Title:

Modular tissue-specific regulation of *doublesex* underpins sexually dimorphic development in *Drosophila*

Running Title:

Tissue-specific regulation of *dsx*

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Summary Statement:

We identify the modular *cis*-regulatory elements that direct expression of *doublesex* in sexually dimorphic structures in *Drosophila* legs and genitalia. This regulatory landscape provides insight into how cells obtain their sex-specific identity.

Abstract:

The ability of a single genome to produce distinct and often dramatically different male and female forms is one of the wonders of animal development. In most animals, sex-specific phenotypes are shaped by interactions between a sex determination pathway and spatial patterning gene networks. In *Drosophila melanogaster,* most sexually dimorphic traits are controlled by sex-specific isoforms of the *doublesex* (*dsx*) transcription factor, and *dsx* expression is mostly limited to cells that give rise to sexually dimorphic traits. However, it is unknown how this mosaic of "sex-naïve" and "sex-aware" tissues arises. Here, we characterize the *cis*-regulatory sequences that control *dsx* expression in the foreleg, which contains multiple types of sex-specific sensory organs. We find that separate modular enhancers are responsible for *dsx* expression in each sexually dimorphic organ. Expression of *dsx* in the sex comb is co-regulated by two enhancers with distinct spatial and temporal specificities that are separated by a genitalia-specific enhancer. Thus, the mosaic of sexually dimorphic and monomorphic organs depends on modular regulation of *dsx* transcription by dedicated cell type-specific enhancers.

50 **Introduction**:

51 Most animals are mosaics of sexually dimorphic and monomorphic tissues. Despite the
52 multitude of traits that distinguish males from females, many tissues and organs lack overt sexual
53 dimorphism. To understand how this mosaic pattern is produced, the action of sex determination
54 pathways needs to be understood at the cellular level.

55 Sexually dimorphic morphologies are specified by diverse molecular mechanisms in
56 different animal phyla (Kopp, 2012; Matson and Zarkower, 2012). One of the best studied
57 mechanisms is found in the fruit fly, *Drosophila melanogaster*, where sex-specific development
58 of most somatic cells is controlled by an alternative pre-mRNA splicing pathway (reviewed in
59 Christiansen et al., 2002; Cline, 1993; Erickson and Quintero, 2007; McKeown, 1992). In
60 *Drosophila*, the number of X chromosomes sets off a cell-autonomous cascade of sex-specific
61 splicing that leads to the production of functional Transformer (Tra) protein in females, but not
62 in males. Tra controls alternative splicing of *doublesex* (*dsx*) pre-mRNAs, such that the presence
63 of functional Tra in females leads to a female-specific *dsx* isoform, and the absence of functional
64 Tra leads to the production of a male-specific isoform (dsx^F and dsx^M, respectively). *dsx* encodes
65 transcription factors involved in the establishment of almost all morphological traits that differ
66 between males and females (Baker and Ridge, 1980; Hildreth, 1965). The Dsx^M and Dsx^F
67 proteins share a common DNA binding domain and bind to the same target sequences, but have
68 different and sometimes opposite effects on gene expression, leading to sex-specific cell
69 differentiation (Arbeitman et al., 2004; Arbeitman et al., 2016; Burtis et al., 1991; Goldman and
70 Arbeitman, 2007; Lebo et al., 2009; Li and Baker, 1998; Yang et al., 2008). For instance, the
71 *yolk protein 1, bric-à-brac*, *Fmo-2,* and *desat-F* genes are directly regulated by Dsx and are
72 expressed at higher levels in females compared to males. *dsx* mutants show intermediate
73 expression of *yolk protein 1, bric-à-brac*, and *Fmo-2* in both sexes, indicating that Dsx^F is likely
74 activating, and Dsx^M repressing, these genes (Coschigano and Wensink, 1993; Luo and Baker,
75 2015; Williams et al., 2008). *desat-F,* on the other hand, is activated by Dsx^F but is not affected
76 by Dsx^M (Shirangi et al., 2009). At the morphological level, loss-of-function *dsx* mutants
77 develop as intersexes with a mixture of male, female, and intermediate traits (Baker and Ridge,
78 1980; Hildreth, 1965).

79 The details of the *tra*/*dsx* splicing cascade were characterized in the 1980s (Boggs et al.,
80 1987; Burtis and Baker, 1989; Butler et al., 2018; McKeown et al., 1987; McKeown et al., 1988;

81 Nagoshi et al., 1988), and are a textbook example of the role of alternative splicing in
82 development. In contrast, the role of transcriptional regulation of *dsx* in *Drosophila* sexual
83 differentiation was realized only recently. Although it may seem that every cell would need to
84 "know" its sex, *dsx* is only transcribed in cells that are associated with sexually dimorphic organs
85 (Camara et al., 2008; Rideout et al., 2010; Robinett et al., 2010; Tanaka et al., 2011). Thus, both
86 males and females are mosaics of sexually dimorphic and monomorphic cells. This mosaicism
87 makes it all the more remarkable that *dsx* regulates the development of so many different sex-
88 specific traits, from pigmentation and genital morphology to brain neural circuits and gene
89 expression in the gut (Goldman and Arbeitman, 2007; Keisman et al., 2001; Luo and Baker,
90 2015; Sanders and Arbeitman, 2008; Williams et al., 2008). To understand how this mosaic
91 pattern is specified, we need to elucidate the mechanisms that establish *dsx* transcription.
92 Among the best studied sexually dimorphic organs in *Drosophila* are the foreleg bristles.
93 The foreleg of *D. melanogaster* displays two distinct sex-specific features. First, the sex comb –
94 a row of strongly modified mechanosensory bristles – is present only in males, while in females
95 the homologous bristles retain the typical mechanosensory bristle morphology (Kopp, 2011;
96 Tokunaga, 1962). Second, males have a greater number of foreleg chemosensory bristles
97 compared to females (Mellert et al., 2012; Tokunaga, 1962). The sex comb and chemosensory
98 bristles are found in close proximity to each other, and both are involved in mating behavior (Fan
99 et al., 2013; Hurtado-Gonzales et al., 2015; Ng and Kopp, 2008; Spieth, 1952). The male-
100 specific chemosensory bristles are used early in the stereotypic courtship sequence to taste the
101 female cuticular pheromones (Fan et al., 2013; Spieth, 1952), while the sex comb assists in
102 grasping the female prior to copulation (Hurtado-Gonzales et al., 2015; Ng and Kopp, 2008;
103 Spieth, 1952).
104 *dsx* is required both for the distinctive sex comb morphology and for the higher number
105 of chemosensory bristles in males (Belote and Baker, 1982; Mellert et al., 2012). Expression of
106 *dsx* in the developing foreleg starts in the late 3rd instar larva (Tanaka et al., 2011). During
107 prepupal and early pupal development, this pattern resolves into several distinct clusters of cells
108 corresponding to chemosensory bristle precursors, sex comb bristle precursors, and surrounding
109 epithelial cells (Mellert et al., 2012; Robinett et al., 2010; Tanaka et al., 2011). This raises a
110 question – is *dsx* expression in the foreleg controlled by a different, modular enhancer for each
111 sex-specific bristle type, or by a common leg enhancer? More generally, is *dsx*, despite its

112 apparently global function as a binary sex switch, subject to modular transcriptional control, with
113 a dedicated enhancer for every cell population that expresses *dsx*?
114 To address this question, we identified and characterized the enhancers that control *dsx*
115 expression in the sex comb and the foreleg chemosensory bristles. We found that three separate
116 enhancers are responsible for regulating *dsx* expression in the foreleg. One enhancer drives broad
117 foreleg expression that encompasses both sex comb and chemosensory organ primordia, while
118 the other two have mutually exclusive activities – one in the sex comb, and the other in sex-
119 specific chemosensory bristles. The two enhancers that contribute to sex comb development are
120 separated by a genitalia-specific enhancer that has no activity in the leg. This complex *cis*-
121 regulatory architecture suggests that *dsx* transcription, like that of other developmentally
122 regulated transcription factors, is controlled by modular tissue-specific enhancers that can have
123 both overlapping and non-overlapping spatial activities.

