Evolution of Sex-Specific Traits through Changes in HOX-Dependent doublesex Expression

Kohtaro Tanaka\(^1\)\(^a\), Olga Barmina\(^1\), Laura E. Sanders\(^2\), Michelle N. Arbeitman\(^2\)\(^b\), Artyom Kopp\(^1\)\(^e\)

\(^1\)Department of Evolution and Ecology, University of California–Davis, Davis, California, United States of America, \(^2\)Section of Molecular and Computational Biology, Department of Biological Sciences, University of Southern California, Los Angeles, California, United States of America

Abstract

Almost every animal lineage is characterized by unique sex-specific traits, implying that such traits are gained and lost frequently in evolution. However, the genetic mechanisms responsible for these changes are not understood. In Drosophila, the activity of the sex determination pathway is restricted to sexually dimorphic tissues, suggesting that spatial regulation of this pathway may contribute to the evolution of sex-specific traits. We examine the regulation and function of doublesex (\textit{dsx}), the main transcriptional effector of the sex determination pathway, in the development and evolution of Drosophila sex combs. Sex combs are a recent evolutionary innovation and show dramatic diversity in the relatively few Drosophila species that have them. We show that \textit{dsx} expression in the presumptive sex comb region is activated by the HOX gene Sex combs reduced (Scr), and that the male isoform of \textit{dsx} up-regulates Scr so that both genes become expressed at high levels in this region in males but not in females. Precise spatial regulation of \textit{dsx} is essential for defining sex comb position and morphology. Comparative analysis of \textit{Scr} and \textit{dsx} expression reveals a tight correlation between sex comb morphology and the expression patterns of both genes. In species that primitively lack sex combs, no \textit{dsx} expression is observed in the homologous region, suggesting that the origin and diversification of this structure were linked to the gain of a new \textit{dsx} expression domain. Two other, distantly related fly lineages that independently evolved novel male-specific structures show evolutionary gains of \textit{dsx} expression in the corresponding tissues, where \textit{dsx} may also be controlled by \textit{Scr}. These findings suggest that changes in the spatial regulation of sex-determining genes are a key mechanism that enables the evolution of new sex-specific traits, contributing to some of the most dramatic examples of phenotypic diversification in nature.

Introduction

Sexual dimorphism is a common feature of animal morphology. Most lineages display unique sets of sex-specific traits, indicating that new sexual characters are gained and old ones are lost, frequently in evolution. At the genetic level, the origin of sex-specific structures from sexually monomorphic precursors implies the evolution of new, sexually dimorphic regulatory pathways. One way in which this could occur is through the emergence of novel interactions between the sex determination pathway and an ancestrally monomorphic genetic network that controls pattern formation and morphogenesis in the evolving tissue. The nature and origin of such interactions can best be understood by characterizing the development of recently evolved sex-specific traits that have sexually monomorphic homologs in closely related species [1–5].

One such trait is the \textit{Drosophila} sex comb, a male-specific structure that develops on the first pair of legs (T1) from stereotypically arranged mechanosensory bristles. The sex comb is a recent evolutionary innovation, present in a relatively small subset of \textit{Drosophila} species including the \textit{melanogaster} and \textit{obscura} species groups [6–8]. Following their origin, sex combs have undergone dramatic morphological diversification with many examples of rapid divergence between closely related species and convergent evolution in distantly related ones [9,10]. This pattern may be caused by sexual selection, since sex combs are used by males for grasping and stimulating females during mating [11–14]. Sex combs can develop by different cellular mechanisms, including a coordinated rotation of the surrounding epithelium [15–18]. The presence of sex combs in the model species \textit{D. melanogaster}, their diversity among close relatives of this species, and the existence of more distant \textit{Drosophila} lineages that primitively lack sex combs make this structure an excellent model for investigating the developmental mechanisms responsible for the origin and diversification of novel sex-specific traits.

In \textit{Drosophila}, sexual differentiation of most somatic tissues is controlled by the sex-specific transcription factors encoded by \textit{doublesex} (\textit{dsx}), an effector of the sex determination pathway that is regulated by alternative pre-mRNA splicing [19–21]. The male isoform (\textit{dsxM}) promotes the development of male-specific
Author Summary

Most animals are sexually dimorphic, yet each species has a different set of sex-specific traits. Much of evolutionary biology since Darwin has focused on explaining these differences. In contrast to the well-developed theories of sexual selection (how and why males compete for females), we are still far from understanding the molecular mechanisms underlying the rapid gain and loss of sexually dimorphic phenotypes. In Drosophila melanogaster, the development of most sex-specific traits is controlled by the doublesex transcription factor. One of these traits is the sex comb, a group of modified bristles that develops on the front legs of males, which they use during mating to grasp the female’s abdomen and genitalia. Sex combs are a recent innovation that evolved within the genus Drosophila but show dramatic diversity in the relatively few species that have them. In this study, we show that the origin and diversification of sex combs were associated with an evolutionary gain of a new doublesex expression domain and novel regulatory interactions between doublesex and the HOX gene Sex combs reduced, best known for its role in the specification of the labial and first thoracic segments. We find that other sex-specific structures that evolved in separate Drosophila lineages are also linked to new doublesex expression domains, suggesting that changes in the spatial regulation of doublesex may be a general mechanism enabling the evolutionary turnover of sex-specific traits.

We propose that similar mechanisms based on the spatial regulation of sex-determining genes may contribute to the origin of new sex-specific traits in other animals. Consistent with this hypothesis, dsx shows specific, derived expression patterns in two other Drosophilid lineages that independently evolved different male-specific structures on their legs.

Results

dsx Expression Is Regulated in a Spatio-Temporal and Sex-Specific Manner

In D. melanogaster, the anterior-ventral side of the first tarsal segment (ta1) is covered with tightly packed transverse bristle rows (TBRs) in both sexes. In the male, the most distal TBR is modified into the sex comb that rotates 90 degrees to align along the proximo-distal leg axis (Figure 1P,Q). In T1 leg imaginal discs of third instar larvae (L3), Scr is strongly expressed in the anterior-ventral region of the presumptive distal tibia (Ti) and ta1 corresponding to the future location of TBRs and the sex comb, and at a lower level in the rest of the disc [10,39,40]. dsx is expressed in an apparently more restricted domain in the T1 imaginal disc, as indicated by a dsx-Gal4 reporter [35]. To characterize the expression pattern of dsx during sex comb development in greater detail, we co-stained T1 legs at different stages with antibodies against Scr [39] and the common domain of Dsx (DsxC), which is shared by the male- and female-specific protein isoforms [33].

