase (New England Biolabs) in a 50  $\mu$ l total reaction  
376 volume at 37°C for 1 h. Samples were run through columns packed with Sephadex G-50 fine gel (Amersham  
377 Biosciences) to separate free ATP from the labeled probes. The volume of the sample eluted from the column  
378 was adjusted to 100  $\mu$ l using deionized water so that the final concentration of the probe was 10 fmol/  $\mu$ l.  
379 Binding reactions were performed in a 20  $\mu$ l volume using the conditions described previously [34] except for  
380 the concentration of the non-specific competitor poly(dA-dT):poly(dA-dT) in the binding reaction. The final

381 concentration of poly(dA-dT):poly(dA-dT) was varied between 0.1 and 0.25 mg/ml depending on the DNA  
382 probe used. 1  $\mu$ l of nuclear extract (corresponding to about 20 ng of protein) or an equal volume of 360 mM  
383 nuclear extraction buffer (for negative control) was used. 2 or 3  $\mu$ l of nuclear extract was used when indicated.

384 In some reactions, unlabeled competitor DNA was included so that the final concentration of the competitor  
385 would be in 25- to 100-fold excess. The reaction mixtures containing the  $\gamma$ -<sup>32</sup>P-labeled DNA probes were  
386 incubated for 30 min at room temperature.

387 For supershift experiments, pre-immune rabbit serum or antibodies against different proteins were pre-  
388 incubated in the reaction mixtures described above with the nuclear extract or gel column fractions for 30 min at  
389 room temperature to allow the protein-antibody association, followed by an incubation with <sup>32</sup>P- labeled DNA  
390 probes for 30 min at room temperature. Either 4  $\mu$ l of rabbit polyclonal anti-CLAMP antibody [58], 1  $\mu$ l of  
391 rabbit polyclonal antibodies against GAF and Mod(mdg4) was used.

392 Binding reactions were electrophoresed using the conditions described previously [34]. The gels were run at  
393 180 V for 3 to 4 h at 4°C, dried, and imaged using a Typhoon 9410 scanner and Image Gauge software or X-ray  
394 film.

### 395 **Antibodies**

396 ChIP antibodies against GAF (full length) were raised in rats and purified from the sera by ammonium sulfate  
397 fractionation followed by affinity purification on the CNBr-activated Sepharose (GE Healthcare, United States)  
398 according to standard protocols. Anti-Ph rabbit antibodies used in ChIP experiments were a gift from Maxim  
399 Erokhin. EMSA antibodies against GAF were obtained as gift from Carl Wu and David Gilmour, against  
400 Mod(mdg4) – from Anton Golovnin and Elissa Lei.

### 401 **Chromatin Immunoprecipitation**

402 Chromatin for the subsequent immunoprecipitations was prepared from 12-24 h embryos and mid-late pupae as  
403 described in [39,59]. Aliquots of chromatin were incubated with antibodies against GAF (1:200), and Ph  
404 (1:500), or with nonspecific rat or rabbit IgG (control). At least three independent biological replicates were  
405 made for each chromatin sample. The results of the ChIP experiments are presented as a percentage of the input

406 genomic DNA after triplicate PCR measurements and normalized to a positive genomic site for the appropriate  
407 protein, in order to correctly compare different transgenic lines with each other. The  $\gamma$ *Tub37C* coding region  
408 (devoid of binding sites for the tested proteins) was used as negative control; *Hsp70* region was used as positive  
409 control for GAF binding, PRE of *engrailed* was used as positive control for Ph binding. The sequences of used  
410 primers are presented in Supporting Table S1.

411

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417 and Education of the Russian Federation.

418

419

## 420 Figure Legends

421 **Fig. 1 HS1 alone is not sufficient for boundary function.** (A) Map of the bithorax complex showing the  
422 location of the three homeotic genes and the parasegment-specific regulatory domains. (B) Map of *Fab-7*  
423 region showing the four hypersensitive sites, HS\*, HS1, HS2, and HS3. The locations of recognition motifs for  
424 proteins known to be associated with *Fab-7* are indicated. Replacement fragments are shown below the map,  
425 with a summary of their cuticle (tergite and sternite) phenotypes. (C) Bright field (top) and dark field (bottom)  
426 images of cuticles prepared from wild type (*wt*), *Fab-7 attP* (replacement platform), *HS1+HS3*, *HS1*, and *HS3*  
427 male flies. As described in the text, the *HS1+HS3* replacement resembles wild type. In contrast, the *HS1* and  
428 *HS3* replacements have a strong GOF transformation. In those instances in which residual A6 (PS11) cuticle is  
429 present, it shows evidence of a LOF transformation.

430 **Fig. 2 HS1 combinations with either HS2 or HS\* are not fully functional.** (A) Map of *Fab-7* region  
431 showing the four hypersensitive sites, HS\*, HS1, HS2, and HS3 and the locations of recognition motifs for  
432 proteins known to be associated with *Fab-7*. Replacement fragments are shown below the map with a summary  
433 of their cuticle (tergite and sternite) phenotypes. (B) Bright field (top) and dark field images (bottom) of  
434 cuticles prepared from *HS1+HS2*, *HS\*+HS1+HS3*, *HS\*+HS1*, *HS\*+HS1+HS2*, and *[HS\*+HS1+HS2]R*  
435 (reverse) male flies. As detailed in the text, the *HS1+HS2* replacement lacks a sternite (GOF), but has a nearly  
436 normal tergite size with wild type morphology. The *HS\*+HS1+HS3* looks like wild type. The *HS\*+HS1*  
437 replacement lacks a sternite (GOF), while there is a variable reduction in the size of tergite. The morphology of  
438 the residual tergite suggests that it has the appropriate A6 (PS11) identity. *HS\*+HS1+HS2* males frequently  
439 show some A6 cuticle defects indicative of a weak A6(PS11)→A7(PS12) (also see Figure S2).  
440 *[HS\*+HS1+HS2]R* flies have a normal A6 tergite; however, the sternite shows evidence of a weak LOF  
441 transformation (bristles). wt\* -- minor deviations in phenotype. wt\*<sup>GOF</sup> – variable phenotype between wt and  
442 GOF.