124 **Results:**

125 ***Three enhancers with distinct spatial and temporal activities contribute to complex* dsx**
126 ***expression in the foreleg***

127 To identify the enhancers that specify the complex expression pattern of *dsx* during foreleg
128 development, we generated a series of pPTGAL GAL4 reporter lines covering the nearly 50 kb of
129 the upstream and intronic regions of the *dsx* locus (brown boxes, Fig. S1). In parallel, we examined
130 an overlapping series of pBPGUw GAL4 reporters generated using a different vector and
131 integration method (Pfeiffer et al., 2008) (grey boxes, Fig. S1). These lines were crossed to UAS-
132 GFP.nls and screened 5 hr and 24 hr after puparium formation (APF). Using both sets of reporters,
133 we identified three DNA fragments that drove expression patterns consistent with known *dsx*
134 expression in the foreleg (Rideout et al., 2010; Robinett et al., 2010; Tanaka et al., 2011). We
135 identified a chemosensory enhancer in a ~4 kb fragment of the first *dsx* intron (Fig. 1, F,G and Sup
136 Fig. 1B, blue text), a sex comb enhancer in a ~3 kb section of the second intron (Sup Fig. 1C, red
137 and green text), and an early foreleg enhancer in a ~4 kb section of the second intron downstream
138 from the sex comb enhancer (Sup Fig. 1C, purple and green text). In parallel, another research
139 group had identified a genital enhancer in the overlap between the 40F03 and 42D04 reporters
140 (Sup Fig. 1C) (John Yoder, University of Alabama, personal communication), both of which
141 showed foreleg expression in our screen. To test whether the sex comb and early foreleg enhancers
142 were separate, and to reduce the temporal lag between enhancer activation and reporter detection,
143 we cloned smaller sub-fragments of the *dsx* second intron into a nuclear GFP expression vector.
144 We found that both the sex comb and the early foreleg enhancers drove the same expression
145 patterns in this assay as in the larger GAL4 reporter constructs (Fig. 1 B-E).

146 The three enhancers direct distinct spatial and temporal expression of reporter genes,
147 consistent with known *dsx* expression. *dsx* expression in the foreleg first becomes visible in
148 wandering third instar larvae and is well developed in prepupal legs (Tanaka et al., 2011). Leg
149 bristles are specified over several developmental periods. Most chemosensory bristles and the
150 largest mechanosensory bristles are specified at the end of the 3rd larval instar or early in prepupal
151 development, while most mechanosensory bristles (including sex comb teeth) are specified
152 between 6 and 15 hr APF (Belote and Baker, 1982; Held, 2002). At 5 hr APF, Dsx is present in
153 an anterior-ventral region of tarsal segments 1 through 4 (ta1-4), where the sex comb and sex-
154 specific chemosensory bristles will form (Tanaka et al., 2011). At this stage, the early foreleg

155 enhancer drives reporter expression in a pattern similar to Dsx protein (Fig 1B). Although Dsx
156 expression is already becoming sexually dimorphic by 5 hr APF (Tanaka et al., 2011), the GFP
157 reporter intensity at this stage is roughly monomorphic, possibly due to the higher stability of the
158 GFP protein (Fig 1B). The sex comb enhancer is not active at 5 hrs APF (Fig 1D), while the
159 chemosensory enhancer is expressed in both sexes in small clusters of cells that match the pattern
160 of developing chemosensory bristles (Fig 1F).

161 By 28 hr APF, Dsx expression is strongly dimorphic; in males, high Dsx expression is
162 seen in sex comb bristles and surrounding epithelial cells, whereas in females, the homologous
163 region of ta1 shows much lower Dsx expression (Tanaka et al., 2011; Fig 1C). In the rest of the
164 tarsus, epithelial expression of Dsx disappears by 28 hr APF. At this stage in males, the early
165 foreleg enhancer still shows weak activity in epithelial cells around the sex comb, especially on
166 the distal-ventral side; however, it is not visibly active in sex comb bristles (Fig 1C). The sex comb
167 enhancer drives a complementary pattern, with strong expression in sex comb bristles and weak
168 expression in the surrounding epithelial cells (Fig 1E). In females, neither of these enhancers
169 shows significant expression at 28 hr APF, consistent with the sexually dimorphic expression of
170 Dsx (Fig 1C,E). For the chemosensory enhancer, cell clusters that reflect the pattern of
171 chemosensory bristles continue to be observed at 28 hr APF in both males and females (Fig 1G).
172 Thus, each of the three enhancers directs a distinct expression pattern in the foreleg.
173

174 ***All three enhancers contribute to sex-specific sensory organ development:***

175 The patterns of GFP reporter expression suggest that the sex comb enhancer controls sex
176 comb development and the chemosensory enhancer controls sex-specific chemosensory bristle
177 development, while the early foreleg enhancer could potentially contribute to the development of
178 both types of sex-specific bristles. To test these hypotheses, we used the chemosensory (42C06),
179 foreleg (42D04), and sex comb (40F03) pBPGUw-GAL4 lines to drive a UAS-RNAi construct
180 targeting the *shaven*/*Pax2* gene (*sv*). Knockdown of *sv* in sensory organ precursors causes severe
181 truncation of bristle shafts (Kavaler et al., 1999), allowing us to identify the bristles where each
182 *dsx* enhancer is active during prepupal and pupal development. We collected and analyzed 8 male
183 and 5 female samples for each of the three GAL4 lines. This approach also allowed us to test for
184 enhancer activity at developmental stages that were not observed by visual inspection of GFP

185 reporters. The chaetotaxy of ta1 is particularly stereotypic and has been thoroughly mapped
186 (Tokunaga, 1962), allowing us to compare the male and female knockdown phenotypes.
187 First, to identify all *dsx*-expressing bristles, we expressed UAS-*sv*RNAi using a *dsx*-GAL4
188 knock-in line, which was generated by inserting the GAL4 coding sequence into the *dsx* locus and
189 reflects the full expression pattern of *dsx* (Robinett et al., 2010). We found that the ta1
190 chemosensory bristles fall into three classes (Fig 2). First, the dorsal-posterior chemosensory
191 bristles are not affected by *dsx*-GAL4/UAS-*sv*RNAi; in the wild type these bristles are sexually
192 monomorphic in size and position, suggesting they do not express or require *dsx* for their
193 development (Fig 2A, B). Second, on the dorsal-anterior side, males have four chemosensory
194 bristles on the distal half of ta1 (dark orange triangles in Fig 2A), compared to only one in females
195 (Fig 2B); these bristles were always strongly affected by *sv* RNAi in both sexes, indicating that
196 they express *dsx* (Fig 2A,B). The third class, two chemosensory bristles (yellow triangles in Fig
197 2A,B) were affected weakly and with more variability among individuals, suggesting that they
198 may express *dsx* at lower levels or for a briefer period.
199 The male sex comb was always severely affected by *dsx*-GAL4/UAS-*sv*RNAi, whereas the
200 homologous female bristles were affected weakly and with more variability (Fig 2A,B), consistent
201 with a lower level of *dsx* expression in females compared to males (Tanaka et al., 2011). With the
202 exception of the sex comb and homologous female bristles, no mechanosensory bristles were
203 affected by *dsx*-GAL4/UAS-*sv*RNAi (Fig 2A,B), confirming their sexually monomorphic nature.
204 Next, we used each of the three *dsx* leg enhancers to drive UAS-*sv*RNAi and compared the
205 resulting phenotypes in ta1 to that of the *dsx* knock-in. The early foreleg enhancer was similar to
206 the *dsx* knock-in in having a strong and consistent effect on the male sex comb, but only a weak
207 and variable effect on homologous bristles in females (Fig 2C,D). It also affected a single
208 chemosensory bristle in proximal ta1 in both sexes (Fig 2C,D). As expected, the sex comb
209 enhancer had a clear effect on sex comb development in males, but did not affect chemosensory
210 bristles or any bristles in females (Fig 2E,F). The chemosensory enhancer consistently affected all
211 anterior-ventral chemosensory bristles in males, including the two proximal bristles that were only
212 weakly affected by the *dsx* knock-in; it did not affect any mechanosensory bristles (Fig 2G).
213 Surprisingly, the chemosensory enhancer had no effect in females (Fig 2H), even though some
214 female chemosensory bristles express *dsx* (Fig 2B), and the chemosensory enhancer drives UAS-
215 GFP expression in ta1 in females (Fig 1C).