In late non-wandering L3 T1 leg discs, high levels of Scr are already detectable in the Ti and ta1 region (Figure 1A). In contrast, no Dsx expression is observed at this time in the leg discs of either sex (Figure 1A). By the wandering L3 and white prepupal stages, Dsx expression is apparent in both male and female T1 discs in an anterior-ventral crescent that overlaps the distal but not the proximal part of the high Scr expression domain (Figure 1B,C). In some males, Dsx expression also extends more distally and posteriorly into the region of low Scr expression (Figure 1B); this variability may reflect subtle temporal differences. Dsx expression was not detected in T2 or T3 leg discs (Figure 1D and unpublished data). In the prepup leg at 5 h after pupariation (5 h AP), Dsx expression is clearly seen in the distal ta1 in both male and female T1 legs (Figure 1E–H). However, the overlap with high Scr expression, which extends more proximally, is more extensive in males than in females (Figure 1G,H). In males, but not in females, Dsx expression is also seen in clusters of cells in the more distal tarsal segments (ta2–ta5) (Figure 1F,G). Thus, Dsx expression becomes sexually dimorphic at the prepupal stage, before the future sex comb bristles are determined.

At 16 h AP, when the sex comb begins its rotation, DsxC expression in the distal ta1 is obviously dimorphic. In males, Dsx is expressed strongly in and around the presumptive sex comb, while female expression is consistently lower (Figure 1I–K). Male-specific expression of Dsx in ta2–ta5 disappears by this time (Figure 1I and unpublished data). By 24 h AP, when sex comb rotation is complete, Dsx and Scr develop roughly complementary expression patterns in the male leg (Figure 1L,M). Dsx is expressed at a high level in sex comb teeth and surrounding epidermal cells, whereas Scr expression is low or absent in sex comb teeth but highest in the adjacent epidermal cells (Figure 1L,M). This pattern is maintained at later stages (Figure 1O). In females, Dsx expression becomes very low or undetectable, and Scr expression in the distal ta1 is much weaker than in males, by 24 h AP (Figure 1N). These observations show that both Dsx and Scr are expressed in tightly restricted and sex-specific patterns in the sex comb at the critical time in its development.
doublesex and the Evolution of Sexual Dimorphism
dsx Regulation Is Transcriptional

The similarities between Dsx protein (Figure 1B,C) and dsx-Gal4 [35] expression patterns suggest that the spatially restricted expression of dsx in the T1 leg is due to transcriptional regulation. To confirm this, we used a RNA probe directed against the male-specific dsx exon to examine dsxM expression by in situ hybridization. In wandering L3 and white prepupal leg discs, dsxM transcript is present in the same pattern as the Dsx protein (Figure 2A, Figure 1B). This transcript is undetectable either in the male T2 and T3 discs or in the female T1 (Figure 2B). At 24 h AP, dsxM transcript in the male T1 leg is confined to the presumptive sex comb region (Figure 2C), similar to the protein distribution (Figure 1M). To confirm that the restriction of dsxM to the sex comb region is not due to post-transcriptional regulation, we drove ectopic UAS-dsxM expression in both males and females using the m-Gal4 driver, which is expressed around the entire circumference of the pupal leg from distal ta1 to ta4 [10]. The UAS-dsxM construct [30] contains most of the male-specific 3’UTR, including a predicted recognition site for the bantam miRNA. Both in situ hybridization with a dsxM-specific probe and immunostaining with the antibody specific to DsxM [31] revealed ectopic dsxM expression throughout the tarsus in all three legs, with no detectable difference between males and females (Figure 2D,E). Thus, we find no evidence for a post-transcriptional mechanism confining dsx expression to the sex comb region. Finally, quantitative rt-PCR with primers flanking male-specific and female-specific exon junctions did not reveal any differences in dsx splicing between T1 and T2 legs (unpublished data). We conclude that the spatially restricted expression of dsx in the sex comb region is caused by its precise transcriptional regulation.

The Roles of dsx and Scr in Sex Comb Development

To determine the significance of the spatial regulation of dsx in sex comb development, we examined the effects of loss and ectopic expression of the male-specific dsx isoform (dsxM) in different cell types. Knocking down dsx in both bristle precursors and epidermal cells in m-Gal4/UAS-dsxRNAi males resulted in an intersex phenotype with small, partially rotated sex combs composed of bristles that were intermediate in morphology between normal sex comb teeth and female TBR bristles (Figure 3C). A similar intersex phenotype was observed in females (not shown). In both sexes, the number of bristles in the partially formed sex comb was intermediate between a wild-type sex comb and the distal-most female TBR phenotype. In males, the transformation was strongest toward the distal end of ta1, but only in regions that express high levels of Scr. The number of bristles in the distal-most female TBR was unchanged despite the changes in bristle morphology. Interestingly, the ventral-posterior region of ta1 in the T3 leg, which also carries TBRs, also

Figure 1. Dsx and Scr expression during sex comb development in D. melanogaster. Immunostaining with anti-Scr (red) and anti-DsxC (green) antibodies. Ti, tibia; ta, tarsus; AP, after pupariation. All panels except (E) and (F) are merged images. (A) L3 male T1 leg disc. Anterior is to the left, dorsal is up. Scr is expressed at a high level in the anterior part of distal Ti and ta1 (arrow) and in a more proximal region corresponding to the presumptive body wall (arrowhead). Low expression is present in the rest of the disc. (B) Wandering male T1 leg disc. Dsx is expressed in the distal part of the Scr domain (overlap in yellow) and in the more central region (arrow), inset shows a magnified view of the boxed area. (C) Wandering female T1 leg disc. (D) Wandering male T2 leg disc. The only detectable Scr expression is subepidermal. (E–H) 5 h AP T1 leg (E–G, male; H, female). The tarsal segments are numbered. Dsx is strongly expressed on the ventral-anterior side of distal ta1 in both sexes (arrow). (F) In the male, Dsx is expressed in the distal ta1 and in small dorsal and ventral patches in ta2–4 (asterisks). (G) In the female, Dsx expression is only in the distal ta1 and is weaker than in the male. (I–K) 16 h AP T1 leg (I, J, male; K, female). Arrows point to the rotating sex comb. Note the absence of Dsx expression in ta2. High background staining is caused by the pupal cuticle, which is still attached to the epidermis at this stage. (L–N) 24 h AP T1 leg. (L, M, male; N, female). (O) 36 h AP male T1 leg. (P, Q) Scanning electron micrographs of the distal ta1 in the adult male (P) and female (Q). Ventral is to the right and anterior is facing out of the page.

