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ORIGINAL ARTICLE

Developmental competence and the induction of ectopic proboscises in *Drosophila melanogaster*

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Abstract Developmental competence is the response of a cell(s) to information. Determination of adult labial identity in *Drosophila* requires Proboscipedia (PB) and Sex combs reduced (SCR); however, co-ectopic expression of PB and SCR is not sufficient for induction of ectopic adult labial identity, because the developmental information supplied by PB and SCR is suppressed. The evolutionarily conserved LASCY, DYTQL, NANGE motifs, and the C-terminal domain of SCR are sequence elements that mediate some, or all, of the suppression of ectopic proboscis determination. Therefore, the developmentally competent primordial proboscis cells provide an environment devoid of suppression, allowing PB and SCR to determine proboscis identity. SCR derivatives lacking suppression sequences weakly induce ectopic proboscis transformations independently of PB, suggesting that SCR may be the activity required for induction of adult labial identity, as is the case for larval labial identity. A possible explanation for PB independence of SCR in determination of adult and embryonic labial identity is PB operates as a competence factor that switches SCR from determining T1 identity to labial identity during metamorphosis. Lastly, labial determination is not conserved between SCR and murine HOXA5, suggesting that SCR has acquired this activity during evolution.

Keywords *Hox* · Sex combs reduced · Proboscipedia · Conservation of Hox function · Segmental identity · Developmental competence · *Hoxa5*

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Introduction

The HOX transcription factors pattern the anterior–posterior axis of bilaterians by specifically regulating sets of genes required for differentiating segmental identity (Capovilla et al. 1994; Carroll 1995; Hueber et al. 2007). The *Drosophila* HOX protein Sex combs reduced (SCR) is required for the patterning of the labial and prothoracic segments of the larva and imago (Struhl 1982; Pattatucci et al. 1991; Pederson et al. 1996). In the prothorax (T1), SCR is required during embryogenesis for the formation of a full larval T1 beard and during morphogenesis for patterning first leg bristles, the male sex comb, and the prothoracic body wall (Lewis et al. 1980). In the embryonic labial segment, SCR is required for development of salivary glands and other larval head structures. Although expressed, Proboscipedia (PB) is dispensable for development of the embryonic labial segment (Pultz et al. 1988; Panzer et al. 1992; Pederson et al. 1996). During metamorphosis, SCR is required with PB for determination of proboscis identity (Percival-Smith et al. 1997). Ectopic expression of SCR during embryogenesis results in the formation of ectopic salivary glands and ectopic T1 beards on T2 and T3 (Gibson et al. 1990). Ectopic expression of SCR during larval development results in an arista to tarsus transformation, transformation of the head toward the thorax, loss of wings and ectopic sex combs on the second and third legs in males (Gibson et al. 1990; Zhao et al. 1993; Prud'homme et al. 2011).

The SCR protein is highly conserved across bilaterian phyla (Fig. 1a). In addition to the HOX5 class homeodomain (HD), the octapeptide, YPWM and KMAS motifs are universally conserved across bilaterian SCR homologs. The LASCY motif is conserved in protostome SCR homologs. The SCKY, PQDL, and NANGE motifs are conserved in arthropod SCR homologs. The DYTQL motif and C-terminal domain (CTD) are conserved in insect SCR homologs (Curtis et al. 2001). Hypomorphic *Scr* alleles with altered octapeptide, DYTQL, YPWM, HD, or CTD sequences indicate that these conserved

regions are required for SCR activity (Sivanantharajah and Percival-Smith 2009). In contrast, ectopic expression of SCR, HOXA5, and synthetic SCR all result in ectopic T1 beards, ectopic salivary glands, transformation of the head toward the thorax, and reduction of eyes and arista to tarsus transformations, suggesting that the YPWM, HD, and KMAS sequences constitute the central core of SCR activity while the octapeptide, DYTQL, NANGE, and CTD sequences provide "fine-tuning" of SCR activity (Gibson et al. 1990; Zhao et al. 1993; Papadopoulos et al. 2010).

In most bilaterian phyla, the HOX proteins are expressed in spatially restricted domains that overlap; however, in insects, HOX proteins are generally expressed in spatially restricted domains that show little overlap of expression with the exception of PB which overlaps the expression domains of Deformed (DFD) and SCR (Carroll 1995; Hughes and Kaufman 2002). Therefore, understanding the interaction between PB and SCR in proboscis determination may be generally applicable to other potential HOX–HOX interactions. The requirement of both PB and SCR for the determination of adult proboscis/labial palp identity is conserved in species of three insect orders: *Drosophila melanogaster*, *Tribolium casteneum*, and *Oncopeltus fasciatus* (Percival-Smith et al. 1997; Beeman et al. 1989; Hughes and Kaufman 2000). Genetic analysis of *pb* and *Scr* in *Drosophila* led to the proposal that a protein complex containing PB and SCR is required for determination of adult labial identity (Percival-Smith et al. 1997). However, this model has problems of which the most important is co-expression of PB and SCR in the maxillary palp and antenna not inducing ectopic proboscis transformations (Percival-Smith et al. 1997). We show here that the conserved LASCY, DYTQL, NANGE motifs, and CTD are targets of a mechanism(s) that suppresses SCR from inducing ectopic proboscises. In addition, PB may be a competence factor that aids SCR in proboscis determination. Finally, ectopic proboscis induction is not evolutionarily conserved in murine HOXA5.

Materials and methods

UAS constructs and *Drosophila* crosses

All constructs were created using standard PCR-based mutagenesis with high-fidelity DNA polymerases, and standard molecular cloning was used to fuse the *Scr* constructs behind the *UAS* promoter (Brand and Perrimon 1993; McPherson and Møller 2000). All constructs were sequenced to confirm the DNA sequence. P-element transformation was used to introduce *UAS* constructs into the germline (Rubin and Spradling 1982). The *Δocta* mutation deletes 1–16 of SCR; *Δocta, LASCY* mutation deletes 1–21 of SCR; *ΔLASCY* mutation deletes 18–21 of SCR; *ΔDYTQL* mutation includes a D111Q substitution and deletes 112–118 of SCR; *ΔNANGE* mutation replaces 316–319 of SCR with ala (ST**VNANGE** to AAAA**ANGE;** the residues in bold are the conserved motif); *ΔTS* mutation replaces 329 and 330 with ala (Berry and Gehring 2000); *ΔKMAS* mutation replaces 384–387 of SCR with ala (**KMASMN** to AA**A**A**M**N); *ΔKMAS, CTD* mutation is a stop codon at 384 of the *SCR* ORF; *ΔCTD* is a stop codon at 390 of the *SCR* ORF. Both the triple tag (TT: **DYKDHD** G**DYKDHD** I**DYKDDD** DKENKYFQSNW*SHPQFEK***HHHHHH** which contains three **FLAG** tags, a TEV cleavage site, a *strep* tag, and a **His** tag) and eBFP were fused after 417 of SCR (Tiefenbach et al. 2010). The HOXA5 and SCR fusion proteins were fusions of 1–324 of SCR to 195–270 of HOXA5 (SCR::HOXA5) and 1–194 of HOXA5 to 325–417 of SCR (HOXA5::SCR). The construction of PBGFP is described in Percival-Smith et al. (2005). PBTT and Fushi tarazu triple tag (FTZTT) are fusions of the TT to the C terminus of PB and FTZ expressed from a heat shock promoter (*hsp*) and *UAS* promoter, respectively (Thummel and Pirrotta 1992; Tiefenbach et al. 2010). All *Drosophila* genetics was performed with standard crosses. All experiments with ectopic expression of PBGFP were performed with the same insertion of *P{UASpbGFP*, w^+*}* on the fourth chromosome. For expression of SCR or SCR derivatives in a *pb* mutant background, *y w*; P{*UASScr*^{x}}; *pb*^{20}*/TM6B,* P{*wal*Ly} were crossed with *y w*; P{*pbGAL4*}, P{*UASlacZ*}*/CyO*; *pb*^{27}*/TM6B*, {P{*wal*Ly} and non-Hu, non-Cy, y and pb progeny were collected for phenotypic analysis.