216 In addition to their function in ta1, the foreleg and chemosensory enhancers show
217 expression in the more distal segments (ta2-ta4) (Fig 1B, F). These segments also contain
218 chemosensory bristles, some of which are sexually dimorphic (Mellert et al., 2012; Tokunaga,
219 1962). In ta2 and ta3, the *dsx* knock-in and the chemosensory enhancer affect the same subset of
220 four chemosensory bristles, while the sex comb enhancer has no effect (Fig Sup 2). The early
221 foreleg enhancer affected some but not all of the bristles affected by the *dsx* knock-in, as well as
222 one of the chemosensory bristles that was not affected by the *dsx* knock-in or the chemosensory
223 enhancer (Fig Sup 2). Pupal expression of the early foreleg enhancer is epithelial, not bristle-
224 specific (Fig 1E); thus, it is difficult to say whether this phenotype reflects a true developmental
225 function of the early foreleg enhancer in sensory organs, or the perdurance of the GAL4 protein or
226 the RNAi effect from an earlier time in development.
227 Examination of adult bristle phenotypes caused by UAS-*sv*RNAi expression suggests that
228 the three leg enhancers of *dsx* make distinct contributions to sex-specific bristle development. The
229 foreleg and sex comb enhancers function in sex comb development, while the chemosensory
230 enhancer functions in the development of sexually dimorphic chemosensory organs. We also
231 cannot rule out some contribution of the early foreleg enhancer to chemosensory bristle
232 development.
233
234 ***The foreleg and sex comb enhancers are modular and flank a separate genital enhancer***
235 The foreleg and sex comb enhancers are located in close proximity within the second intron
236 of *dsx* and are both active in the sex comb, although in different spatial patterns and at different
237 times (Fig 1B-E). The same broad region also contains an enhancer that is active in the third instar
238 larval genital disc (John Yoder, University of Alabama, personal communication). Mutations in
239 *dsx* have been previously shown to disrupt clasper (also known as the surstylus) bristles (Hildreth,
240 1965), which have a morphology similar to sex comb teeth. Through antibody staining, we found
241 that Dsx is expressed in several regions of the pupal male genitalia (Fig. S3). In the developing
242 clasper, Dsx is enriched in the bristle precursor cells, similar to its enrichment in sex comb bristles.
243 Analysis of the genital enhancer showed weak expression in the clasper as well as strong
244 expression in the dorsal postgonites (Fig. S3, S4). These observations led us to inquire whether
245 the foreleg, genital, and sex comb enhancers were fully modular and independent, or parts of a
246 broader pleiotropic enhancer with overlapping activities in multiple tissues. To distinguish

247 between these possibilities, we generated non-overlapping reporter constructs for each enhancer
248 and examined each reporter both in the foreleg and in the genitalia. In addition, we generated larger
249 constructs where either the sex comb or the early foreleg enhancer was excluded (Fig 3).
250 We first confirmed that a 5.5 kb genomic fragment containing all three candidate enhancers
251 (sex comb, genital, and foreleg) was able to drive both early and late expression in the foreleg, as
252 well as genital expression (Fig. 3 and Fig. S4). Each separate enhancer can be seen as driving a
253 distinct subset of that overall pattern. The late sex comb enhancer is active in the sex comb bristle
254 cells but not in the genitalia, while the genital enhancer drives expression in the genitalia but has
255 no activity in the foreleg (Fig. 3 and Fig. S4). The construct encompassing the early foreleg
256 enhancer is active in the epithelial cells surrounding the sex comb, as well as in the genitalia in a
257 pattern that resembles the genital enhancer (Fig. S4). However, further dissection of this region
258 shows that the foreleg and genital activities are largely separable. The sequences necessary for
259 accurate foreleg expression are located on the 3' side of the foreleg enhancer and do not drive
260 genital expression, while the sequences that activate expression in the genitalia are located on the
261 5' side and only drive ectopic expression in the foreleg (Fig. 4 G-H and Fig. S3). Pairwise
262 combinations of enhancers behave in a predictably modular fashion: a construct including the sex
263 comb and genital enhancers drives genital and late sex comb expression, but no early epithelial
264 expression in the foreleg, while the construct including the genital and early foreleg enhancers
265 drives early foreleg and genital expression but no expression in sex comb teeth (Fig. 3 and Fig.
266 S4). The chemosensory enhancer, located in a different intron and thus clearly distinct from the
267 foreleg and sex comb enhancers (Fig 3). In summary, we conclude that the three leg enhancers are
268 all fully separable, modular elements, but we cannot rule out some minor overlap between the
269 foreleg and genital enhancers.
270
271 ***Sequences in the foreleg and sex comb enhancers confine their activity to sexually dimorphic***
272 ***primordia***
273 To identify the minimal sequences required for correct activity, we subdivided the sex
274 comb and early foreleg enhancers into smaller reporter constructs (Fig 4A). While the ~3kb sex
275 comb enhancer drives specific expression in sex comb bristles and the epithelial cells distal to the
276 sex comb (Fig 1E), shorter sequences are unable to recapitulate this pattern. The 3' ~1.5 kb of the
277 sex comb enhancer (*sex comb enhancer sub1*) drives specific expression in sex comb bristles but

278 is not expressed in the epithelial cells (Fig 4B). A shorter ~1 kb 3' fragment (*sex comb enhancer*
279 *sub2*) retains expression in the sex comb bristles, but also causes ectopic expression in the most
280 distal TBR (Fig 4C). A core sequence of ~0.5 kb (*sex comb enhancer sub3*) expands expression to
281 all ta1 TBRs (Fig 4D). The TBRs are serially homologous to the sex comb (Kopp, 2011; Tokunaga,
282 1962), but are sexually monomorphic and do not express Dsx (Robinett et al., 2010; Tanaka et al.,
283 2011).

284 The 5' ~1.5kb of the sex comb enhancer (*sex comb enhancer sub4*) drives expression in
285 some of the epithelial cells surrounding the sex comb, but this expression does not encompass all
286 of the epithelial cells where the full sex comb enhancer is active (Fig. 4E). Reduction of this
287 sequence to a ~1kb 5' fragment (*sex comb enhancer sub5*) causes ectopic expression in proximal
288 epithelial cells (Fig 4F) that do not express Dsx (Robinett et al., 2010; Tanaka et al., 2011). Thus,
289 sequences within the sex comb enhancer are necessary to prevent its activity in sexually
290 monomorphic bristle and epithelial cells.