doi:10.1371/journal.pbio.1001131.g002

Figure 2. Transcriptional regulation of dsx. (A–C) Localization of dsx transcripts by in situ hybridization. Anterior is to the left, dorsal is up. (A) In the wandering L3 male T1 leg disc, dsxM is expressed in an anterior proximal (arrowhead) and a distal (arrow) crescent domain. (B) No detectable signal is seen in female T2. (C) dsxM expression in a male T1 leg. The only epidermal expression is in the presumptive sex comb region (arrow). Strong staining in the center of the leg is non-specific. (D, E) DsxM immunostaining in m-Gal4/UAS-dsxM male (D) and female (E) T1 leg discs is seen throughout the m-expression domain.

doi:10.1371/journal.pbio.1001131.g002
developed bristles with sex comb-like morphology; in contrast, no changes were observed in T2 legs (not shown). The T3 TBRs are specified by the HOX gene \textit{Ubx} [40], while epidermal cells in the T2 leg do not express any HOX genes at the late larval and pupal stages. Thus, it appears that \textit{Ubx} or its downstream targets can induce tooth-like bristles in all three legs and in regions outside of the high Scr domain in T1 [24]. This difference may be explained by the fact that the \textit{hds} constructs were expressed throughout development in both bristle and epithelial cells.

Despite the changes in bristle shaft morphology, the proximal TBRs showed little or no rotation in \textit{tub-Gal80}\textsubscript{ts}; \textit{neur-Gal4/UAS-dsxM} flies. We next drove ectopic \textit{dsxM} expression in both the bristles and the epidermal cells in the distal \textit{ta1}–\textit{ta4} in \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-dsxM} flies. In the female, this treatment transformed two to four distal TBRs into small sex combs that underwent complete or partial rotation (Figure 3G). However, the number of bristles per TBR was unchanged. A similar phenotype was observed in males (not shown). No effects were observed in the more distal tarsal segments (Figure 3G). These results confirm that sex comb rotation is driven by the surrounding epidermal cells [16,18] and that these cells require high levels of both Dsx and Scr. In summary, ectopic expression experiments indicate that \textit{dsxM} acts in bristle precursor cells to specify sex comb tooth morphology and in the surrounding epidermal cells to promote sex comb rotation, and that precise spatial regulation of \textit{dsx} is essential for determining the location and size of this structure.

Next, we investigated cell type-specific requirements for \textit{Scr} in sex comb development. As previously reported [10], uniform \textit{Scr} expression in the distal tarsus in \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-dsxM} flies results in the formation of ectopic, non-rotating sex combs in \textit{ta2}–\textit{ta4} in the male T1 leg (Figure 3I). Knocking down \textit{Scr} in \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-ScrRNAi} flies results in the complete loss of the sex comb and TBRs in the distal \textit{ta1}, indicating a homeotic transformation to the T2 identity (Figure 3J). However, when \textit{Scr} function was knocked down specifically in bristle precursor cells in \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-ScrRNAi} flies, the number of sex comb teeth was reduced to ~50% of normal, but tooth morphology and rotation were not affected (Figure 3K). This phenotype was not significantly enhanced by the addition of \textit{UAS-Gal4} (not shown). Since \textit{Scr} expression in \textit{ta1} is sexually dimorphic and the sex comb contains more bristles than the homologous female TBR, it is possible that \textit{Scr} levels determine the number of bristle precursors during larval or prepupal stages. \textit{Scr} may also be required in epidermal cells for sex comb rotation, but is dispensable for the male-specific differentiation of sex comb teeth, at later stages. This is consistent with the observation that \textit{Scr} protein disappears from the sex comb precursor bristles by 16 h AP (Figure 1LI,J). Thus, many functions of \textit{Scr} in sex comb development may be mediated by the activation of \textit{dsx} expression (see below).

\textbf{Scr Activates dsx Expression in T1}

Based on the observations that Dsx is expressed only in the T1 leg disc overlapping the high Scr domain and that Scr expression precedes that of Dsx (Figure 1A–C), we hypothesized that Scr positively regulates \textit{dsx} expression. To test this hypothesis, we first

---

**Figure 3. dsx and Scr control sex comb development.** (A) Wild-type male adult T1 leg, \textit{ta}, tarsus; bracket, TBRs; arrow, sex combs. (B) \textit{tub-Gal80}\textsubscript{ts}; \textit{neur-Gal4/UAS-dsxM} male. The bristles in TBRs are transformed into ectopic sex comb teeth (bracket). Arrow points to the normal sex comb. (C) \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-dsxRNAi} male. The sex comb is only partially rotated and has fewer and thinner teeth (arrow). (D) \textit{Scr} expression in \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-dsxRNAi} male at 24 h AP. \textit{Scr} is down-regulated except in the cells distal to the sex comb (arrow). (E) Wild-type female adult T1 leg. Bracket, TBRs. (F) \textit{tub-Gal80}\textsubscript{ts}; \textit{neur-Gal4/UAS-dsxM} female. As in the male of the same genotype (B), TBR bristles assume sex comb-like morphology (bracket). (G) \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-dsxRNAi} female. The two most distal TBRs develop into partially rotated sex combs (arrows). (H) \textit{Scr} expression in \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-dsxRNAi} female T1 leg at 24 h AP. \textit{Scr} is down-regulated except in the cells distal to the ectopic sex comb (arrow); compare to (D). (I) \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-ScrRNAi} male. Ectopic sex combs are formed on distal tarsal segments (arrow). (J) \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-ScrRNAi} male. The number of teeth is reduced, but tooth morphology is normal (arrow). (K) \textit{tub-Gal80}\textsubscript{ts}; \textit{neur-Gal4/UAS-ScrRNAi} male. The number of teeth is reduced, but tooth morphology is normal (arrow). (L) T1 leg disc of \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-ScrRNAi} male. No Dsx is detectable. (M) T2 leg disc of \textit{tub-Gal80}\textsubscript{ts}; \textit{rn-Gal4/UAS-ScrRNAi} male at the wandering stage. Ectopic Dsx expression is detected throughout the \textit{m} expression domain.