443 **Fig. 3 dHS1 but not pHs1 can function as a boundary together with HS3.** (A) Map of *Fab-7* region  
444 showing the four hypersensitive sites, HS\*, HS1, HS2, and HS3 and the location of recognition motifs for  
445 proteins known to be associated with *Fab-7*. Replacement fragments are shown below the map with a summary  
446 of their cuticle (tergite and sternite) phenotypes. (B) Bright field (top) and dark field images of cuticles prepared  
447 from *HS1+HS3*, *pHS1+HS3*, *dHS1+HS3*, and *dHS1* male flies. As described in text, the *HS1+HS3* and  
448 *dHS1+HS3* replacements resemble wild type males, while *pHS1+HS3* and *dHS1* flies have strong GOF  
449 phenotypes. In flies that have residual A6 cuticle (typically, a tergite), there are LOF transformations. GOF\* --  
450 incomplete GOF phenotype in most males.

451 **Fig. 4 LBC binding to dHS1 and HS3 requires the GAGAG motifs.** Nuclear extracts prepared from 6-18 hr  
452 embryos were used for EMSA experiments: (-) no extract, (+) with extract. (A) EMSA experiments with a wild  
453 type and GAGAG mutant (as indicated) dHS1 probe. (B) EMSA competition experiments with a probe

454 spanning HS3. Control lanes on the left show the LBC shift of HS3 in the absence of cold competitors. As  
455 illustrated by the triangles, increasing concentrations (25x, 50x, 100x) of cold competitor were added. The cold  
456 competitor used in each set of three lanes is indicated below. (C) EMSA of wild type and mutant HS3 probes.  
457 The two HS3 GAGAG sites are mutant in the HS3<sup>mGAF</sup> probe. The three HS3 Pho sites are mutant in the HS3<sup>mP</sup>  
458 probe. (D) Antibody supershift experiments using fractions from a gel filtration column. Fraction numbers (45,  
459 47, and 67) are indicated above each lane. 45 and 47 are two of LBC peak fractions while fraction 67 doesn't  
460 have LBC activity. The antibody used for each set of supershift experiments is indicated below. Note: after  
461 fractionation by gel filtration, the LBC shift is typically slightly stimulated by the inclusion of non-specific  
462 serum.

463 **Fig. 5 HS3 boundary activity requires the two GAGAG motifs but not the three Pho recognition**  
464 **sequences.** (A) Map of *Fab-7* region showing the four hypersensitive sites, HS\*, HS1, HS2, and HS3 and the  
465 locations of recognition motifs for proteins known to be associated with *Fab-7*. Replacement fragments are  
466 shown below the map with a summary of their cuticle (tergite and sternite) phenotypes. (B) Bright field (top)  
467 and dark field (bottom) images of cuticles prepared from *dHS1+HS3<sup>mGAF</sup>*, *dHS1+HS3<sup>mPho</sup>*, *Trl<sup>R85</sup>*  
468 *dHS1+HS3<sup>mPho</sup>*,/ *dHS1+HS3<sup>mPho</sup>*, and *dHS1<sup>mGAF</sup>+HS3* male flies. As described in text, most *dHS1+HS3<sup>mPho</sup>*  
469 flies have a wild type phenotype, while the other mutant replacements typically exhibit strong GOF  
470 transformations. While nearly all *dHS1+HS3<sup>mPho</sup>* males are wild type, reducing the dose of the *Trl* gene in half  
471 induces a GOF transformation. The sternite is usually absent while the tergite had an irregular shape and is  
472 reduced in size. wt\* -- minor deviations in phenotype.

473 **Fig.6 ChIPs with GAF and Ph antibodies at sites across the *Fab-7* region.** Binding of GAF and Ph across  
474 the *Fab-7* region (*iab-6*, HS1, HS3, and *iab-7*) in different *Fab-7* replacements in embryos and pupae. The  
475 results of ChIPs are presented as a percentage of the input DNA normalized to a positive genomic site (*Hsp70*  
476 region – for GAF binding, PRE of *engrailed* - for Ph binding. Negative control is the *γTub37C (tub)* gene.

477 Error bars indicate standard deviations of triplicate PCR measurements from three independent biological  
478 samples of chromatin.

479

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610

611 **Supporting Figures**

612 **Fig. S1 Variations in morphology of the abdominal segments of the *HS3* males.** Brightfield and darkfield  
613 images of male cuticles, as indicated. *Fab-7<sup>attP</sup>*: A6 is absent, indicating that PS11 is transformed into a  
614 duplicate copy of PS12. *HS3*: Two different classes of phenotypes are observed. The first class (I) resembles  
615 the GOF transformation of the starting *Fab-7<sup>attP</sup>* replacement platform. The second class (II) has a small  
616 residual tergite that (based on trichome hairs) appears to have an appropriate A6 (PS11) identity. Sternites are  
617 not observed in either class. *HS1*: Three classes, I, II, and III, are observed. These classes differ in the size of  
618 the tergite. Class II is the most frequent.

619 **Fig. S2 Variations in morphology of the abdominal segments of *HS\*+HS1* and *HS\*+HS1+HS2* males.**

620 Brightfield and darkfield images of male cuticles, as indicated. *HS\*+HS1*: The cuticular phenotypes fall into

621 three different classes depending on the size of the A6 tergite. In the most frequent class, class I, the tergite is  
622 significantly reduced in size and misshapen. There is a modest reduction in the size of the tergite in class II,  
623 while in class III, which is the least frequent, there is only a slight reduction in the size of the tergite compared  
624 to wild type. In these flies, trichome pattern in the A6 tergite resembles that in wild type, suggesting that  
625 surviving histoblasts that give rise to the dorsal cuticle are properly specified. In all *HS\*+HS1* male flies the  
626 A6 sternite is missing. *HS\*+HS1+HS2*: Three classes of cuticular phenotypes are observed. In the most  
627 frequent class, class III, the size of tergite is close to that in wild type, though sometimes the edges of the tergite  
628 are irregular. The sternite is present, but typically misshapen. Flies in the next most frequent class, class II,  
629 lack a sternite, while their tergite resembles that of class I. Finally, in class III, the tergite is noticeably reduced  
630 in size, while the sternite is misshapen.