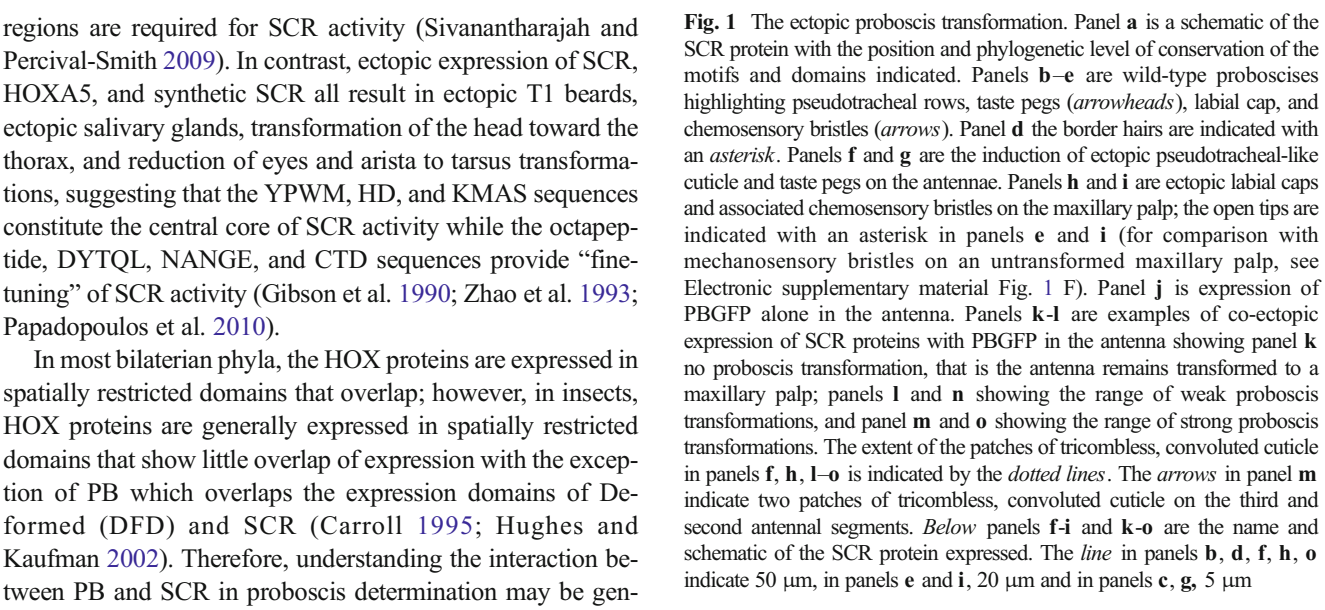
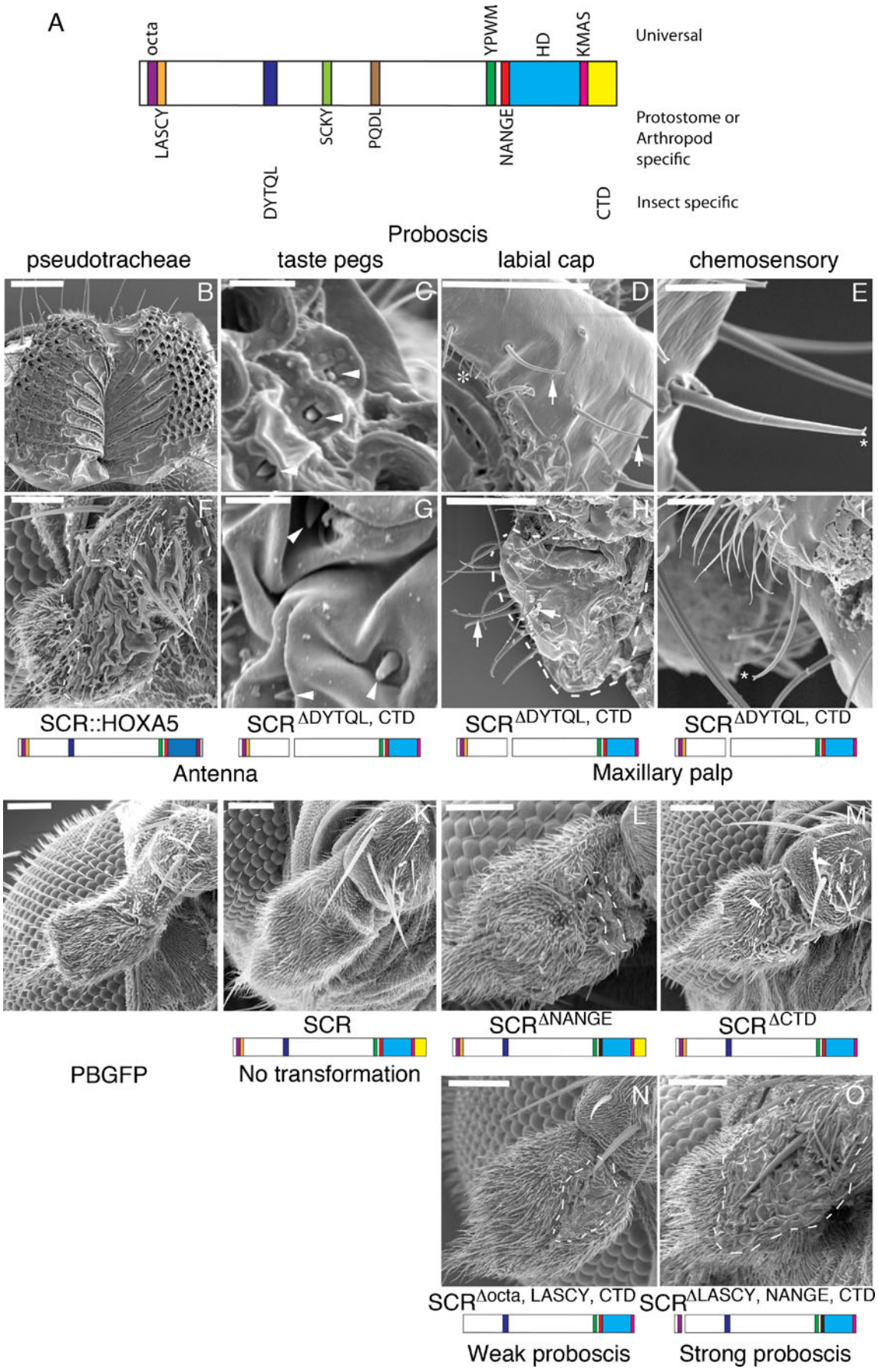