doi:10.1371/journal.pbio.1001131.g003
performed an RNAi knockdown of Scr in tub-Gal80\(^\theta\); \textit{rm-Gal4/UAS-ScrRNAi} and \textit{tub-Gal80\(^\theta\)/UAS-Gal80\(^\theta\)} flies. In both male and female T1 leg discs, DsxC expression was strongly reduced in the former genotype and undetectable in the latter after a 24-h shift to the restrictive temperature (Figure 3L), indicating that Scr is necessary for Dsx expression. In a reciprocal experiment, we expressed Scr around the entire circumference of distal ta1–ta4 in all three legs in tub-Gal80\(^\theta\); \textit{rn-Gal4/UAS-Scr} flies. This resulted in the ectopic expression of Dsx in the same pattern as the ectopic Scr in all three pairs of leg discs (Figure 3M and unpublished data), indicating that Scr is sufficient to activate Dsx in the tarsus. These observations suggest that a major role of Scr in sex comb development is to initiate a sex-specific developmental program by turning on ddx expression.

Consistent with this notion, co-expression of Scr and \textit{dxxM} in \textit{tub-Gal80\(^\theta\); \textit{rn-Gal4/UAS-Scr UAS-dxxM}} flies produces the same phenotype as ectopic expression of Scr alone (not shown).

ddx Modulates Scr Expression in the Sex Comb Region

Scr expression in the T1 leg is sexually dimorphic in \textit{D. melanogaster} and other species with rotated sex combs (Figure 1L–P) [10]. To test whether ddx is responsible for the sex-specific regulation of Scr, we first examined the effects of ddx knockdown in \textit{rn-Gal4/UAS-dxxRNAi} males. At 24 h AP, Scr expression in the distal ta1 was reduced, becoming intermediate between wild-type male and wild-type female (Figure 3D, compare to Figure 1L–N). In a reciprocal experiment, we looked at the effect of dxxM expression in \textit{rn-Gal4/UAS-dxxM} females. In this genotype, Scr expression was induced in the distal ta1 in a pattern identical to the \textit{rn-Gal4/UAS-dxxRNAi} males (Figure 3H). These results are consistent with the effects of ddx on adult morphology: in the absence of either \textit{dxxM} or \textit{dxxF}, or in the presence of both isoforms, the distal-most TBR assumes a morphology intermediate between a sex comb and a female TBR in both XX and XY flies (Figure 3C) [22]. We conclude that in the absence of ddx, Scr is expressed at an intermediate level and that this level is sufficient to induce partial sex comb development in \textit{D. melanogaster}. DsxM up-regulates and DsxF down-regulates Scr relative to this default level, so that both isoforms are actively involved in sexually dimorphic development.

Correlated Evolutionary Changes in Scr and ddx Expression Are Associated with Sex Comb Diversification

The size and location of sex combs in the \textit{melanogaster} and \textit{obscura} species groups correlate with the domain of high Scr expression [10,41]. Moreover, Scr expression is sexually dimorphic in species with rotated sex combs, but not in species in which sex comb teeth remain organized into TBRs [10]. We used DsxC and DsxM antibodies to examine ddx expression in the presumptive sex comb region in \textit{melanogaster} group species with diverse sex comb morphologies (Figure 4). Importantly, these species represent several independent phylogenetic contrasts, since distantly related species have evolved similar sex combs independently (Figure 4H) [10]. In all species, Dsx expression is strongest in sex comb teeth and is also present in the adjacent epidermal cells, while Scr expression is low or absent in sex comb teeth but highest in the surrounding cells (Figure 4). In \textit{D. ficusphila} and \textit{D. kikkawai}, which independently evolved large sex combs spanning the entire ta1 and ta2, Scr and Dsx are expressed throughout the anterior-ventral surface of these segments (Figure 4A,E). In \textit{D. bipunctata} and \textit{D. breamipes}, which independently evolved rotated sex combs derived from two separate TBRs, Dsx and Scr are expressed in and around both rows of teeth (Figure 4B,F). In the closest relatives of these species that have transverse sex combs (\textit{D. malerkotliana} and \textit{D. takahashii}, respectively), Dsx expression in epidermal cells is lower than in the species with rotated sex combs, and is only seen in a few cells immediately adjacent to the sex comb (Figure 4C,G). In \textit{D. nikanum}, whose sex comb is secondarily reduced from a \textit{D. kikkawai}-like ancestral state, Dsx expression is also confined to a smaller domain that resembles the \textit{D. melanogaster} pattern (Figure 4D). Thus, the spatial correlation of Dsx and Scr expression is maintained in all species and reflects sex comb morphology rather than phylogenetic history. This pervasive pattern of convergent evolution suggests that the cross-regulatory relationship between Dsx and Scr is conserved throughout the \textit{melanogaster} species group and may contribute to the rapid evolution of sex comb morphology.

ddx Expression in the Presumptive Sex Comb Region Is an Evolutionary Innovation

The sex comb is a recent evolutionary innovation that is absent in most \textit{Drosophila} species. In the ancestral condition, the pattern of mechanosensory bristles is similar in males and females. To understand the role of ddx regulation in the origin of sex combs, we examined Dsx expression in several distantly related species of \textit{Drosophila} and related genera (Figures 5, 6). The \textit{melanogaster} and \textit{obscura} species groups form a monophyletic lineage characterized by the presence of sex combs (Figure 5A). In \textit{D. pseudoobscura}, a representative of the \textit{obscura} group, Dsx is expressed in the presumptive sex comb region (Figure 5D,E), suggesting that this expression domain was already present in the last common ancestor of both species groups.