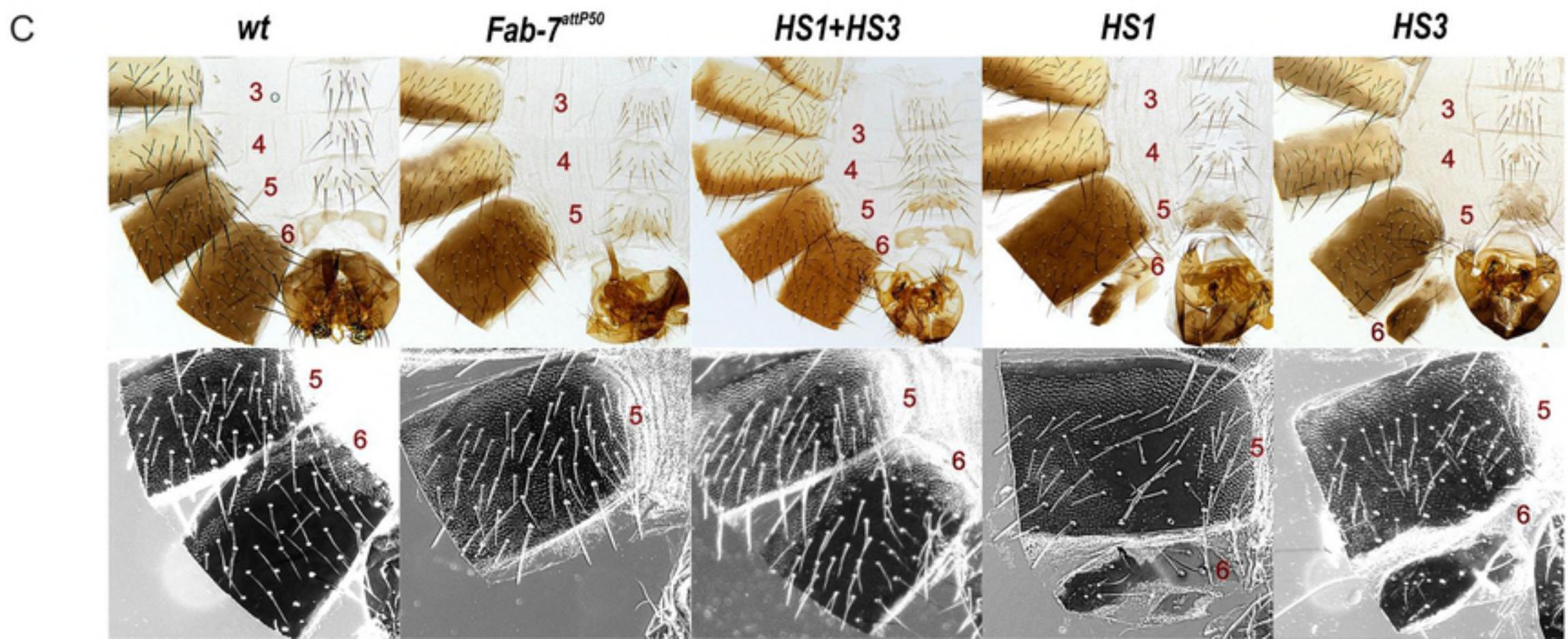
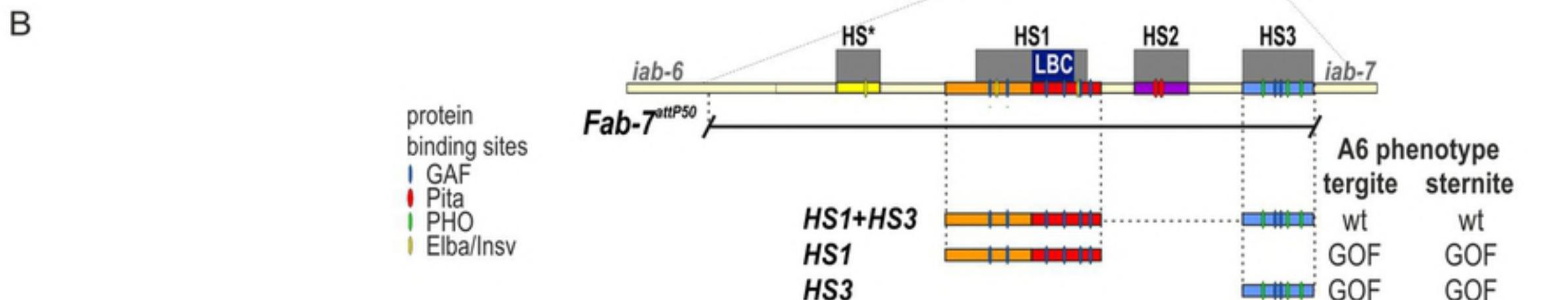
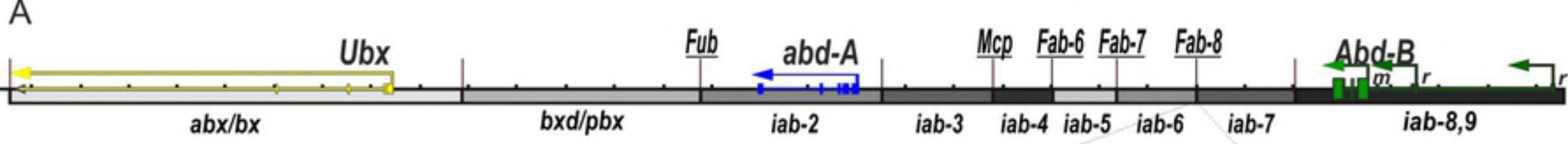
631 **Fig. S3 Variations in morphology of the abdominal segments of the *pHS1+HS3* males.** Two roughly equal  
632 classes of phenotypes are observed for the *pHS1+HS3* replacement. In class I, A6 is transformed into a  
633 duplicate copy of A7, and is absent. In class II, the transformation is not complete, and a small residual A6  
634 tergite is observed.

635 **Fig. S4 LBC binds preferentially to larger fragments.** Nuclear extracts prepared from 6-18 hr embryos were  
636 used for EMSA experiments: (-) no extract, (+) with extract. Comparison of LBC binding to probes spanning  
637 just GAGA3 (G3) or GAGA4 (G4) to probes spanning both GAGA3 and GAGA4 (G3+4). (A) EMASAs of G3,  
638 G4, and G3+4. (B) EMASAs of G3 and G3+4 with increasing amount of extract (1  $\mu$ l, 2  $\mu$ l, 3  $\mu$ l). (C)  
639 Competition experiments with probe G3+G4 and excess cold G3+G4 or G3+LacZ (left to right: 100x, 75x, 50x,  
640 25x, and 10x).