Fig. 1 The ectopic proboscis transformation. Panel **a** is a schematic of the SCR protein with the position and phylogenetic level of conservation of the motifs and domains indicated. Panels **b**–**e** are wild-type proboscises highlighting pseudotracheal rows, taste pegs (*arrowheads*), labial cap, and chemosensory bristles (*arrows*). Panel **d** the border hairs are indicated with an *asterisk*. Panels **f** and **g** are the induction of ectopic pseudotracheal-like cuticle and taste pegs on the antennae. Panels **h** and **i** are ectopic labial caps and associated chemosensory bristles on the maxillary palp; the open tips are indicated with an asterisk in panels **e** and **i** (for comparison with mechanosensory bristles on an untransformed maxillary palp, see Electronic supplementary material Fig. 1 F). Panel **j** is expression of PBGFP alone in the antenna. Panels **k**-**l** are examples of co-ectopic expression of SCR proteins with PBGFP in the antenna showing panel **k** no proboscis transformation, that is the antenna remains transformed to a maxillary palp; panels **l** and **n** showing the range of weak proboscis transformations, and panel **m** and **o** showing the range of strong proboscis transformations. The extent of the patches of tricombless, convoluted cuticle in panels **f**, **h**, **l**–**o** is indicated by the *dotted lines*. The *arrows* in panel **m** indicate two patches of tricombless, convoluted cuticle on the third and second antennal segments. *Below* panels **f**-**i** and **k**-**o** are the name and schematic of the SCR protein expressed. The *line* in panels **b**, **d**, **f**, **h**, **o** indicate 50 μm, in panels **e** and **i**, 20 μm and in panels **c**, **g**, 5 μm

Phenotypic analysis

Expression of PBGFP using *pbGAL4* is toxic; therefore, for most phenotypic analysis, pharate adults were dissected from the pupal cases. The adults and pharate adults were critical


A
octa
YPWM
HD
KMAS
Universal
LASCY
SCKY
PQDL
NANGE
Protostome or Arthropod specific
DYTQL
CTD
Insect specific
Proboscis
pseudotracheae
taste pegs
labial cap
chemosensory
B
C
D
E
F
G
H
I
SCR::HOXA5
SCR ΔDYTQL, CTD
SCR ΔDYTQL, CTD
SCR ΔDYTQL, CTD
Antenna
Maxillary palp
J
K
L
M
PBGFP
SCR
SCR ΔNANGE
SCR ΔCTD
No transformation
N
O
SCR Δocta, LASCY, CTD
SCR ΔLASCY, NANGE, CTD
Weak proboscis
Strong proboscis

point-dried and images collected with a scanning electron microscope. The legs of adults expressing SCR or SCR derivatives using the *rnGAL4* driver were dissected and mounted in Hoyer's mountant (Wieschaus and Nüsslein-Volhard 1986). The number of ectopic sex comb bristles on the second leg was counted using bright field optics. First instar larva expressing SCR or SCR derivatives using the *armGAL4* driver were mounted in Hoyer's. The number of ectopic T1 beard setae in T2 of the larval cuticle was counted using dark field optics. Embryos that expressed a SCR molecule using the *armGAL4* driver were fixed, and ectopic salivary gland formation was assessed with the rabbit anti-CREB antibody (Andrew et al. 1997). Induction of ectopic tarsi was performed with the *dppGAL4* driver.

Coimmunoprecipitation and Western analysis

For analysis of expression of SCR and SCR derivatives, *y w*, P{*UASScr*^{x}} and *y w*; P{*armGAL4*} flies were crossed, and protein was extracted from progeny embryos collected between 0 and 12 h after egg laying (AEL). For coimmunoprecipitation of SCR with PBTT, protein was extracted from embryos collected between 3 and 15 h AEL that had been heat-shocked at 37 °C for 30 min and allowed to recover for 10 min with the genotypes: *y w;* P{*armGAL4*}/+; P{ *hsp pbTT* }/+, *y w;* P{*UASScr*}/P{*armGAL4*}; P{*hsp pbTT* }/+ or *y w;* P{*UASScr*}/P{*armGAL4*}. Anti-FLAG agarose beads were added to immunoprecipitate the PBTT protein. On the Western analyses, SCR was detected with a mouse monoclonal anti-SCR antibody. Tubulin was detected with a mouse monoclonal anti-tubulin antibody, and PBTT was detected with a mouse monoclonal anti-FLAG antibody (Glicksman and Brower 1988).

Results

Transformation of the antennae and maxillary palps toward proboscis identity

In this study, proteins were ectopically expressed using *UAS* constructs and the GAL4 driver *pbGAL4*. The *pbGAL4* driver is expressed in the larval labial imaginal discs as well as in a ring of cells in the arista primordia of the eye antennal disc. During metamorphosis, *pbGAL4* is expressed in the maxillary palps and proboscis primordium as well as throughout the antenna (Benassayag et al. 2003; Percival-Smith unpublished results). Ectopic expression of PBGFP alone with *pbGAL4* resulted in an antenna toward maxillary palp transformation as previously described for ectopic expression of PB (Fig. 1j) (Cribbs et al. 1995). The observation that initiated this study was that co-ectopic expression of PBGFP with SCRBFP using the *pbGAL4* driver resulted in a proboscis transformation of the antenna but that co-ectopic expression of PBGFP with SCR did not. Before presenting the results of the investigation of the root of this difference, the phenotype of the ectopic proboscises is presented.