In \textit{Scaptodrosophila lehmannensis} and \textit{Drosophila (Drosilophia) busecki}, which are among the most distant outgroups in our analysis, no Dsx expression is seen in the L3 leg discs (Figure 5B,C). In \textit{D. hydei} and \textit{D. virilis}, which represent different species groups in the subgenus \textit{Drosophila} and also primitively lack sex combs, Dsx is expressed in the T1 tarsi in two clusters per segment during the larval and prepupal stages (Figure 5N,P and unpublished data). These clusters, which are seen in both males and females but only in the T1 leg (Figure 5O), are not homologous to the presumptive sex comb region, resembling instead the transient expression in the distal tarsal segments of male \textit{D. melanogaster} (Figure 1F). Dsx expression in \textit{D. hydei} and \textit{D. virilis} is also transient: by the time of leg extension in the early pupa, when bristles begin to develop, no Dsx expression is detected in either males or females (Figure 5Q).

The closest well-studied relatives of the \textit{melanogaster} and \textit{obscura} species groups that lack sex combs are the Neotropical \textit{Sophophora} including the \textit{willistoni} and \textit{saltans} species groups (Figure 5A) [42,43]. However, the Neotropical \textit{Sophophora} have recently been shown to be the sister group of the genus \textit{Lordophosa}, some but not all representatives of which have sex combs [8,44–46]. Thus, it is not clear whether the \textit{willistoni} and \textit{saltans} species groups lack sex combs primitively or have lost them secondarily.

In \textit{D. willistoni} and \textit{D. saltans} imaginal discs, the DsxC antibody shows expression around the entire circumference of the tarsus in all three pairs of legs in both sexes, as well as in a more proximal crescent that is only seen in the male T1 disc (Figure 5G–I). Surprisingly, the ring pattern is seen with both DsxC and DsxM antibodies in both males and females, while the male T1 crescent is only detected with the DsxC antibody. Although the DsxC antibody reveals a typical ddx expression pattern in the adult brain of \textit{D. willistoni} (Figure 5J), the DsxM antibody shows a different pattern, suggesting that it may not be specific to Dsx. Thus, it is not clear whether the ring seen in larval leg discs reflects ddx expression. At 5 h AP, this ring can be seen to extend from ta2 to ta4; the crescent pattern can no longer be detected at this stage (Figure 5K). By the time of leg extension in the early pupa (24–27 h AP), the ring pattern also disappears from the T1 legs of both sexes (Figure 5L).

Thus, in contrast to the \textit{melanogaster} and \textit{obscura} species groups, ddx expression is not maintained at the developmental stage when bristle differentiation begins. The morphology and chaetotaxy of T1
and other legs in *D. willistoni* and *D. saltans* are sexually monomorphic, lacking even the male-specific chemosensory bristles that are present in most other *Drosophila* lineages (Figure 5F, not shown). This suggests that *dsx* is not directing sex-specific morphological differentiation in the legs of these species.

Overall, our results show that *dsx* is expressed in temporally dynamic, rapidly evolving, and segment-specific patterns in *Drosophila* legs. However, *dsx* expression in the presumptive sex comb region appears to be an evolutionary innovation that coincides with the origin of the sex comb.

**Independent Origin of Other Sex-Specific Structures Correlates with Gain of *dsx* Expression**

Sex combs are only one example of sex-specific structures that decorate the legs of many Drosophilidae and other Diptera [47]. For example, T1 TBRs show strong sexual dimorphism in the *immigrans* species group, a member of the *Drosophila* subgenus that is distantly related to the *melanogaster* and *obscura* groups and other *Sophophora* (Figure 5A). The females of *D. immigrans* have the same arrangement of TBRs as other *Drosophila* species, while in males the anterior-ventral surface of ta1 and ta2 is covered with smaller but much more numerous and densely packed bristles (Figure 6A,B). The corresponding region of the L3 imaginal disc shows *Dsx* expression in both males and females (Figure 6C, not shown); in contrast, no expression is seen in T2 and T3 legs (Figure 6D). By 5 h AP, this expression remains strong in males but begins to fade in females (Figure 6E, not shown). In extended pupal legs, when bristles begin to differentiate, all of the densely packed bristles are expressing high levels of *Dsx* in males, whereas no expression is seen in the homologous region in females (Figure 6F,G).

**Figure 4. Dsx and Scr expression in the *melanogaster* species group.** Ta1–2 of adult male T1 legs are shown on the left. Scr (red) and Dsx (green) immunostaining of the same segments in mid-pupal male T1 legs are shown in the right panels. Developing sex combs are indicated by arrows (longitudinal combs) or arrowheads (small and transverse combs). In all species, Dsx expression is highest in the sex comb teeth, while Scr is low in the sex comb teeth but high in the surrounding cells. (A) *D. ficusphila*. (B) *D. biarmipes*. (C) *D. takahashii*. (D) *D. nikananu*. (E) *D. kikkawai*. (F) *D. bipectinata*. (G) *D. malerkotliana*. (H) Phylogenetic relationships among the species shown in this figure. The latest common ancestor of *D. kikkawai* and *D. nikananu* had a sex comb similar to that of *D. kikkawai*; the latest common ancestor of *D. malerkotliana* and *D. bipectinata* had a sex comb similar to *D. malerkotliana* (Barmina and Kopp 2007) [10].

doi:10.1371/journal.pbio.1001131.g004
Figure 5. Dsx expression in distantly related lineages. Dsx immunostaining is in green. (A) Simplified phylogeny of the species shown in this figure and Figure 6. (B) Male T1 leg disc of S. lebanonensis. (C) Male T1 leg disc of D. busckii. (D) Adult male T1 leg of D. pseudoobscura carries sex combs on the ta1 and ta2 segments. (E) Dsx expression in the corresponding segments of the male T1 leg at the early pupal stage. (F–L) D. willistoni. (F) Adult male T1 leg. Note the absence of sex combs and the very small number of long and curved chemosensory bristles (compare to M). (G) Male T2 leg disc stained with the DsxC antibody. Arrowhead, an expression domain unique to the male T1 disc. (H) Male T2 leg disc stained with the DsxC antibody. (I) Female T1 leg disc stained with the DsxC antibody. (J) Adult male brain stained with the DsxC antibody, showing the PC1 (arrow) and
In most species of the genus *Zaprionus*, TBRs on the distal ta1 of the T1 leg are replaced with much thinner and more numerous bristles that form a densely packed brush [48,49]. This structure is only observed in males, while females retain standard T1 leg morphology (Figure 6H–J). As in the *melanogaster* and *immigrans* species groups, we find that this sex-specific pattern is prefigured by Dsx expression in the corresponding region of the T1 leg (Figure 6K,L), but no expression is seen in the T2 and T3 legs (not shown).