291 Dissection of the early foreleg enhancer identified a shorter 3' fragment (*early foreleg*
292 *enhancer sub2*) capable of driving expression in the correct region of the foreleg (Fig. 4H). A
293 partially overlapping fragment (*early foreleg enhancer sub1*) shows broad ectopic activity
294 throughout the tarsal segments (Fig 4G). Thus, similar to the late sex comb enhancer, both
295 activating and repressive sequences are needed to limit this enhancer's activity to sexually
296 dimorphic cell populations.

297 **Discussion**:

298 ***dsx expression in the foreleg is controlled by modular enhancers with both distinct and***
299 ***overlapping activities***

300 In this study, we have characterized the *cis*-regulatory architecture directing the precise
301 spatiotemporal and cell type-specific expression of *dsx* in the foreleg. Our results help explain the
302 origin of the developmental mosaicism that generates a mix of sex-specific and sexually
303 monomorphic organs in *Drosophila*, and possibly in other insects. Given the largely cell-
304 autonomous control of insect sexual differentiation, the essential function of *dsx* enhancers is to
305 provide spatial landmarks that establish which organs will become sexually dimorphic. In
306 principle, *dsx* expression in different types of foreleg bristles could be controlled either by a single,
307 broadly acting leg enhancer, or by multiple enhancers, each dedicated to a different bristle type.
308 For example, the proneural genes *achaete* and *scute*, which control bristle specification, have very
309 complex *cis*-regulatory regions with dozens of modular enhancers responsible for different,
310 stereotypically patterned bristles (Garcia-Bellido and de Celis, 2009). Other enhancers are
311 pleiotropic and active in multiple tissues, reflecting a history of evolutionary co-option from the
312 more ancient organs to the more recently evolved (Glassford et al., 2015; Nagy et al., 2018; Rice
313 and Rebeiz, 2019). In fact, some genes contain both modular (tissue-specific) and pleiotropic
314 (multi-tissue) enhancers (Preger-Ben Noon et al., 2018).

315 We observe a partial overlap in the temporal and spatial activities of the three leg enhancers
316 (Fig 1-3). However, these are not functionally redundant "shadow enhancers" of the type found in
317 some other genes (Frankel et al., 2010; Hong et al., 2008). During prepupal development, the early
318 foreleg enhancer is expressed in cells that give rise both to the sex comb and to the chemosensory
319 bristles (Fig 2, Sup Fig 2). Later, the early foreleg enhancer expression in the sex comb becomes
320 complementary to the activity of the sex comb enhancer: the former is expressed only in the
321 epithelial cells, and the latter predominantly in sex comb bristles and only weakly in the epithelial
322 cells. At pupal stages, when the early foreleg enhancer is no longer active in bristle cells, the late-
323 acting sex comb and chemosensory enhancers have completely non-overlapping expression
324 patterns. Overall, the existence of these three modular enhancers suggests that *dsx* expression in
325 the foreleg goes through two successive stages: relatively broad expression early in development,
326 followed by tightly limited, cell type specific expression later on.

327 These phases of *dsx* expression may have different developmental functions. For example,
328 *dsx* knockdown in chemosensory bristles late in bristle development causes a defect in the axonal
329 midline crossing by the bristle neurons (Mellert et al., 2012). Early *dsx* expression in the foreleg
330 induces a male-specific increase in the number of bristles both in the sex comb and in the
331 chemosensory system (Belote and Baker, 1982; Mellert et al., 2012). The broad early foreleg
332 expression of *dsx* is established prior to the formation of mechanosensory bristles (Tanaka et al.,
333 2011), suggesting that the early foreleg enhancer may control sex comb size by modulating the
334 specification of sensory organ precursors. On the other hand, the later-acting sex comb enhancer
335 is more likely to be involved in controlling the morphology of the individual sex comb teeth, which
336 develop from mechanosensory bristle shafts but, in contrast to most such bristles, are thick, blunt,
337 and darkly pigmented. Indeed, knockdown of *dsx* late in development affects the morphology but
338 not the number of sex comb teeth, which become masculinized in females and feminized in males
339 (Belote and Baker, 1982). Both the foreleg and the sex comb enhancers are active in the epithelial
340 cells around the sex comb, suggesting that both could contribute to sex comb rotation – a
341 coordinated sequence of cell shape changes that moves the sex comb from an initial transverse to
342 the final longitudinal orientation (Atallah et al., 2009; Ho et al., 2018; Malagón et al., 2014; Tanaka
343 et al., 2011). Since sex comb development involves so many different cellular processes spread
344 over multiple developmental stages, it is perhaps not surprising that it requires multiple *dsx*
345 enhancers.

346 Not all tissues and cell types behave in a modular fashion with respect to *dsx* expression.
347 In the central nervous system, only one of the pPTGAL reporters tiling the *dsx* region showed an
348 overlap with endogenous *dsx* expression (in 4 neurons in the PC2 cluster) (M. Arbeitman,
349 unpublished). A separate study identified a *dsx* enhancer upstream of the transcription start with
350 an activity overlapping Dsx protein expression in the brain (Zhou et al., 2014). However, most of
351 the Dsx CNS expression pattern is not accounted for by any of the *dsx* reporter constructs (M.
352 Arbeitman, unpublished). This suggests that for most *dsx*-expressing cells in the CNS, *dsx*
353 regulation may not be due to modular enhancers acting independently, but rather to more complex,
354 long-distance sequence interactions that are not captured by ~5 kb reporter fragments. If true, such
355 "entangled" regulatory architecture could be more constraining than the modular enhancer
356 organization we observe in the foreleg, which could in turn lead to slower evolution of *dsx*
357 expression in the brain compared to other tissues.

358 ***cis-regulatory information limits dsx expression to sexually dimorphic cells***

359 In contrast to some enhancers where very short sequences are sufficient to convey correct
360 regulatory information (Grieder et al., 1997; Manak et al., 1994; Swanson et al., 2010), both the
361 sex comb and the early foreleg enhancers are quite large and cannot be reduced to a core region
362 without perturbing their activities. Interestingly, dissecting these enhancers further leads not to the
363 loss of activity, but rather to its expansion. This is similar to the abdominal enhancer of the *ebony*
364 gene, where a core 0.7 kb sequence is sufficient for abdominal activity, but a much broader
365 genomic region is necessary to prevent ectopic expression (Rebeiz et al., 2009). We find a similar
366 pattern in our study: both the foreleg and the late sex comb enhancers of *dsx* have core sequences
367 of less than 1 kb capable of driving expression in the foreleg, but require additional sequences to
368 repress ectopic activity.

369 The late sex comb enhancer is particularly illuminating in this respect. The sex comb
370 develops from the most distal transverse bristle row on ta1 (Atallah et al., 2009; Tanaka et al.,
371 2009; Tokunaga, 1962), so the bristles that comprise it are serially homologous to the more
372 proximal TBRs. However, while the distal TBR that gives rise to the sex comb undergoes dramatic
373 male-specific modification, the remaining TBRs retain their original, sexually monomorphic
374 morphology. Consistent with this, *dsx* is expressed, and the sex comb enhancer of *dsx* is active,
375 only in the most distal TBR. Loss of sequences at the 5' end of this enhancer leads to an expansion
376 of its activity to the proximal, sexually monomorphic TBRs. Thus, much of the regulatory
377 information contained in the sex comb enhancer is devoted to restricting its activity to sexually
378 dimorphic cells, from a broader population of similar cells. This may reflect a general trend for
379 *dsx* expression: in each tissue or body part (such as the brain, midgut, foreleg, body wall epidermis,
380 etc.), only a subset of cells express *dsx* and undergo sex-specific differentiation (Goldman and
381 Arbeitman, 2007; Luo and Baker, 2015; Rideout et al., 2010; Robinett et al., 2010; Sanders and
382 Arbeitman, 2008; Tanaka et al., 2011). Integrating both activating and repressive inputs in *dsx*
383 enhancers may be necessary to segregate these cells from otherwise similar but sexually
384 monomorphic cells.