The distiproboscis has a number of landmarks (Kumar et al. 1979). The distiproboscis is composed of two major, tricombless elements: the labial palps and the labial caps, which are separated by a single row of tricombs called the border hairs (Fig. 1b–d). A labial palp is composed of six pseudotracheal rows, which are composed of two structurally distinct elements along the mouth proximal distal axis. The distal element is composed of tricombless, highly convoluted cuticle. The area between the rows distal to the mouth has tricombless, convoluted cuticle surrounding the taste pegs (Fig. 1c). Taste pegs are flattened, triangular (when viewed from the top) sensilla basiconica and are distinct from sensilla basiconica found on the antenna and maxillary palp, which are cylindrical and rounded at the tip (Electronic supplementary material Fig.1 A, D, G). In addition, taste pegs are sunk into the tricombless cuticle such that only the tip of the sensilla is exposed. The second, proximal pseudotracheal element has a zipper-like structure, and the rows are separated by less convoluted, tricombless cuticle lacking taste pegs (Fig. 1b). The labial cap is also tricombless, but the cuticle is not convoluted and has chemosensory bristles. Short, intermediate, and long chemosensory bristles are organized in a stereotypic manner on the labial cap (Shanbhag et al. 2001). Unlike many mechanosensory bristles that are fluted with ridges and valleys (Electronic supplementary material Fig. 1 F), these bristles are smooth, curved like a scythe and open at the tip (Fig. 1e).

When SCR was co-ectopically expressed with PBGFP, no transformation of the antenna to proboscis was observed (Fig. 1k). When some SCR derivatives, such as SCR::HOXA5, were co-ectopically expressed with PBGFP, we observed patches of convoluted, tricombless cuticle on the antennal segments suggesting a pseudotracheal transformation (Fig. 1f). Some of the convolutions form structures similar to those found in the distal element of the pseudotracheal row; a perfect pseudotracheal row containing the two elements was not observed, and no labial cap transformation or definitive row of border hairs was observed in the transformed antennae. The extent of this pseudotracheal transformation varied with the SCR protein co-ectopically expressed with PBGFP, and the strength of this transformation was qualitatively assigned as no transformation (Fig. 1k), as weak if only a scar or small patch of convoluted, tricombless cuticle on the third antennal segment were observed (Fig. 1l and n), or as strong if patches of convoluted, tricombless cuticle on the second and third segments or a continuous patch of convoluted, tricombless cuticle across both the second and third antennal segments were observed (Fig. 1m and o). In addition, taste peg like sensilla basiconia surrounded by convoluted, tricombless cuticle were observed in antenna toward proboscis transformations (Fig. 1g).

Table 1 Phenotypes of SCR proteins

	Ectopic proboscis						
Protein	None	Weak	Strong	Sex combs on 2nd leg	Ectopic T1 setae on T2	Arista to tarsus	Salivary gland
SCR	4[a]			16±2[b] (5) [c]	146±19[b] (4) [c]	+	+
SCRTT	3	1		*23±3* (1)	3.7±1.2 (2)	+	ND[d]
SCRBFP			2	0±0 (2)	*98±5* (1)	+	ND
Single							
$SCR^{\Delta octa}$	2			*24±8* (1)	*40±7* (1)	+	+
$SCR^{\Delta LASCY}$	1	2		12±1 (3)	74±20 (3)	+	ND
$SCR^{\Delta DYTQL}$	1	2		10±4 (4)	137±22 (3)	+	+
$SCR^{\Delta NANGE}$		4		17±4 (2)	91±1 (3)	+	+
$SCR^{\Delta TS}$	2	3		10±2 (5)	20±11 (2)	+	ND
$SCR^{\Delta KMAS}$	1	1		9±3 (2)	98±11 (2)	+	ND
$SCR^{\Delta CTD}$			5	0.3±0.3 (2)	35±17 (3)	+	+
Double							
$SCR^{\Delta octa,\ CTD}$	1	1		*0±0* (1)	31±13 (2)	+	+
$SCR^{\Delta LASCY,\ DYTQL}$			2	17±3 (3)	119±7 (3)	+	+
$SCR^{\Delta DYTQL,\ CTD}$			2	0±0 (2)	55±27 (2)	+	ND
$SCR^{\Delta KMAS,\ CTD}$	3			0±0 (2)	*83±6* (1)	+	ND
Triple							
$SCR^{\Delta octa,\ LASCY,\ CTD}$		3	1	*0±0* (1)	99±16 (5)	+	+
$SCR^{\Delta octa,\ DYTQL,\ CTD}$	1	1	1	*0±0* (1)	102±36 (2)	+	+
$SCR^{\Delta LASCY,\ DYTQL,\ CTD}$		3	1	*0±0* (1)	95±17 (4)	+	+
$SCR^{\Delta LASCY,\ NANGE,\ CTD}$			2	0±0 (3)	50±4 (3)	+	+
$SCR^{\Delta DYTQL,\ TS,\ CTD}$	4			0±0 (2)	83±40 (2)	+	ND
$SCR^{\Delta DYTQL,\ KMAS,\ CTD}$			2	0±0 (2)	14±9 (2)	+	+
Quadruple							
$SCR^{\Delta octa,\ LASCY,\ DYTQL,\ CTD}$	3			0±0 (3)	79±20 (3)	+	+
$SCR^{\Delta LASCY,\ DYTQL,\ TS,\ CTD}$	2	1	1	0±0 (3)	95±8 (2)	+	ND
$SCR^{\Delta DYTQL,\ NANGE,\ TS,\ CTD}$		2		*0±0* (1)	27±1 (2)	+	ND
Quintuple							
$SCR^{\Delta LASCY,\ DYTQL,\ NANGE,\ TS,\ CTD}$	3	1		0±0 (2)	20±20 (2)	+	ND
SCR HOXA5 fusions							
HOXA5	3			0±0 (3)	67±29 (2)	+	ND
SCR::HOXA5			3	0±0 (3)	46±13 (3)	+	ND
HOXA5::SCR	3			2±1 (3)	8±1 (2)	+	ND

[a] Number of independent transformants exhibiting the phenotype

[b] Average ± SEM for multiple independent transformants and in italics *Average ± SEM* within a single transformant line

[c] Number of independent transformants examined (*n*)

[d] Not determined

When some SCR derivatives were ectopically expressed alone or co-ectopically expressed with PBGFP in the maxillary palps, tricombless, convoluted cuticle was observed (Fig. 1g). In addition, non-convoluted, tricombless cuticle that had curved, scythe-like, non-fluted, open-tipped chemosensory bristles were observed (Fig. 1g and h), which were not observed on wild-type maxillary palps (Electronic supplementary material Fig. 1 F). No definitive row of border hairs was observed. The antennae and maxillary palps in which some SCR derivatives and PBGFP were co-expressed are transformed toward proboscis identity.