Phylogenetic analysis suggests that male-specific morphological structures originated independently in the *immigrans* species group, *Zaprionus*, and the *melanogaster*+*obscura* clade (Figure 5A). In each case, these morphological innovations correlate with newly evolved, T1-specific patterns of *dsx* expression. These observations suggest that the evolutionary gain of new *dsx* expression domains through a regulatory link between *Scr* and *dsx* has been a key step in the origin of novel sexually dimorphic structures.

**Discussion**

Localized Activation of *dsx* Induces the Formation of a Sex-Specific Structure

Traditional models of sexually dimorphic development in *Drosophila* have assumed that the sex determination pathway functions ubiquitously, and emphasized the joint regulation of downstream targets by *dsx* and the genes that establish positional information [19]. Indeed, co-regulation of downstream targets by *dsx* and spatial selector genes and signaling pathways plays a key role in the development of sex-specific morphological structures.

---

**Figure 6. Dsx expression in species that evolved lineage-specific sexually dimorphic structures.** Dsx immunostaining is in green. (A–G) *D. immigrans*. (A, B) Adult male and female T1 legs, respectively. (C) Male T1 leg disc. (D) Male T2 leg disc. (E) Male T1 prepupal leg. (F, G) Male and female T1 pupal legs, respectively, at 48 h AP. (H–L) *Zaprionus tuberculatus*. (H, I) Adult male and female T1 legs, respectively. (J) Male-specific brush structure shown at higher magnification. (K) Male T1 leg disc. Arrow, dsx expression domain. (L) Male T1 pupal leg at 48 h AP.

doi:10.1371/journal.pbio.1001131.g005
including genitalia [50–52], posterior abdominal segments [1,2], and oenocytes [53]. However, recent work has shown that \( dsx \) is expressed in tightly restricted spatial patterns [30–35], suggesting that sexually dimorphic development may also be regulated through localized deployment of \( dsx \). Here, we show that localized transcriptional activation of \( dsx \) in the T1 leg initiates the development of a sex-specific structure, and that the spatial pattern of \( dsx \) defines the position and morphology of this structure. For the first time, we also identify an upstream regulator of \( dsx \) transcription, the HOX gene \( Scr \). Our results indicate that \( Scr \) is responsible for activating \( dsx \) expression in the T1 leg, and thus for restricting sexually dimorphic chaetotaxy to a single thoracic segment. Since \( Dsx \) expression is more restricted than that of \( Scr \), we suspect that \( dsx \) is also regulated by one or more of the transcription factors that establish the proximo-distal leg axis.

In turn, \( dsx \) up-regulates \( Scr \) in males in the presumptive sex comb region prior to and during sex comb rotation. Thus, the HOX and sex determination genes establish a positive autoregulatory loop (Figure 7). The mutual up-regulation of \( Scr \) and \( dsxM \) may explain why \( Dsx \) levels become much higher in males than in females as sex comb development progresses. The loss of \( Dsx \) expression in the homologous region in females is caused by the gradual reduction of protein levels in both epithelial and bristle cells; we do not observe large amounts of cell death in this region at the pupal stage. In contrast, \( Dsx \)-expressing domains in the central nervous system (CNS) become sexually dimorphic through programmed cell death and cell division. In one set of \( Dsx \)-expressing neurons, \( DsxF \) directs cell death in females, while in another \( DsxM \) contributes to an increase in cell division in males [33]. In the embryonic gonad, sex differences in the number of \( Dsx \)-expressing cells also result from the activation of cell death by \( DsxF \) [54]. Taken together, these results demonstrate that differences in \( dsx \) transcription, functional differences between \( Dsx \) isoforms, and the cellular context in which these isoforms are expressed can lead to sex-specific differentiation through a variety of cellular processes.

The molecular mechanisms responsible for the \( Scr-dsx \) feedback loop may be different at different stages. The initial activation of \( dsx \) by \( Scr \) in the late L3 leg disc may be direct, since the two proteins accumulate in the same cells. However, once the bristle precursor and epithelial cells are segregated at the pupal stage, \( Scr \) and \( Dsx \) domains become complementary and cell type-specific: \( Dsx \) expression is highest in the sex comb teeth while \( Scr \) is excluded from the bristle cells but is strongly up-regulated in the epithelial cells immediately adjacent to the sex comb. These patterns suggest that the cross-regulation between \( Dsx \) and \( Scr \) at this stage may be mediated by cell-cell signaling.

The regulation of \( dsx \) and \( Scr \) in precise spatial and cell type-specific patterns casts the roles of HOX and sex determination genes in development in a new light. Instead of modulating the output of a patterning network from the outside as “master regulators,” both \( dsx \) and \( Scr \) are intimately integrated into the middle of this network (Figure 7). Akam [55] has suggested that HOX genes may act more as “micromanagers” than master regulators in many developmental contexts. It now appears that the main determinant of sex-specific development may have to be demoted to a similar position.

**Evolutionary Origin of a Sex-Specific Developmental Pathway**

New sex-specific traits may arise in two different ways. If the sex determination pathway is already active in the relevant tissue, the origin of a novel trait requires only the acquisition of new joint downstream targets by the sex determination and spatial patterning genes. This may happen either through evolution of \( Dsx \) binding sites in a previously sexually monomorphic enhancer or through the co-option of a pre-existing dimorphic enhancer into a new tissue [2,53]. In contrast, a tissue that shows no sexual dimorphism in the ancestral condition may not express \( dsx \) at all. In this case, a new sex-specific trait cannot arise without the evolution of a new \( dsx \) expression domain. To our knowledge, the sex comb is the first example of an evolutionary change of this kind. We suggest that in the common ancestor of the *melanogaster* and *obscura* species groups, \( dsx \) was recruited into a previously sexually monomorphic developmental pathway, resulting in the gain of a novel expression domain in the presumptive sex comb region (Figure 7). This cooption may have been facilitated by the fact that \( dsx \) is already expressed in segment-specific, and presumably \( Scr \)-regulated patterns in some species that primitively lack sex combs. In parallel, \( Scr \) and \( dsx \) must have acquired new joint downstream targets that mediate different aspects of sex comb morphogenesis including bristle patterning, tissue rotation, and modification of bristle shafts. Subsequent changes in the spatial regulation and cross-regulation of \( dsx \) and \( Scr \), as well as gains and losses of downstream targets, have likely contributed to the dramatic evolutionary diversification of sex combs.