385

386 ***Modular dsx enhancers may allow different sex-specific traits to evolve independently***

387 While the sex combs are present in only a subset of *Drosophila* species, particularly in the
388 *melanogaster* and *obscura* species groups, most though not all *Drosophila* species have sex-

389 specific chemosensory bristles on their forelegs. Thus, one could imagine a more ancestral
390 chemosensory foreleg enhancer becoming co-opted for sex comb development in the *melanogaster*
391 and *obscura* groups. A more extreme scenario would involve a co-option of a genital enhancer for
392 sex-specific foreleg development. Male genitalia and legs are serially homologous and share
393 similar developmental programs involving some of the same transcription factors and signaling
394 pathways (Gorfinkiel et al., 1999; Keisman et al., 2001; Sánchez et al., 1997), and the male
395 genitalia of most *Drosophila* species have strongly modified clasper bristles reminiscent of sex
396 comb teeth. Indeed, we find that Dsx is expressed in the clasper during late genital development.
397 However, our results argue against a simple co-option scenario. The chemosensory bristle
398 enhancer of *dsx* is clearly distinct from both enhancers that contribute to sex comb development,
399 being located in a different intron. The situation is less clear with the early foreleg enhancer, where
400 we cannot rule out a minor overlap with the genital enhancer. However, the late sex comb
401 enhancer, which appears to play the dominant role in the development of sex comb teeth, is clearly
402 separate from the genital enhancer.
403 Both the sex comb and the chemosensory bristles play important roles during courtship
404 (Hurtado-Gonzales et al., 2015; Mellert et al., 2012; Ng and Kopp, 2008) and have undergone
405 extensive changes in number, size, morphology, and spatial distribution in *Drosophila* evolution.
406 The ability of all three foreleg enhancers to function and evolve independently of each other may
407 have been a key element driving this evolutionary diversification. Analysis of these enhancers in
408 other *Drosophila* species will provide important insights into the role of *dsx* in the evolution of sex
409 combs and chemosensory bristles. The new GAL4 drivers that mark different types of sex-specific
410 sensory organs may also prove valuable for dissecting the neural mechanisms of courtship
411 behavior.
412
413 **Modular transcriptional regulation of *dsx* may reflect an evolutionarily ancient mode of**
414 **sexual development.**
415 Dsx-related transcription factors (the *Dmrt* gene family) play important roles in sexual
416 differentiation in animals as different as vertebrates, insects, and nematodes (Kopp, 2012; Matson
417 and Zarkower, 2012). Outside of insects, however, *Dmrt* genes do not produce distinct male and
418 female isoforms, but are regulated instead at the transcriptional level. Sex-specific splicing of *dsx*,
419 and more generally the mode of sexual differentiation based on alternative splicing, is an insect

420 innovation (Kopp, 2012; Wexler et al.). In crustaceans and chelicerates, *dsx* homologs are
421 transcribed exclusively or predominantly in males, particularly in male-specific organs, and are
422 required for male but not for female sexual development (Kato et al., 2011; Li et al., 2018;
423 Pomerantz and Hoy, 2015). In *C. elegans*, the *Dmrt* genes *mab3, mab23,* and *dmd3* are expressed
424 in tightly restricted cell lineages and are essential for male-specific differentiation of these cells,
425 but are dispensable in females (Lints and Emmons, 2002; Mason et al., 2008; Ross et al., 2005;
426 Serrano-Saiz et al., 2017; Yi et al., 2000). In vertebrates, the *Dmrt1* gene is highly expressed in
427 the male but not in the female gonad, and is necessary to direct or maintain male-specific
428 differentiation of the initially bipotential gonad (Matson et al., 2011).

429 These similarities suggest that sexual differentiation based on male-specific transcription
430 of *Dmrt* genes in restricted cell populations is an evolutionarily ancient mode of establishing sexual
431 dimorphism, and that in insects, sex-specific splicing of *dsx* was overlaid on this ancestral
432 mechanism (Kopp, 2012; Wexler et al.). The finding that *dsx* transcription in *Drosophila* is
433 controlled by multiple modular enhancers may reflect this more ancestral mode of sexual
434 development. We predict that a similar modular control of *dsx* expression will be found in other
435 arthropods, from basal insects and crustaceans to chelicerates.

436 **Materials and Methods:**

437 *Fly stocks:*

438 Fly strains were obtained from the Bloomington Stock Center, including UAS-GFP.nls
439 (Bloomington stock #4775, genotype: w[1118]; P{w[+mC]=UAS-GFP.nls}14), UAS-svRNAi
440 P{TRiP.JF02582}attP2 (Bloomington stock #27269, genotype: y[1] v[1]; P{y[+t7.7]
441 v[+t1.8]=TRiP.JF02582}attP2), and *dsx*-GAL4 (Robinett et al., 2010) (Bloomington stock
442 #66674, genotype: w[1118]; TI{GAL4}dsx[GAL4]/TM6B, Tb[1])

443 *Transgenic reporter constructs:*

444 Candidate enhancer regions were cloned into the GAL4 reporter vector pPTGAL (Sharma et al.,
445 2002), and randomly integrated into the *D. melanogaster* genome by P-element transformation.
446 Three independent lines from each of ten overlapping fragments were examined. In addition, we
447 analyzed 15 independently generated GAL4 lines which span the same non-coding regions of
448 *dsx* but have different fragment boundaries (Pfeiffer et al., 2008). These lines were constructed
449 using the site-specific pBPGUw vector (Pfeiffer et al., 2008) and integrated into either the attP2
450 or attP40 genomic landing sites via the PhiC31 integrase system (Groth et al., 2004; Venken et
451 al., 2006). Each line from both sets was crossed to a UAS-GFP.nls reporter and GFP expression
452 was examined in the developing foreleg at 5 and at 24 hours after puparium formation (APF).

453 While the high level of GFP expression driven by the GAL4/UAS system was useful for
454 the preliminary screening of genomic fragments, subsequent analysis was performed using direct
455 GFP reporters, which enable more accurate temporal resolution. Genomic fragments were
456 amplified by PCR, cloned into either pCR2 or pCR8 vectors (Invitrogen), transferred into the
457 pGreenFriend GFP destination vector (Miller et al., 2014) using either restriction/ ligation or
458 Gateway cloning, and integrated into the attP2 or attP40 genomic landing sites using the PhiC31
459 integrase system. Germline transformation was performed either in the Kopp lab or by BestGene
460 (http://www.thebestgene.com/). In tests performed on a subset of constructs, we found little
461 difference between attP2 and attP40; we subsequently used the attP40 site, which gave higher
462 integration efficiency. In designing the sub-fragments of the sex comb and early foreleg
463 enhancers, we used Evo-printer (Odenwald et al., 2005) and VISTA (Frazer et al., 2004) to
464 identify sequences within these enhancers that were conserved between *D. melanogaster* and *D.*
465 *pseudoobscura*. Whenever possible, we avoided breaking up highly conserved regions. The

466 boundaries of all reporter fragments and the primers used to amplify them are listed in
467 Supplementary Tables 1 and 2.
468 *Light Microscopy*:
469 Legs were dissected from adult flies and mounted in PVA-Mounting-Medium (BioQuip) or
470 Hoyer's media to clear the samples. Images were taken under Brightfield illumination using a
471 Leica DM500B microscope with a Leica DC500 camera.
472 *Immunohistochemistry and Confocal Microscopy:*
473 Samples were aged, dissected, fixed, stained and imaged as described in Tanaka et al 2009 and
474 Mellert, et al 2012. The primary mouse-Dsx[DBD] antibody (a gift from C. Robinett and B.
475 Baker, later obtained from the Developmental Studies Hybridoma Bank, Cat# DsxDBD,
476 RRID:AB_2617197) was used at 1:10. The secondary Goat anti-Mouse Alexa Fluor 594
477 antibody (Invitrogen Cat# A20185) was used at 1:200. Confocal images were taken using an
478 Olympus FV1000 laser scanning confocal microscope.