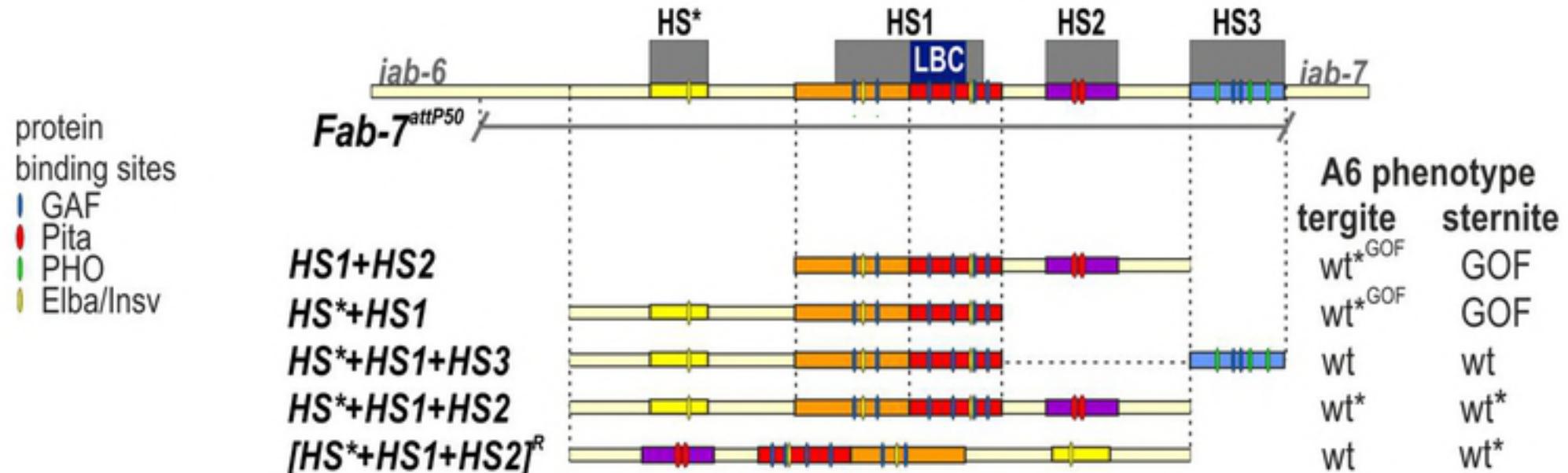
641 **Fig. S5 LBC binding to HS3.** Nuclear extracts prepared from 6-18 hr embryos were used for EMSA  
642 experiments with three overlapping HS3 probes: Probe #1, 100 bp from proximal side of HS3. Probe #2, 100 bp  
643 probe from center of HS3. Probe #3, 88 bp probe from distal side of HS3. \* – unique shifts; arrows – shifts  
644 observed with two or more probes.

645

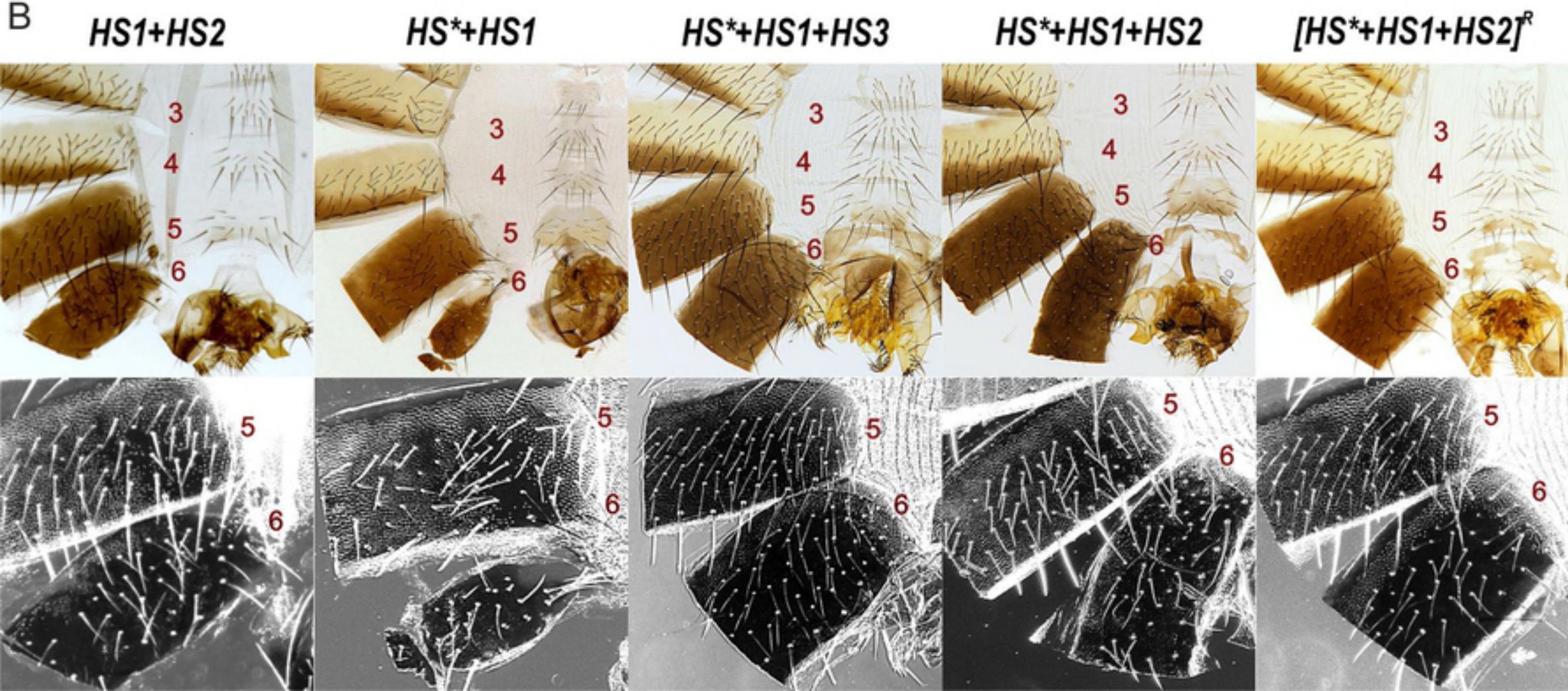
646 **Supporting Table S1. The list of oligonucleotides and DNA fragments.**