Motifs of SCR required for suppression of ectopic proboscis formation

The following mutant analysis suggests that SCR contains peptide sequence information required for suppression of ectopic proboscis formation (Table 1; Electronic supplementary material Fig. 2). Analysis of the *ΔCTD* and *ΔNANGE* showed that the CTD and the NANGE motif were required in the suppression of SCR activity. The strongest proboscis transformation of the antenna for the single mutants was observed with deletion of

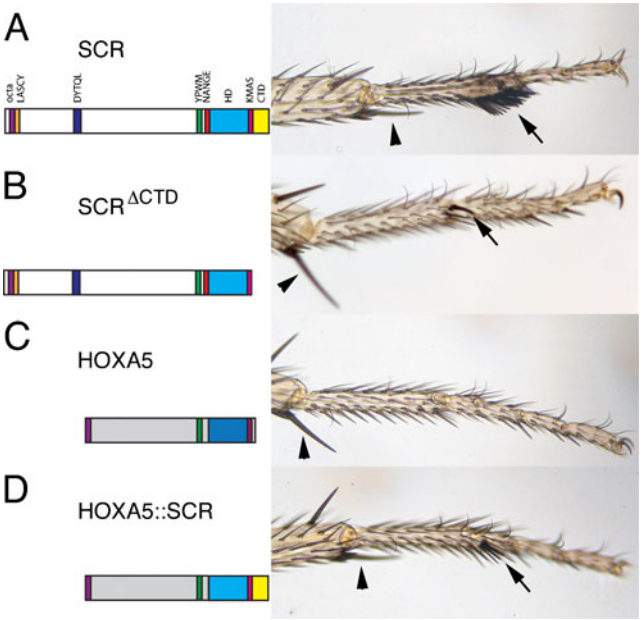


Fig. 2 CTD is partially sufficient for induction of ectopic sex combs on the second leg. The proteins expressed with the *rnGAL4* driver are indicated on the *left* the second leg phenotypes are on the *right*. The tarsi in panels **a** and **d** are composed of three segments rather than five. The *arrows* indicate ectopic sex comb bristles, and the *arrowheads* indicate the second leg apical bristle

the CTD and fusion of the Blue fluorescence protein (BFP) just C-terminal to the CTD (Table 1; Fig. 1m; Electronic supplementary material Fig. 2). In addition, the fusion protein containing the N terminus of SCR and the HOXA5 HD+KMAS motif (SCR::HOXA5), which differs from $SCR^{\Delta CTD}$ by six residues in the HD and three of six residues in the KMAS motif also results in a strong transformation (Fig. 1f). Four out of four independent *UASScr* transformants resulted in no transformation, and four out of four *UASScr*$^{\Delta NANGE}$ transformants resulted in weak proboscis transformations, which is significant ($p<0.05$) using the Fisher's exact test ($p=0.03$) (Fisher 1922). The *ΔNANGE* mutation does not completely remove the NANGE sequence, which may explain the weak transformation. The weak transformations observed for *ΔLASCY*, *ΔDYTQL*, *ΔTS,* and *ΔKMAS* are not significant ($p>0.05$). Two out of three independent transformants expressing $SCR^{\Delta LASCY}$ or $SCR^{\Delta DYTQL}$ resulted in a weak proboscis transformation ($p=0.14$); however, the double-mutant $SCR^{\Delta LASCY,\ DYTQL}$ resulted in strong proboscis transformations suggesting that the LASCY and DYTQL motifs may be additive for suppression of ectopic proboscis formation. One of two $SCR^{\Delta KMAS}$ and three of five $SCR^{\Delta TS}$ expressing lines resulted in weak proboscis transformations ($p=0.33$ and $p=0.17$, respectively). Multiple mutants did not suggest an additive role for TS and KMAS with other motifs, as $SCR^{\Delta KMAS,\ CTD}$ and $SCR^{\Delta DYTQL,\ TS,\ CTD}$ resulted in no transformations. We conclude that LASCY, DYTQL, NANGE, and CTD are required for suppression of SCR activity in ectopic proboscis induction and that we also cannot rule out TS of the HD and KMAS having a role.

To determine whether the suppression of ectopic proboscis formation by these SCR peptide elements was additive with the aim of potentially observing a complete antenna to proboscis transformation, a select number of double up to quintuple mutant combinations were co-expressed with PBGFP. In general, SCR derivatives with double and triple combinations of ΔLASCY, ΔDYTQL, ΔNANGE, and ΔCTD resulted in strong proboscis transformations, but we never observed a complete antenna to proboscis transformation. In some cases, as the number of mutations increased, the strength of the transformation decreased.

The CTD is important and partially sufficient for ectopic sex comb formation

Ectopic expression of SCR with the *rotund GAL4* (*P{GawB}*) driver resulted in a strong induction of ectopic sex combs on both the second and third legs, but ectopic expression of murine HOXA5 from the *UAS* promoter did not induce ectopic sex comb formation (Fig. 2a and c) (Table 1). Deletion of the octapeptide, LASCY, DYTQL, NANGE, TS at position 7, 8 of the HD, and KMAS motifs did not drastically affect the number of ectopic sex combs that formed (Table 1). The deletion of the CTD resulted in a low number of ectopic sex combs (Fig. 2b). The fusion of the N terminus of HOXA5 to the SCR HD, KMAS, and CTD was able to induce a low number of ectopic sex combs, suggesting that the CTD may have an important and partially sufficient role in ectopic sex comb formation (Fig. 2d).

Induction of ectopic T1 beards, tarsi, and salivary glands

Induction of ectopic T1 beards, tarsi, and salivary glands is conserved among SCR homologs (Zhao et al. 1993). Ectopic expression of all SCR derivatives, murine HOXA5, and SCR HOXA5 fusion proteins resulted in ectopic T1 beards and ectopic tarsi (Table 1; Fig 3a–f). In addition, with all SCR derivatives tested, ectopic salivary glands were observed (Table 1). These data suggest that the proteins tested in Table 1 are expressed and appropriately localized. Western analysis of SCR derivatives expressed during embryogenesis that contained the anti-SCR epitope in the DYTQL motif (Glicksman and Brower 1988; Sivanantharajah and Percival-Smith 2009) showed that these proteins were expressed (Fig. 3g). These data collectively suggest that loss of induction of ectopic proboscises and sex combs is due to loss of protein activity and not loss of protein accumulation.

PB-independent proboscis induction

The antennae of flies raised at room temperature expressing $SCR^{\Delta LASCY,\ CTD}$, $SCR^{\Delta octa,\ LASCY,\ CTD}$, $SCR^{\Delta LASCY,\ DYTQL,\ CTD}$, or $SCR^{\Delta DYTQL,\ CTD}$ using *pbGAL4* were not wild-type but