**Positive Feedback and Evolvability**

The positive feedback loop between \( dsx \) and \( Scr \) may play a major role in generating sex comb diversity across species.
Regardless of the exact molecular mechanism, our results suggest that any alteration in Sex comb expression expands or contracts the dsx domain, and vice versa. One can imagine that any mutation that increases Sex comb expression, for example a cis-regulatory mutation in the Sex comb enhancer, would increase the expression of dsx, which in turn would further up-regulate Sex comb in the male, and so on; the effects of any mutation that increases the expression of dsx would be similarly amplified. Conversely, mutations that reduce either Sex comb or dsx expression would also have their effects on both Sex comb and dsx magnified by the autoregulatory loop. This positive-feedback amplification would allow sex comb morphology to respond rapidly to selection for increased or decreased sex comb size. Comparative and experimental analyses show that male secondary sexual traits are lost (or reduced) as frequently as acquired (or exaggerated), and that this pattern may be due to rapidly shifting female preferences [56,57]. It is possible that positive feedback loops similar to the Sex comb-dsx circuit are involved in the rapid gain, diversification, and loss of other exaggerated display characters and sexually selected traits.

A General Role for dsx in Evolutionary Innovations?
The spatial regulation of dsx in Drosophila raises an intriguing question about the evolution of sex-specific traits in general. Sexual selection leads not only to the rapid evolution of existing characters, but also to the frequent origin of novel morphological structures, behaviors, and other phenotypes [58,59]. Almost every lineage of animals has invented its own sex-specific (often, but not always, male-limited) organs. In Diptera, different families and genera have evolved a variety of sex-specific structures and modifications on all three pairs of legs, on the eyes, mouthparts, and the head capsule, on the thorax, abdomen, and generally on every body part imaginable [47,60,61]. Some of these structures reach truly bizarre appearance and proportions, such as the branched and malformed legs of some Dolichopodidae and Platypodidae or the eye stalks that exceed body length in Diopsidae and Platystomatidae, yet they have no clear homologues outside of the lineages that possess them. At the same time, the loss of sex-specific characters occurs at roughly the same rate as the origin of new ones [56]—in other words, there is a constant turnover of sex-specific traits.

Is it possible that the proximate cause of this turnover of sex-specific traits lies in the acquisition and loss of new spatial expression domains of dsx? This model is supported by our observation that different male-specific structures that independently evolved in the immigrans species group and in the genus Zaprionus are, like the sex comb, associated with the origin of new dsx expression patterns. Male-specific reduction of wing size in Nasonia wasps, which is associated with genetic changes near the dsx locus, may represent another example [62]. The modular organization of transcriptional control allows gene expression in different tissues to be decoupled both functionally and evolutionarily through the use of modular, tissue-specific enhancers [63,64], making the gain and loss of discrete expression domains entirely possible. A dsx enhancer responsible for sex comb development in Drosophila was gained and underwent rapid diversification within the genus, raising the possibility that other novel enhancers and expression domains have originated in other lineages on similarly short timescales.

Materials and Methods
Drosophila Strains
The following strains were used: m-Gal4 
[65], near-Gal4[66], UAS-dsxM [Lee et al. 2002] [30], UAS-Scr[67], UAS-dsxRNAi, UAS-ScrRNAi [68], and tub-Gal80[69]. Expression of the UAS constructs was activated at the wandering third instar or white prepupal stage by shifting tub-Gal80[69], Gal4/UAS flies from 18°C to 30°C.

Immunocytochemistry and Imaging
Animals were reared, processed for immunocytochemistry, and imaged as described [16,33]. The primary antibodies used were rat anti-DsxCommon, 1:50 [33], rat anti-DsxM, 1:500 [31], and mouse anti-Scr 6H4:1, 1:10 [39]. The secondary antibodies were AlexaFlour 488 and 594 used at 1:200 (Invitrogen, Carlsbad, CA). In D. melanogaster, both Dsx antibodies showed identical expression patterns in larval leg discs and pupal legs. In species distantly related to D. melanogaster, cross-reactivity of the Dsx antibodies was confirmed by staining adult male and female brains. The DsxC antibody identified neuronal clusters that were similar in size and position to those seen in D. melanogaster (Figure 5), while the DsxM antibody showed variable staining in different species suggesting that it may not be fully specific. With the exception of D. melanogaster, all Dsx expression patterns shown in the figures were determined using the DsxC antibody.

In Situ Hybridization
In situ hybridization on pupal legs and imaginal discs was performed as described [37] using RNA probes directed against the male-specific exon of dsx. Probe template was amplified from genomic DNA by PCR using primers dsxM-Fwd (AATCG-CACTGTAGCCAGATGC) and dsxM-Rev (CTGGAGTCG-GTGGACAAATGC).

Acknowledgments
We thank Guunghee Lee for the UAS-dsxM strain, Brian Oliver for the anti-DsxM antibody, the Bloomington Drosophila Stock Center and the Vienna Drosophila RNAi Center for other fly strains, and Dr. M. Toda for discussions of drosophilid phylogeny. The α-Scr antibody developed by D. Brower was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa Department of Biology.

Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: KT OB MNA. Contributed reagents/materials/analysis tools: LES MNA. Wrote the paper: KT MNA AK.