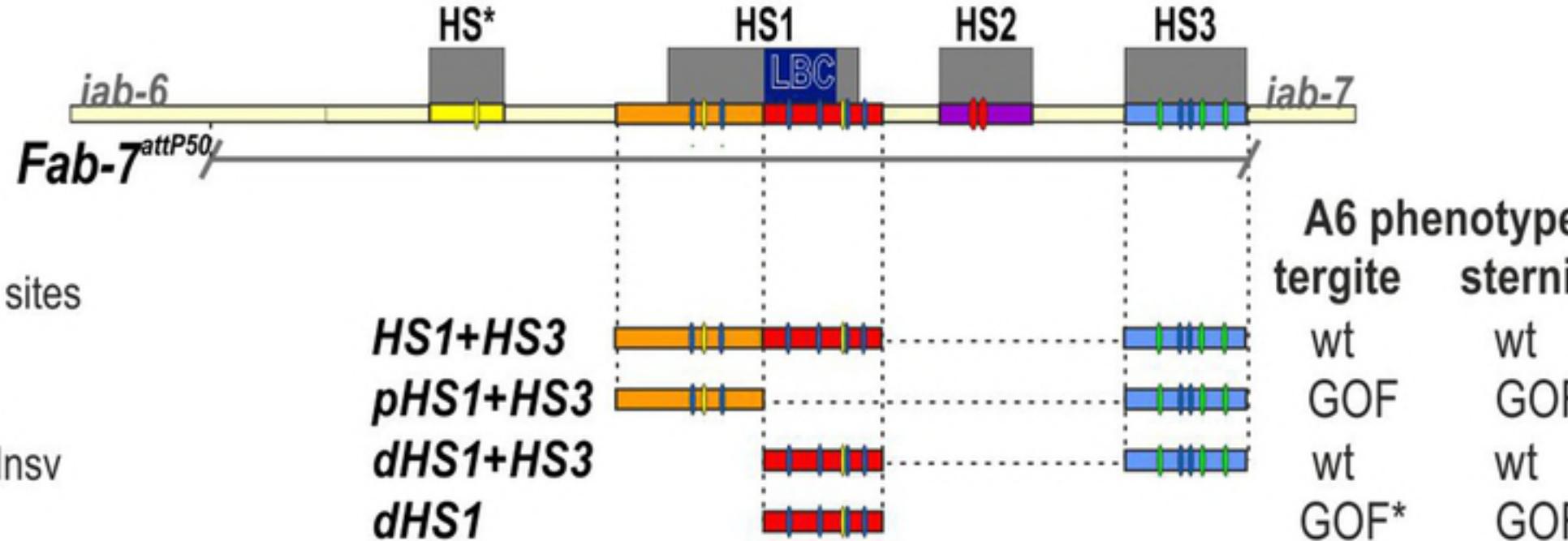
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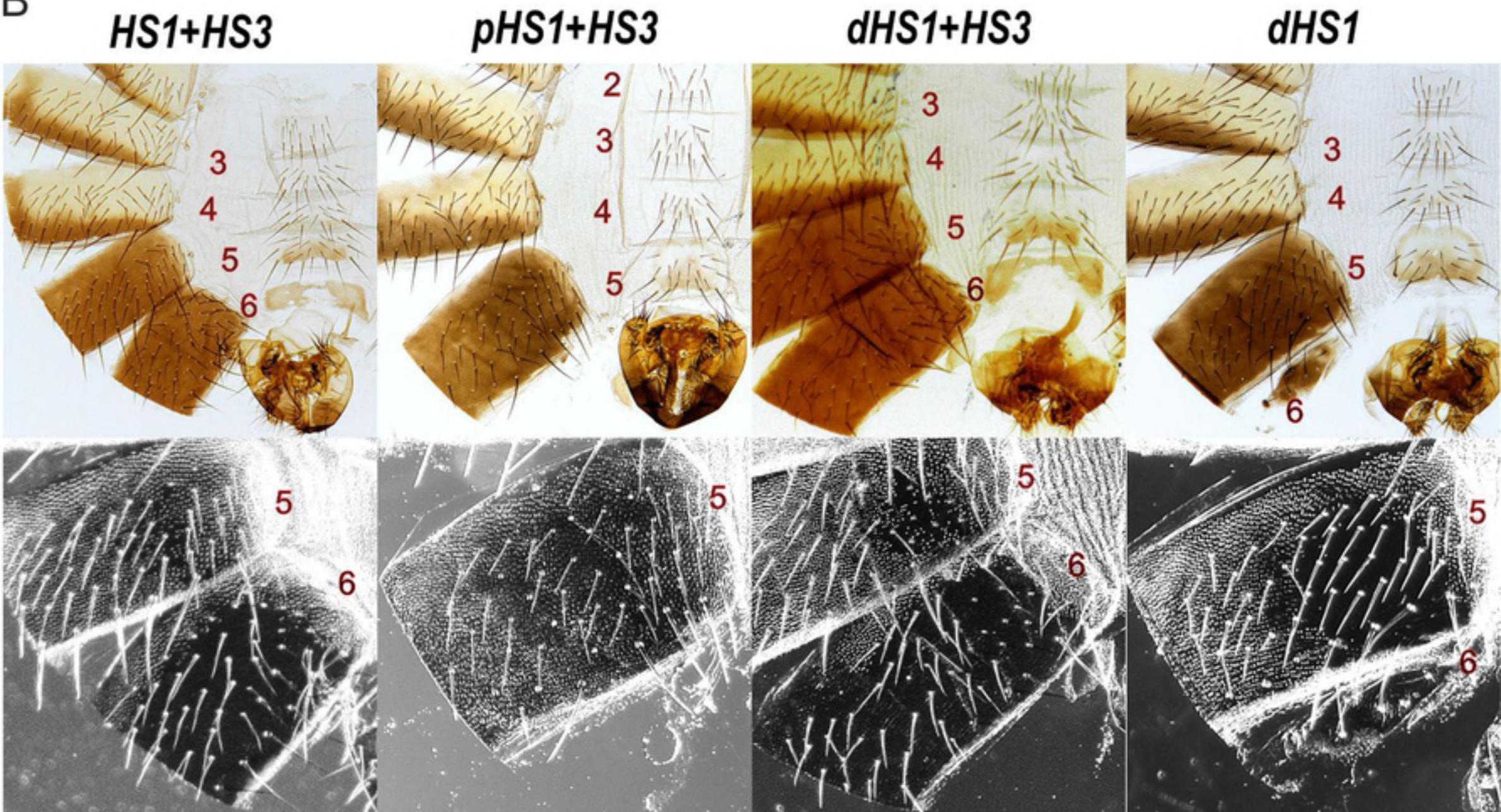
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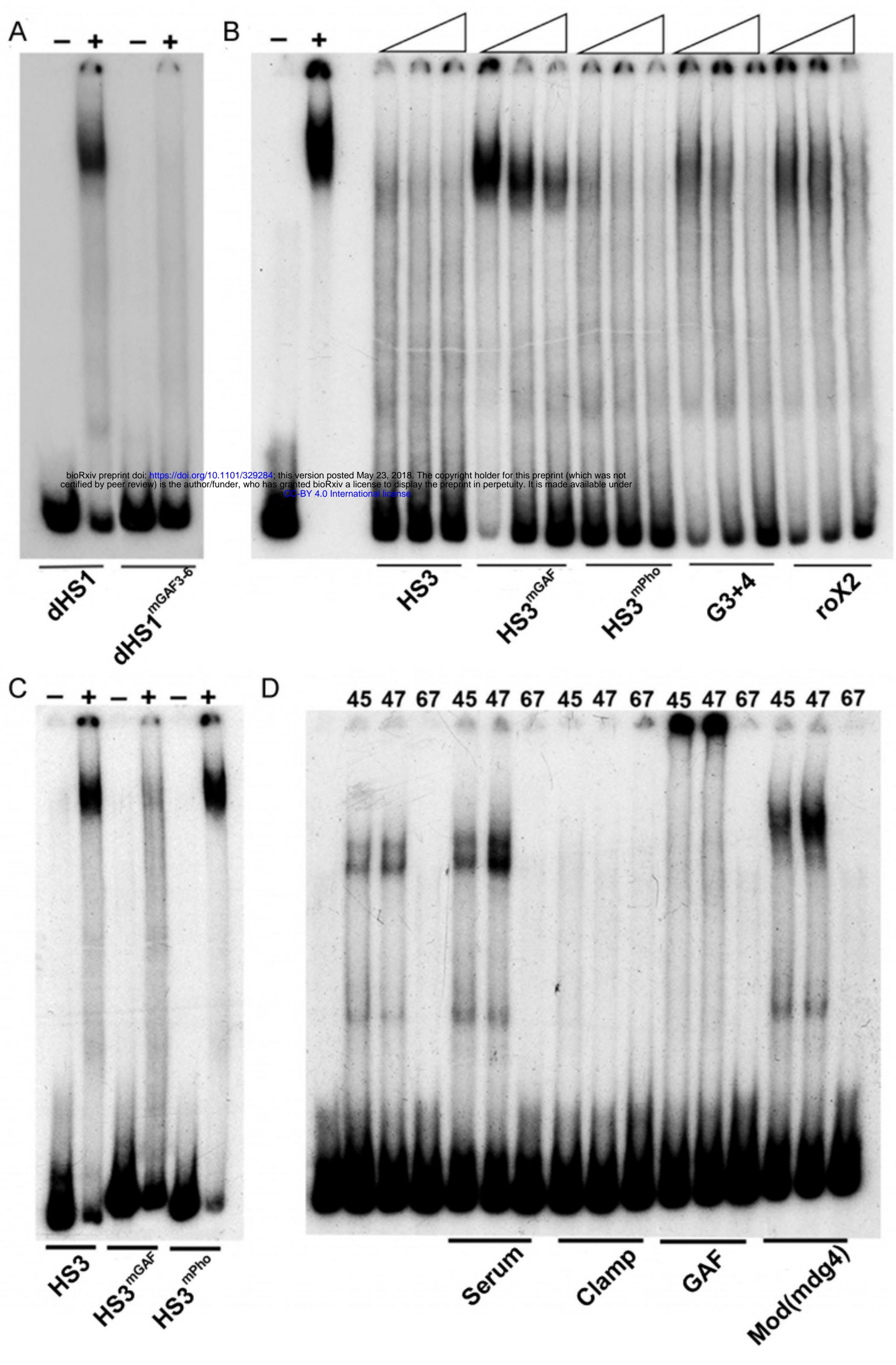