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Competing interests:

No competing interests declared

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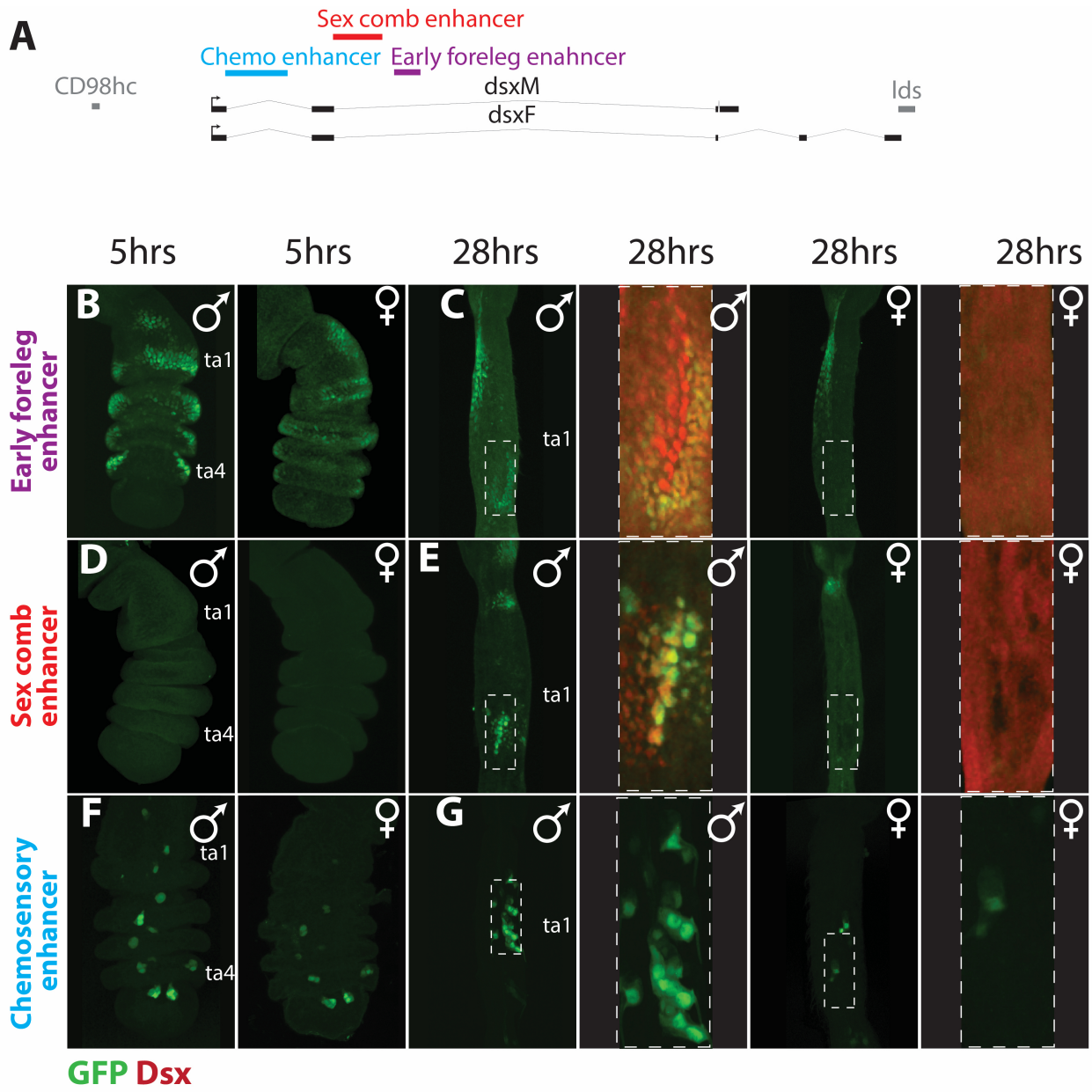
696

697 **Figures:**



698

699 **Figure 1:** Three modular enhancers drive *doublesex* expression in the foreleg

700

701 **A)** A map of the *doublesex* locus with the positions of chemosensory (blue), sex comb (red), and
702 foreleg (purple) *dsx* enhancers. Thick grey lines represent the flanking genes *lds* and *CD98hc*,
703 thick black lines are the exons of *dsx*, and thin black lines are the *dsx* introns. Panels B – G show
704 the foreleg expression patterns of the three *dsx* enhancers, with enhancer-driven GFP in green
705 and magnifications of 28hr samples of the early foreleg and sex comb enhancers show Dsx

706 antibody staining in red. Hours are after puparium formation (APF). The early foreleg and sex
707 comb enhancers constructs are directly adjacent to GFP (Panels B-E), while the chemosensory
708 enhancer (42C06 (Pfeiffer et al., 2008)) construct is a GAL4 driver crossed to a UAS-GFP.nls
709 line. **B)** At 5 hours APF, the early foreleg enhancer recapitulates the Dsx expression pattern in
710 the leg epithelium (Tanaka et al., 2011) and shows a similar expression pattern in males (left)
711 and females (right). Both sexes show ectopic expression in the proximal first tarsal segment
712 (ta1). **C)** At 28 hrs APF, the early foreleg enhancer shows clear sexual dimorphism in the first
713 tarsal segment. Males show strong expression in the epithelial cells surrounding the sex comb
714 and weak to no expression in the sex comb bristle cells. Females show little to no expression in
715 the distal ta1. Both males and females show ectopic expression in the proximal ta1, in the same
716 region as at 5 hrs APF (B), that is not seen by Dsx antibody staining (Tanaka et al., 2011). **D)**
717 The sex comb enhancer is not active in either sex at 5 hrs APF, except for a proximal patch of
718 ectopic expression seen near the joint in some individuals **E)** At 28 hrs APF, the sex comb
719 enhancer is active in the bristle cells of the male sex comb (large nuclei), and weakly in the
720 epithelial cells ventral to the sex comb. Females show weak expression in the distal portion of
721 the segment. Both sexes show ectopic expression in the joint between the tibia and ta1. **F)** At 5
722 hrs APF, the chemosensory enhancer is active in small clusters of cells in ta1-ta5 in both sexes,
723 with more GFP-positive cells in males than in females. **G)** At 28 hrs APF, both sexes show small
724 clusters of expression in ta1-ta5; only ta1 is shown. (No Dsx antibody staining was performed in
725 F-G).