References
1. Kopp A, Duncan I, Godt D, Carroll SB (2000) Genetic control and evolution of sexually dimorphic characters in Drosophila. Nature 408: 553–559.
2. Williams TM, Selegue JE, Werner T, Gompel N, Kopp A, et al. (2008) The regulation and evolution of a genetic switch controlling sexually dimorphic traits in Drosophila. Cell 134: 610–623.
3. Gompel N, Prud‘homme B, Wittkopp PJ, Kassner VA, Carroll SB (2005) Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in Drosophila. Nature 433: 481–487.
4. Bower JR, Nijhout HF (2009) Partial co-option of the appendage patterning pathway in the development of abdominal appendages in theypad fly Thymira biloba. Dev Genes Evol 219: 577–587.
5. Momper AP, Rose DJ (2009) Differential recruitment of limb patterning genes during development and diversification of beetle horns. PNAS, in press.
6. Lemeunier F, David J, Tsacas L, Ashburner M (1986) The melanogaster species group. Ashburner, Carson, Thompson, 1981–1986 e: 147–256.
7. Lakovaara S, Saura A (1982) Evolution and speciation in the Drosophila obscura group. Ashburner, Carson, Thompson, 1981-1986 b: 3-59.
8. Kopp A (2011) Drosophila sex combs as a model of evolutionary innovations. Evolution and Development, (in press).
9. Kopp A, True JR (2009) Evolution of male sexual characters in the oriental Drosophila melanogaster species group. Evol Dev 4: 278–291.
10. Barmina O, Kopp A (2009) Sex-specific expression of a HOX gene associated with rapid morphological evolution. Dev Biol 311: 277–286.
11. Polak M, Steward WT, Wolf LL (2004) Sexual selection for size and symmetry in a diversifying secondary sexual character in Drosophila bipunctata Duda (Diptera: Drosophilidae). Evolution Int J Org Evolution 58: 597–607.
12. Ng C-S, Kopp A (2008) Sex combs are important for male mating success in Drosophila melanogaster. Behav Genet 38: 193–201.
13. Spiehi HT (1952) Mating behavior within the genus Drosophila (Diptera). Bulletin of the American Museum of Natural History 99: 395–474.
14. Cook RM (1977) Behavioral role of the sexcombs in Drosophila melanogaster. Dev Biol 4: 489–516.
15. Atallah J, Liu NH, Dennis P, Hon A, Larsen EW (2009) Developmental constraints and convergent evolution in Drosophila sex comb evolution. Evol Dev 11: 205–218.
16. Atallah J, Liu NH, Dennis P, Hon A, Godt D, et al. (2009) Cell dynamics and developmental bias in the ontogeny of a complex sexually dimorphic trait in Drosophila melanogaster. Evol Dev 11: 191–204.
17. Christiansen AE, Keisman EL, Ahmad SM, Baker BS (2002) Sex combs in the cold: the integration of sex and pattern. Trends Genet 18: 510–516.
18. Burton KC, Baker BS (1989) Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell 56: 997–1010.
19. McKeown M (1992) Sex differentiation: the role of alternative splicing. Curr Opin Genet Dev 2: 299–303.
20. Baker BS, Ridge KA (1980) Sex and the single cell. I. On the action of major loci directing cell number. Dev Biol 320: 378–390.
21. Li H, Baker BS (1996) Hermaphroditic and doublesex function both dependently and independently to control various aspects of sexual differentiation in Drosophila. Development 125: 2641–2651.
22. Jhaveri D, Sen A, Reddy GV, Rodrigues V (2000) Sense organ identity in the Drosophila antenna is specified by the expression of the proneural gene atonal. Proc Natl Acad Sci U S A 106: 4764–4769.
23. Kopp A (2011) Drosophila sex combs as a model of evolutionary innovations. Nature Rev Genet 12: 385–400.
24. Spiehi HT (1952) Mating behavior within the genus Drosophila (Diptera). Bulletin of the American Museum of Natural History 99: 395–474.
25. Drosophila melanogaster. Dev Biol 4: 489–516.
26. Barmina O, Kopp A (2009) Distinct developmental mechanisms underlie the evolutionary diversification of Drosophila sex combs. Proc Natl Acad Sci U S A 106: 4764–4769.
27. Atallah J, Liu NH, Dennis P, Hon A, Larsen EW (2009) Developmental constraints and convergent evolution in Drosophila sex comb evolution. Evol Dev 11: 205–218.
28. Atallah J, Liu NH, Dennis P, Hon A, Godt D, et al. (2009) Cell dynamics and developmental bias in the ontogeny of a complex sexually dimorphic trait in Drosophila melanogaster. Evol Dev 11: 191–204.
29. Christiansen AE, Keisman EL, Ahmad SM, Baker BS (2002) Sex combs in the cold: the integration of sex and pattern. Trends Genet 18: 510–516.
30. Burton KC, Baker BS (1989) Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell 56: 997–1010.
31. McKeown M (1992) Sex differentiation: the role of alternative splicing. Curr Opin Genet Dev 2: 299–303.
32. Baker BS, Ridge KA (1980) Sex and the single cell. I. On the action of major loci directing cell number. Dev Biol 320: 378–390.
33. Li H, Baker BS (1996) Hermaphroditic and doublesex function both dependently and independently to control various aspects of sexual differentiation in Drosophila. Development 125: 2641–2651.
34. Jhaveri D, Sen A, Reddy GV, Rodrigues V (2000) Sense organ identity in the Drosophila antenna is specified by the expression of the proneural gene atonal. Proc Natl Acad Sci U S A 106: 4764–4769.
35. Atallah J, Liu NH, Dennis P, Hon A, Larsen EW (2009) Developmental constraints and convergent evolution in Drosophila sex comb evolution. Evol Dev 11: 205–218.
36. Atallah J, Liu NH, Dennis P, Hon A, Godt D, et al. (2009) Cell dynamics and developmental bias in the ontogeny of a complex sexually dimorphic trait in Drosophila melanogaster. Evol Dev 11: 191–204.
37. Christiansen AE, Keisman EL, Ahmad SM, Baker BS (2002) Sex combs in the cold: the integration of sex and pattern. Trends Genet 18: 510–516.
38. Burton KC, Baker BS (1989) Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell 56: 997–1010.
39. McKeown M (1992) Sex differentiation: the role of alternative splicing. Curr Opin Genet Dev 2: 299–303.
40. Baker BS, Ridge KA (1980) Sex and the single cell. I. On the action of major loci directing cell number. Dev Biol 320: 378–390.
41. Kopp A (2011) Drosophila sex combs as a model of evolutionary innovations. Nature Rev Genet 12: 385–400.