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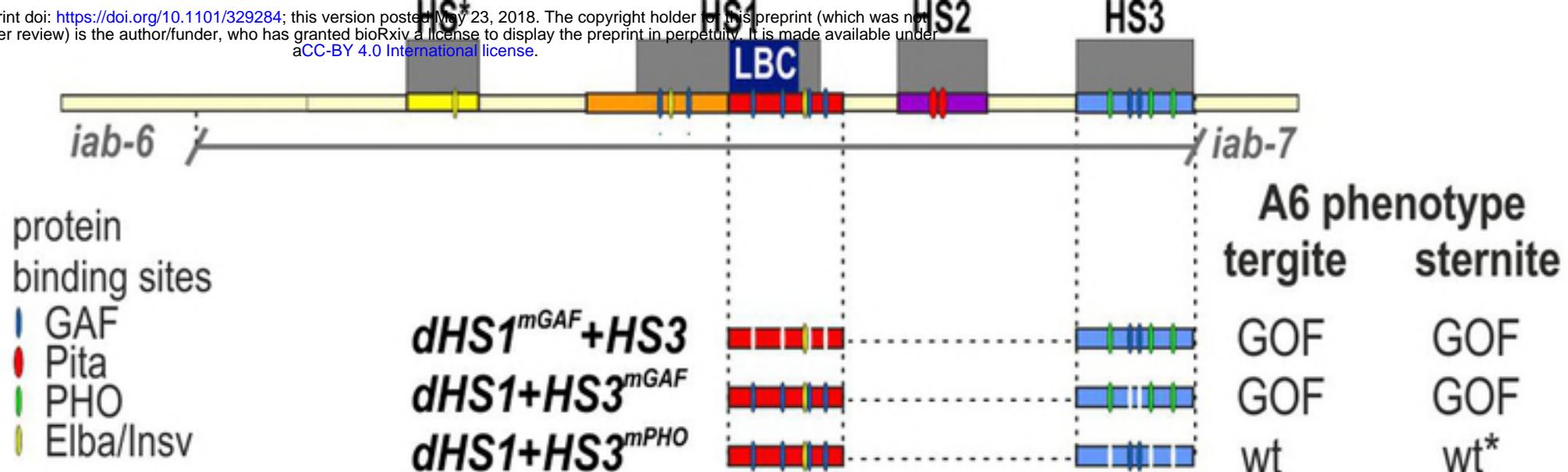