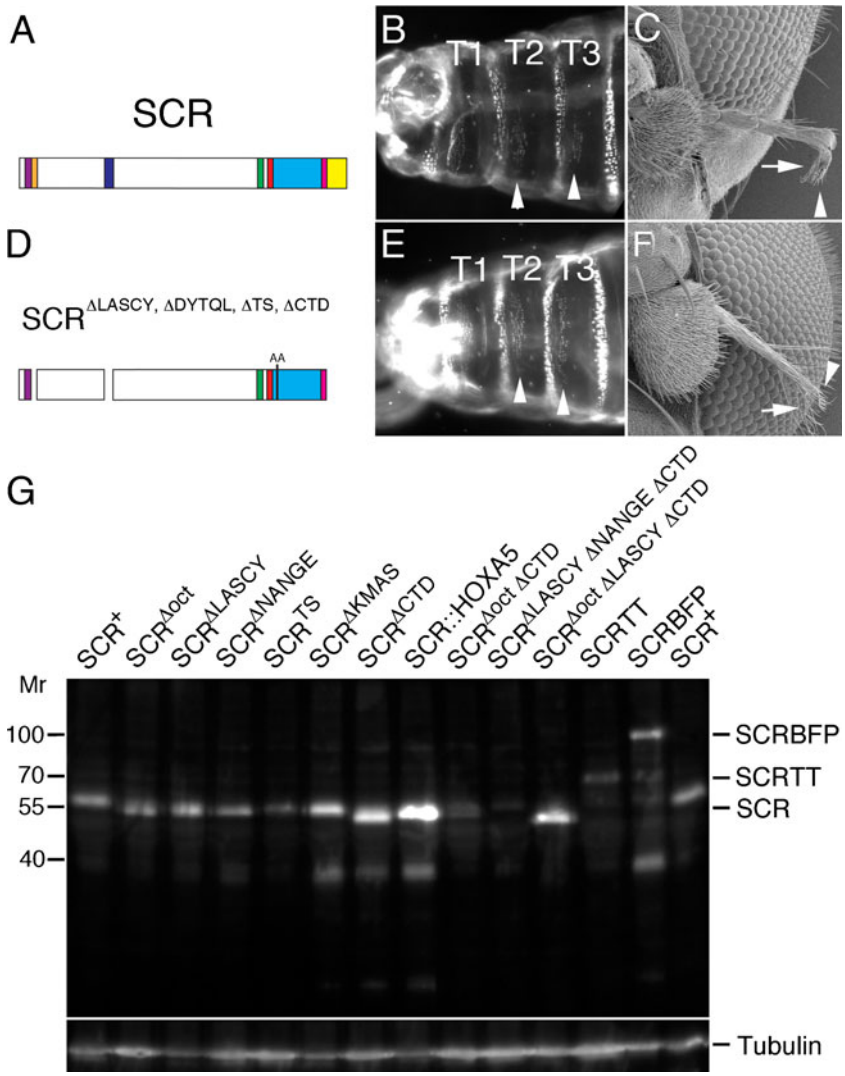


Fig. 3 Phenotypes and expression of SCR proteins. Panels **a** and **d** are schematics of the proteins ectopically expressed with the *armGAL4* driver during embryogenesis **b**, **e** and with the *dppGAL4* driver in the imaginal discs **c**, **f**. Panels **b** and **e** are first instar larval cuticles, and the ectopic T1 beards in T2 and T3 are indicated with *arrowheads*. Panels **c** and **f** are arista to tarsus transformations with the pulvilli and claws indicated by *arrowheads* and *arrows*, respectively. Panel **g** Western analysis. The SCR protein expressed with the *armGAL4* driver during the first 12 h of embryogenesis is *above* the lane. The *top* is SCR detected with a mouse monoclonal antibody, and the *bottom* is the same blot where Tubulin is detected using a mouse monoclonal antibody. The M_r for the markers is indicated on the *left* and the relative positions of SCR proteins indicated on the *right*

did not exhibit an obvious transformation to proboscis identity. To increase the activity of GAL4 expressed from *pbGAL4*, the flies were raised at 28.5 °C (Haerry et al. 1998). The antennae of flies expressing $SCR^{\Delta LASCY,\ CTD}$, $SCR^{\Delta octa,\ LASCY,\ CTD}$, $SCR^{\Delta LASCY,\ DYTQL,\ CTD}$, or $SCR^{\Delta DYTQL,\ CTD}$ but not SCR had patches of tricombless, convoluted cuticle like that shown in Fig. 4b and h. To rule out the possibility that ectopic expression of SCR was activating the expression of endogenous *pb* locus in the antenna, we performed the experiment in a pb^{27}/pb^{20} null mutant background (Randazzo et al. 1991). Ectopic expression of $SCR^{\Delta DYTQL,\ CTD}$ and $SCR^{\Delta LASCY,\ DYTQL}$ resulted in deformed antennae with patches of tricombless, convoluted cuticle whereas ectopic expression of wild-type SCR resulted in a normal antenna (Fig. 4a, b, and h). As a control to test if 28.5 °C affected the transformation, $SCR^{\Delta DYTQL,\ CTD}$ was co-ectopically expressed with PBGFP at 28.5 °C with a resulting strong proboscis transformation similar to that observed at room temperature (Fig. 4c). The maxillary palps of *pb* mutants are reduced to vestigial stumps (a wild-type maxillary palp is shown in Electronic supplementary material Fig. 1 E). The vestigial maxillary palp pb phenotype was not affected by expression of SCR (Fig. 4d and g). However, the expression of $SCR^{\Delta DYTQL,\ CTD}$ and $SCR^{\Delta LASCY,\ DYTQL}$ induced patches of tricombless, convoluted cuticle (Fig. 4e and i). No evidence of a labial cap and chemosensory bristles was observed in the patches of tricombless cuticle as was found with co-ectopic expression of $SCR^{\Delta DYTQL,\ CTD}$ and PBGFP in pb^{+} individuals (Fig. 4f). The induction of tricombless, convoluted cuticle on the antenna and maxillary palp indicates that the SCR derivatives lacking elements that suppress ectopic proboscis formation have a weak, PB-independent, ectopic proboscis induction activity.

SCR does not co-immunoprecipitate with PB

No direct biochemical interaction between PB and SCR was detected in two independent assays (Tayyab et al. 2004). In the genetic model for determination of proboscis identity, hypothetical intermediary factors were proposed to mediate a