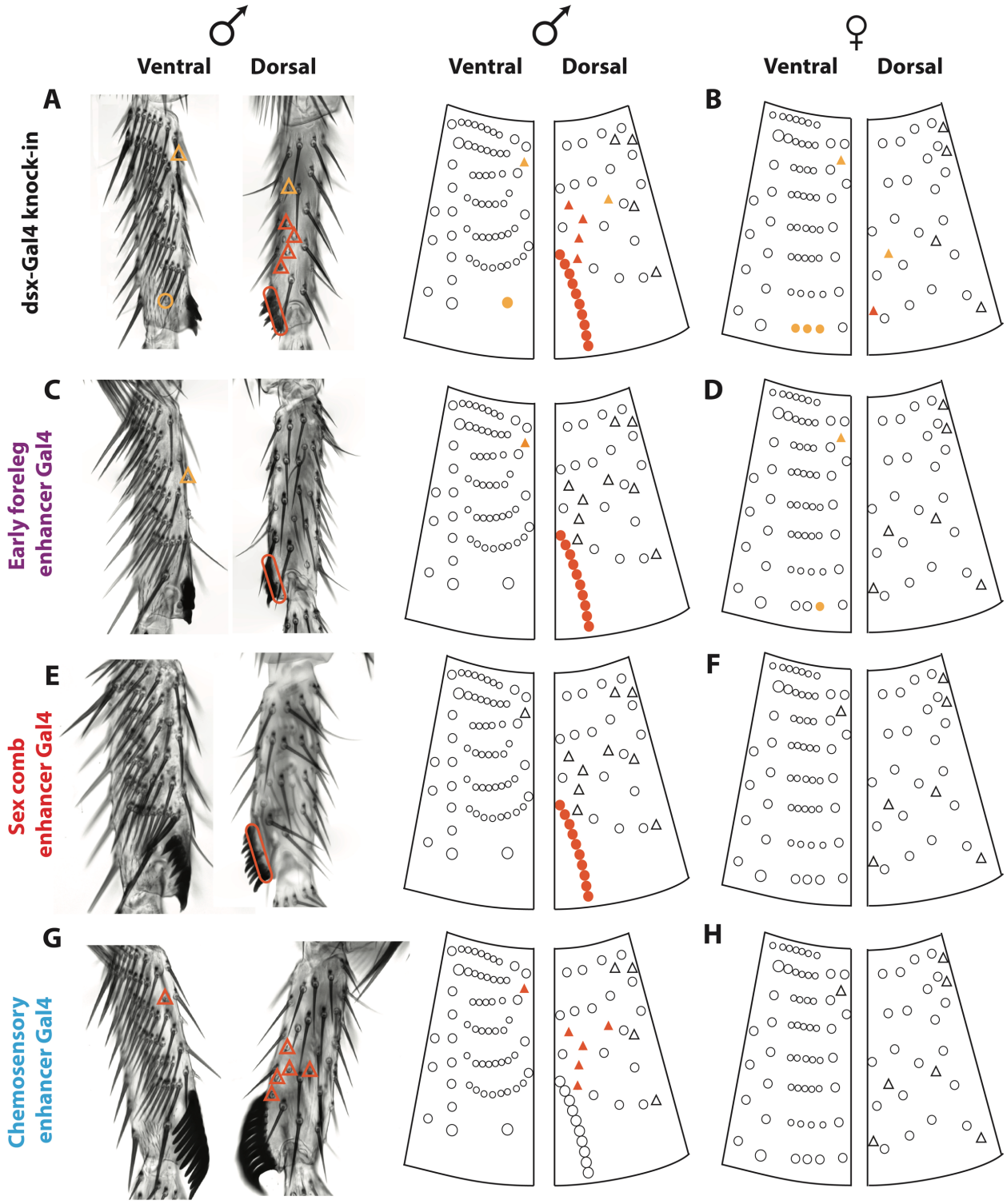


Figure 2: All three leg enhancers contribute to sex-specific bristle development

729 For each enhancer, a GAL4 reporter was used to drive a UAS-*shaven* RNAi construct, truncating
730 the bristles where that enhancer is active. Images of male ta1 are shown next to a schematic
731 representation of male and female ta1 chaetotaxy modified from (Tokunaga 1962). Circles
732 designate mechanosensory bristles, while triangles designate chemosensory bristles. Dark
733 orange symbols mark bristles that were affected in all individuals; bristles marked with yellow
734 varied among individuals of the same genotype raised under standard conditions. **A)** A GAL4
735 knock-in in the *dsx* gene, which reflects the full expression pattern of *dsx* (Robinett et al., 2010),
736 affects both sex comb and sexually dimorphic chemosensory bristles in males. **B)** In females, the
737 *dsx*-GAL4 knock-in has a variable effect on the distal Transverse Bristle Row (TBR), which is
738 homologous to the sex comb, and on some chemosensory bristles. **C)** In males, the early foreleg
739 enhancer affects sex comb bristles in all flies as well as a single chemosensory bristle in some
740 individuals. **D)** In females, the early foreleg enhancer affects one bristle of the distal TBR and
741 the same chemosensory bristle as in males. **E)** In males, the sex comb enhancer affects only the
742 sex comb. **F)** The sex comb enhancer does not affect any bristles in females. **G)** In males, the
743 chemosensory enhancer affects the same chemosensory bristles as the *dsx*-GAL4 knock-in but
744 has no effect on the sex comb. **H)** The chemosensory enhancer does not affect any bristles in
745 females.