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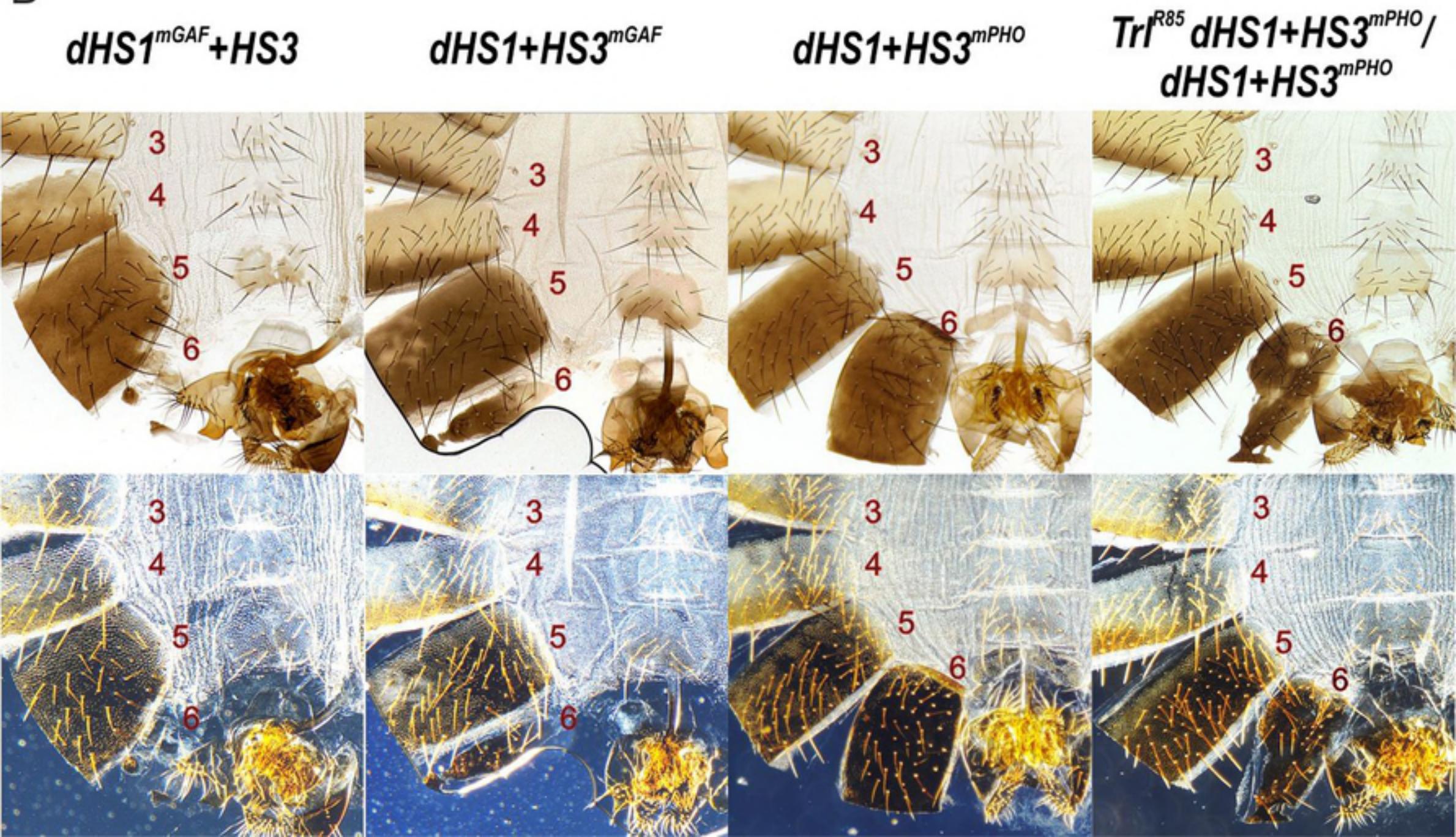