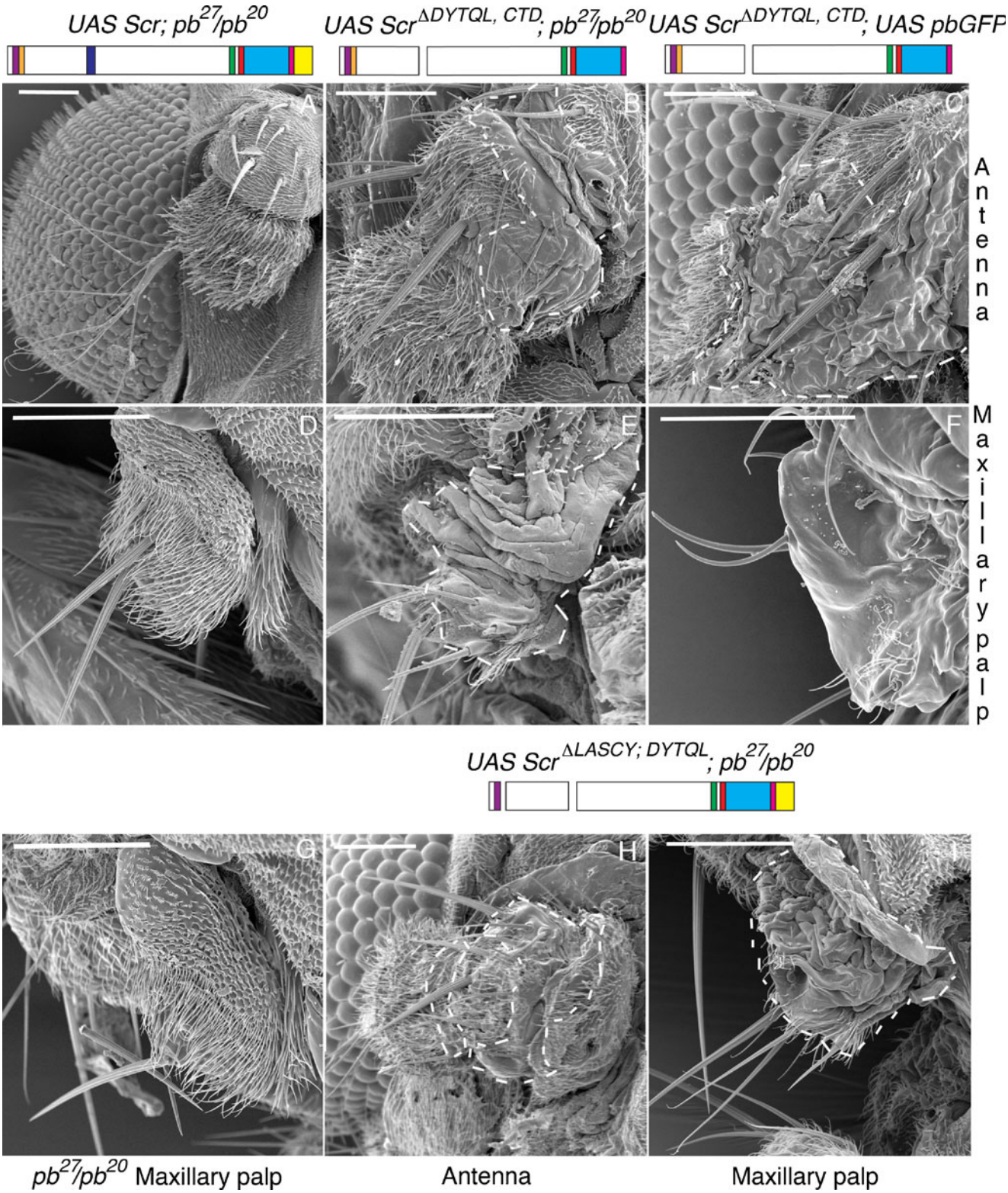


Fig. 4 PB-independent ectopic proboscis transformation. Panels **a**–**c** are the antenna and **d**–**f** are the maxillary palp of *pb* mutants expressing SCR (**a**, **d**), $SCR^{\Delta DYTQL,\ CTD}$ (**b**, **e**), and pb^{+} expressing both $SCR^{\Delta DYTQL,\ CTD}$ and PBGFP at 28.5 C (**c**, **f**). Panel **g** is a vestigial maxillary palp of a *pb* mutant. Panels **h** and **i** are antennae and maxillary palp of *pb* mutants expressing $SCR^{\Delta LASCY,\ DYTQL}$. Schematics of the proteins expressed are shown *below* the name. The *dotted lines* surround tricombless, convoluted cuticle. The *white bars* indicate 50 μm

PB SCR indirect interaction to explain unobserved ectopic proboscis transformations (Percival-Smith et al. 1997). Ectopic expression of PB between 5 and 9 h AEL inhibits SCR in ectopic beard formation; therefore, if the PB SCR

interaction is mediated by intermediary factors, they should be present in protein extracts of embryos between 3 and 15 h AEL of age. PBTT was expressed in all cells from a heat-shock promoter, and SCR was expressed in all cells from the *UAS* promoter using the *armGAL4* driver. PBTT was immunoprecipitated with anti-FLAG agarose. Using embryo extract that expresses both PBTT and SCR and potentially the hypothetical intermediary factors, no co-immunoprecipitation of SCR was observed indicating that PB and SCR do not interact strongly or at all (Fig. 5, lane 5).

Reduction of wings induced by the ectopic expression of HOX proteins

SCR is proposed to specifically suppress wing formation. The suppression of wing formation on T2 by ectopic expression of SCR with the *rnGAL4* driver has been used as an assay of evolutionary conservation of SCR activity (Prud'homme et al. 2011), despite the observation that loss of SCR activity in *Drosophila* does not result in ectopic wing formation on the prothorax (Struhl 1982; Lewis et al. 1980; Percival-Smith et al. 1997; Sivanantharajah and Percival-Smith 2009). In addition, Antennapedia (ANTP) is proposed not to suppress wing formation (Carroll et al. 1995). To test whether induction of ectopic sex combs and suppression of wing formation are specific to SCR, we expressed all HOX proteins with *rnGAL4*. Induction of ectopic sex combs was observed with SCR only (Figs. 2a and 6f). Suppression of wing formation was observed with the ectopic expression of all eight HOX proteins (Fig. 6c–f, h–k). The halteres (Fig. 6) and tarsi (Fig. 2a) were also malformed. Fushi tarazu (FTZTT), a pair-rule protein proposed to have evolved from a HOX ancestor, was found also to suppress wing formation (Fig. 6g) (Lohr and Pick 2005). As controls, ectopic expression of GFP had no effect on wing formation (Fig. 6b) and ectopic expression of Squeeze (SQZ), which is a LIM HD containing transcription factor, resulted in nicks along the wing margin (Fig. 6l) (Allan et al. 2003). These results show that suppression of wing formation by ectopic expression of HOX proteins using *rnGAL4* is non-specific.

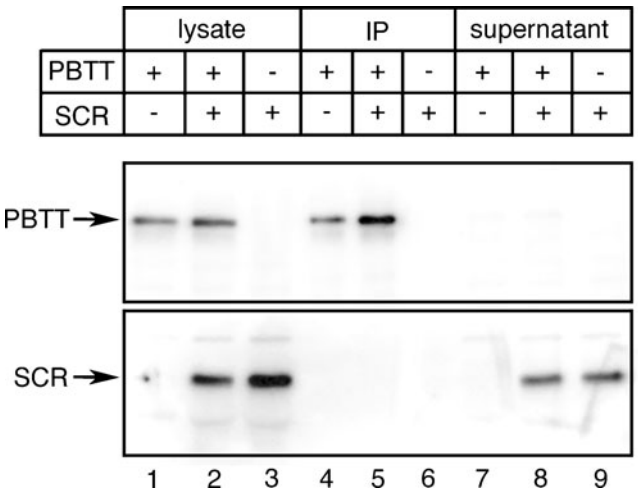


Fig. 5 Immunoprecipitation of PBTT does not co-precipitate SCR. The compositions of the three embryonic protein extracts expressing PBTT and/or SCR are indicated *above* the Western blots. Panel **a** is PBTT detected with mouse anti-FLAG, and panel **b** is SCR detected with mouse anti-SCR. *Lane numbers* are indicated along the *bottom* of the two blots