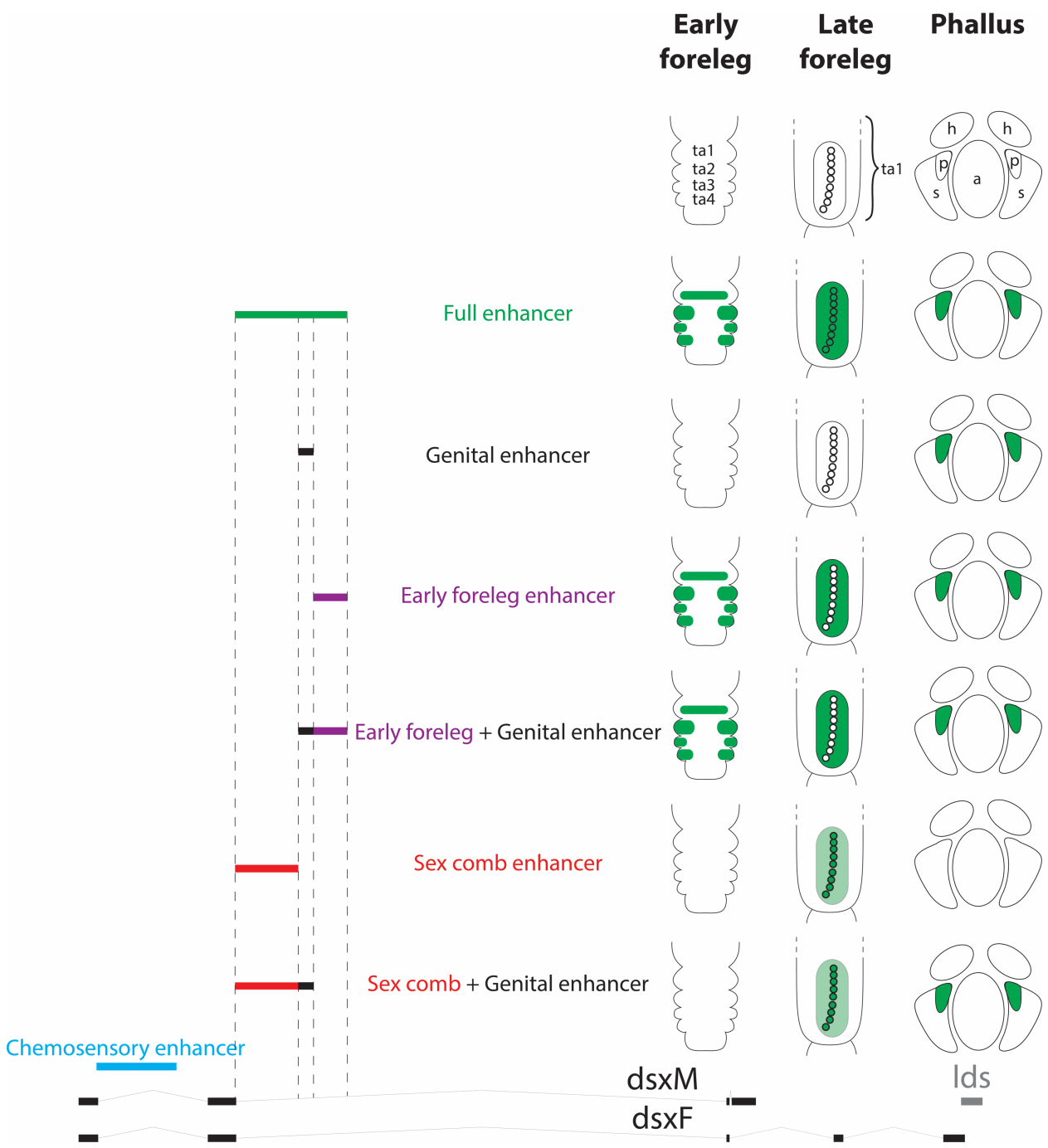


Figure 3: The foreleg and sex comb enhancers are separate and modular

To test for overlap between the foreleg, genital, and sex comb enhancer activities, the region they encompass was split into six constructs: full (all three enhancers, green), genital (black), foreleg (purple), foreleg + genital (purple + black), sex comb (red), and sex comb + genital (red + black). In the drawings, green shading shows reporter expression that matches endogenous Dsx

753 expression, with light green indicating weak expression and white a lack of expression.
754 Representative images of each construct are shown in Fig. S3. Only the constructs that include
755 the early foreleg enhancer drive expression in the forelegs at 5 hrs APF and in the epithelial cells
756 surrounding the sex comb at 24 hrs. Only regions that include the sex comb enhancer drive
757 expression in the sex comb bristles at 24 hrs APF. Both the genital and the early foreleg enhancer
758 constructs are able to drive expression in the genitalia at 48 hrs. ta1-ta4: tarsal segments 1-4; h:
759 hypandrium; a: aedeagus; p: dorsal postgonites; s: aedeagal sheath.

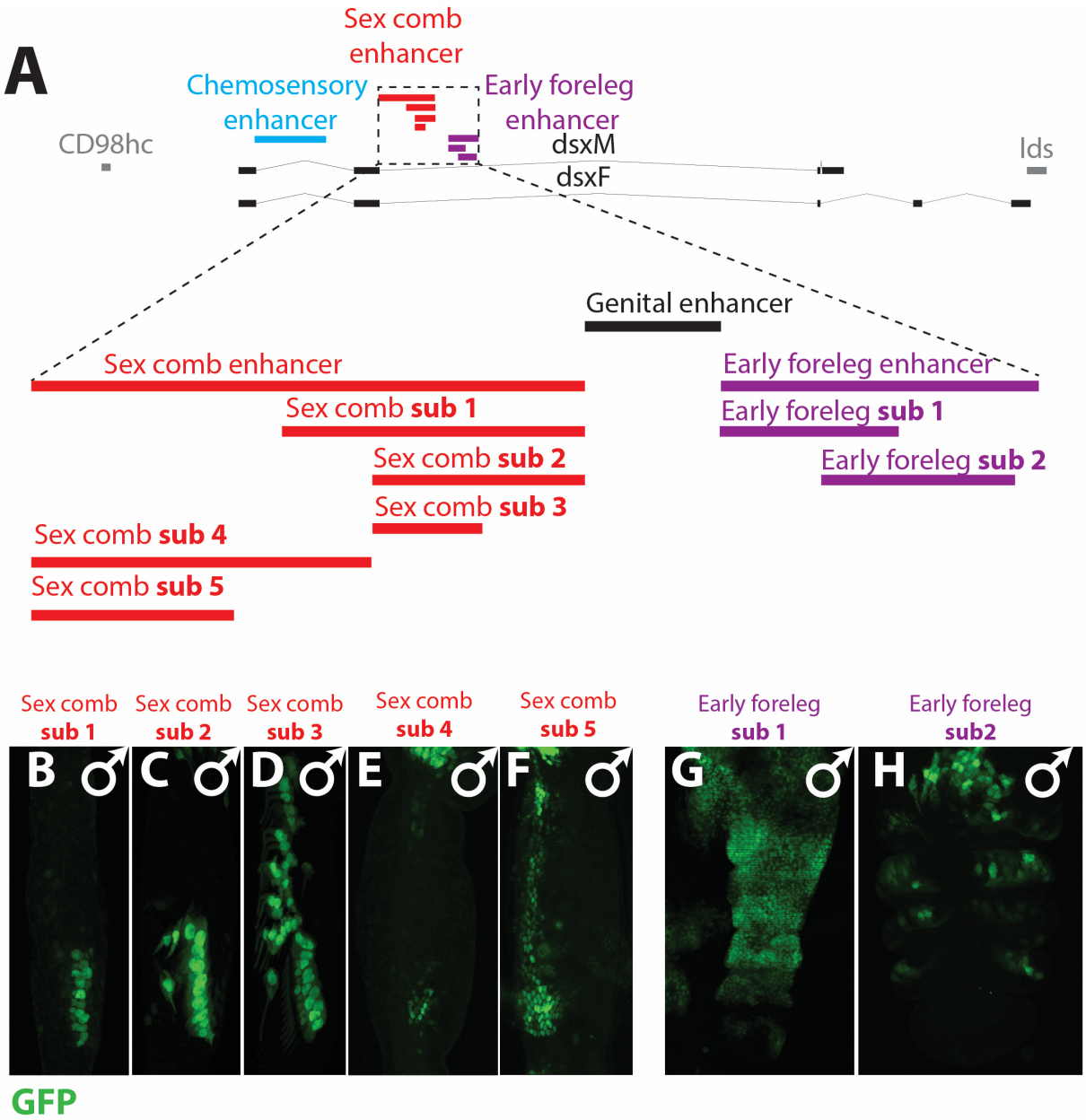


Figure 4: Repressor elements are necessary to limit *dsx* expression to sexually dimorphic cells.

A) A map of the sex comb (red), genital (black), and foreleg (purple) enhancers. **B-E)** Representative images of male forelegs at 36 hrs APF for the *sex comb sub1-sub5* constructs; only the ta1 segment is shown. **B)** The *sex comb sub1* enhancer drives expression in the sex comb bristle cells; **C)** *sex comb sub2* is active in sex comb bristles but has additional ectopic expression in the central bristle and in the second most distal TBR; **D)** *sex comb sub3* is expressed in all TBRs bristle cells in the ta1 segment; **E)** *sex comb sub4* is expressed in some of

769 the epithelial cells that surround the sex comb, but these cells are not directly adjacent to the sex
770 comb bristles; **F)** *sex comb sub5* at 24 hrs AFP is expressed in the epithelial cells surrounding the
771 sex comb, but also shows ectopic expression in more proximal epithelial cells **G-H)** Foreleg
772 expression of the *foreleg sub1* and *sub2* constructs at 5 hrs APF. The *foreleg sub1* region drives
773 broad ectopic expression throughout the ta1-ta4; *foreleg sub2* shows expression in a pattern
774 similar to the complete early foreleg enhancer (Fig. 1B).