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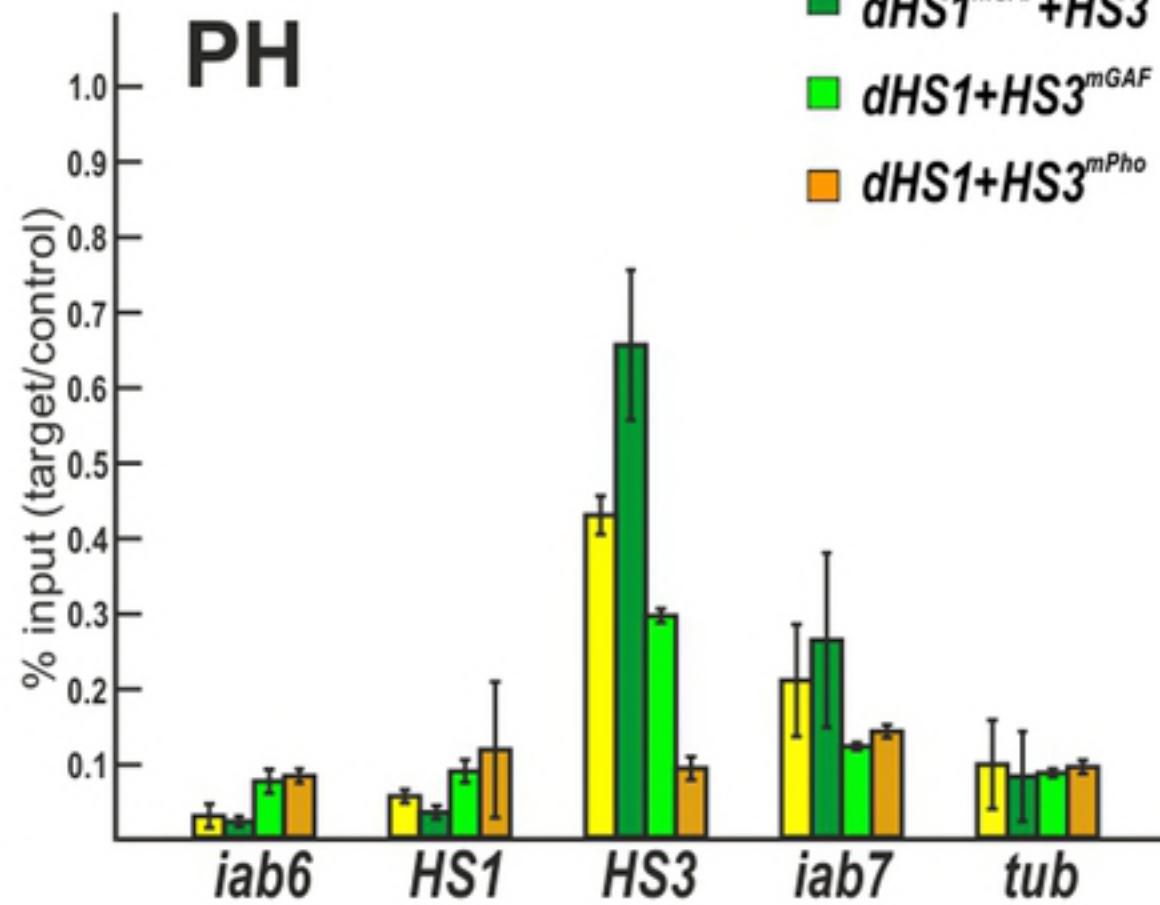
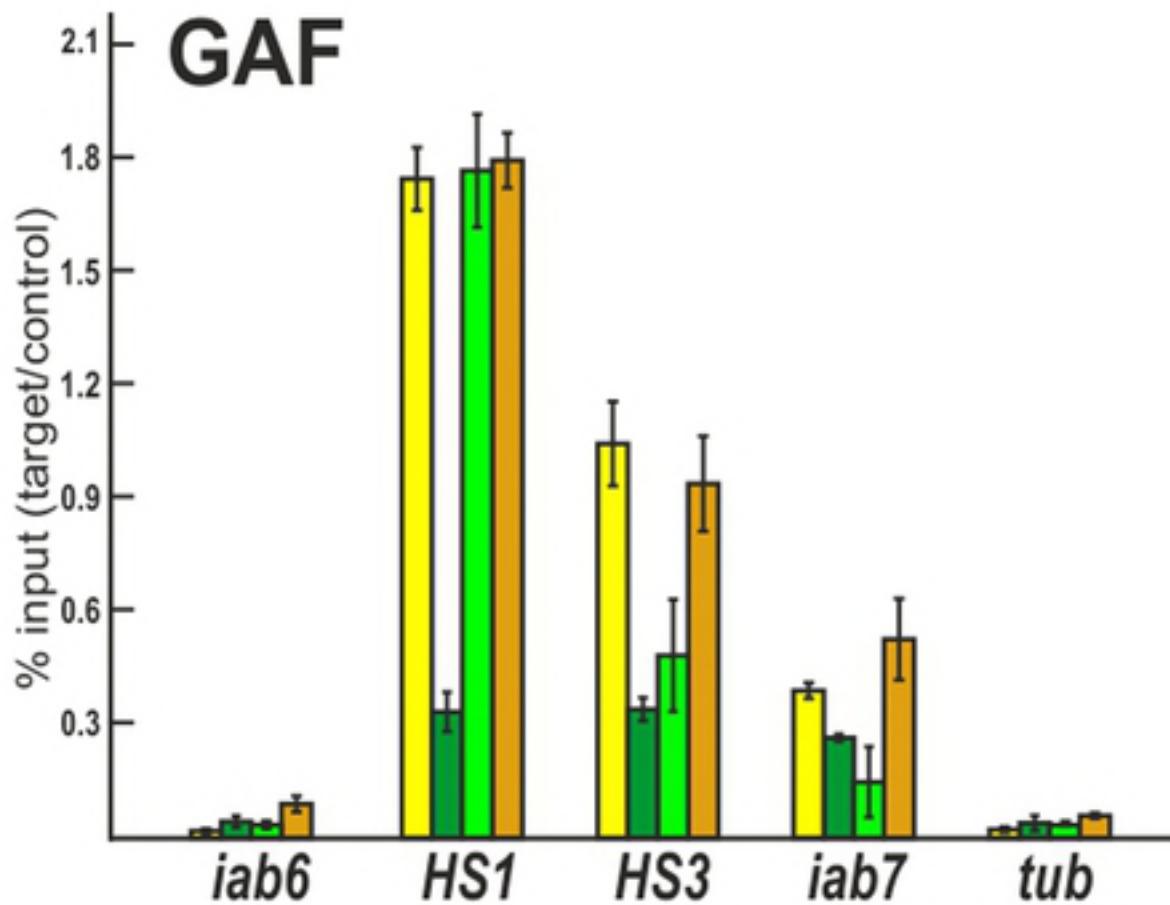


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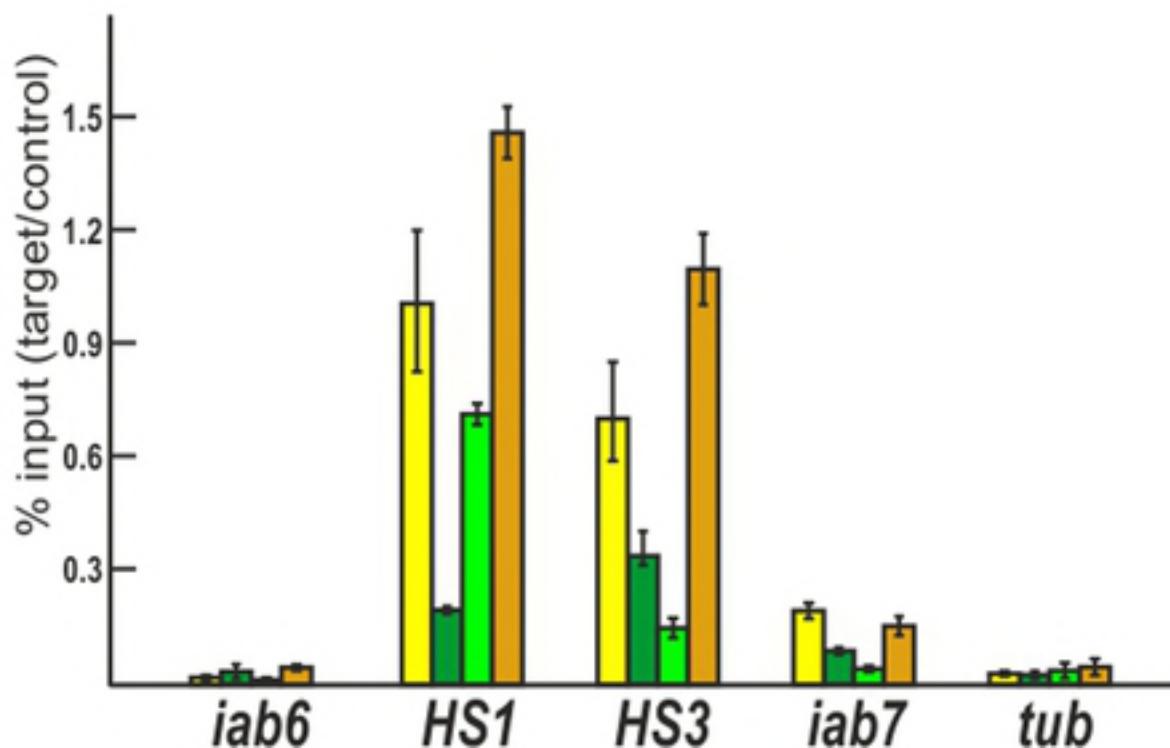
# Embryo

## GAF



# Pupa

## GAF



## PH