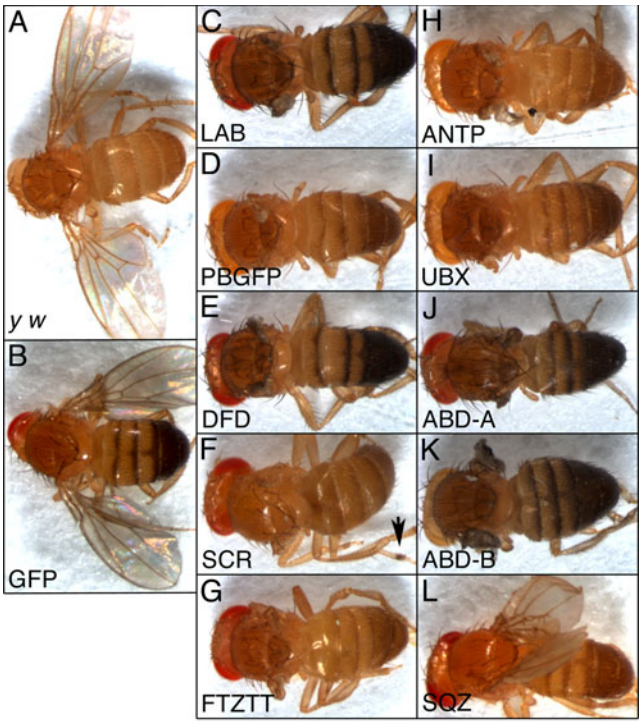


Fig. 6 Common Hox wingless phenotype. Panel **a** is a *y w* fly. In panels **b**–**l**, the protein expression using the *rnGAL4* driver is indicated in the *bottom left* of each panel. The *arrow* in panel **f** indicates ectopic sex combs on the second leg

Discussion

Developmental competence

Developmental competence is the response of cells to information, which can be the presence or absence of any type of molecule ranging from secreted ligands to transcription factors. Two distinct mechanisms of developmental competence are dependent on the cell either having the mechanism to respond to/receive the information or the cell lacking the mechanism that suppresses and cloaks the information. In the latter mechanism, it is possible to bypass the mechanism of suppression by removing elements from the information that mediate the mechanism of suppression, thereby allowing this modified information to act in cells not normally competent to respond. We have shown that the LASCY, DYTQL, NANGE motifs, and the CTD of SCR mediate suppression of induction of ectopic proboscis identity, which would explain why co-expression of PB and SCR is

unable to induce ectopic proboscises. The TS of the HD and KMAS motif may also have (cannot be ruled out from having) a role in suppression of SCR activity. The list of suppression elements may be incomplete because the conserved SCKY, PQDL, and YPWM motifs, non-conserved regions of SCR, and PB were not analyzed. Suppression of ectopic proboscis formation suggests that the primordial cells of the proboscis must provide a special environment, devoid of mechanism(s) of suppression to allow PB and SCR to direct proboscis identity. The cells of the first leg may share this environment because small patches of labial palp identity are observed with co-expression of PB and SCR in the first leg (Aplin and Kaufman 1997). The requirement of cells being developmentally competent to respond to PB and SCR information means that PB and SCR are not sufficient to instruct proboscis identity. This type of HOX insufficiency is observed in phenotypic suppression. Expression of ANTP and Ultrabithorax (UBX) in the same cell results in an Ubx gain-of-function phenotype; the phenotype induced by ANTP is suppressed by UBX, and therefore, ANTP information is not sufficient (Gonzalez-Reyes et al. 1990). SCR is also subject to phenotypic suppression. Teashirt (TSH) suppresses SCR function for abdominal ectopic salivary gland formation, and the Bithorax complex proteins (BX) proteins suppress abdominal ectopic T1 beard formation (Andrew et al. 1994). Suppression of SCR in induction of ectopic proboscises is mediated by the LASCY, DYTQL, NANGE motifs, and CTD, but the ectopic expression phenotype of HOXA5 is the same as SCR suggesting that these sequences, which are absent in HOXA5, do not mediate TSH and BX suppression of SCR activity (Zhao et al. 1993).

The HOX proteins seem to exhibit great specificity in the developmental pathways that they control but do not show great specificity in the DNA sequences which the DNA binding HD recognizes. The common explanation for specificity of HOX proteins is that, by binding the co-factor EXD, HOX proteins do have specific DNA binding properties (Gehring et al. 1994; Chan et al. 1994; Joshi et al. 2007; Slattery et al. 2011). However, EXD is not nuclear in all cells, and a number of EXD-independent processes have been described that includes adult labial identity and sex comb formation (Aspland and White 1997; Percival-Smith and Hayden 1998; Casares and Mann 2000; Galant et al. 2002; Joulia et al. 2006). In addition, as might be expected from HOX HDs having a common DNA binding specificity, there are a number of phenotypes shared among HOX proteins. All *Drosophila* HOX proteins with the exception of PB induce ectopic tarsi (Percival-Smith et al. 2005). PB suppresses ectopic tarsi induced by DFD, SCR, ANTP, UBX, Abdominal-A (ABD-A), and Abdominal-B (ABD-B). SCR, ANTP, UBX, ABD-A, and ABD-B reduce the eye and interact with Eyeless via the HD (Plaza et al. 2001). We show that ectopic expression of *Drosophila* HOX proteins and FTZTT results in a common wingless phenotype. These common phenotypes may be the result of the expression of HOX proteins in tissues that lack mechanisms for HOX suppression allowing HOX proteins to function as unrestricted, non-specific HD transcription factors. Therefore, the HOX specificity problem, rather than being just a positive mechanism of recruiting HOX factors to regulatory regions with co-factors, may also be a negative mechanism where the HOX information is cloaked and unable to act (Slattery et al. 2011; Sorge et al. 2012). Patterns of specific suppression of HOX activities and cell type-specific transcription factors could also give rise to differential patterns of gene expression (Hueber et al. 2007).

Evolutionary conservation of SCR function

The conservation of HOX function across bilaterian species is well documented (Zhao et al. 1993; Carroll 1995; Percival-Smith and Laing Bondy 1999). The conservation of HOX amino acid sequence between species shows multiple levels of conservation of specific motifs from phyla- to class-specific (Curtis et al. 2001). The sequence motifs outside the YPWM motif and HD confer novel activities to HOX proteins. For example, the gain of the FTZ-F1 binding site and loss of the YPWM motif results in a gain of segmentation and loss of homeotic function in FTZ (Lohr and Pick 2005). The insect-specific QA motif of UBX confers repression of limb formation in ectopic expression experiments, although loss of the QA motif at the endogenous *Ubx* locus does not result in the loss of repression of limb formation (Galant and Carroll 2002; Ronshaugen et al. 2002; Hittinger et al. 2005). Although the YPWM, KMAS motifs, and HD of SCR homologs, which constitute 18 % of SCR sequence, may be the core sequences required for induction of ectopic tarsi, ectopic larval T1 beards, and ectopic salivary glands (Zhao et al. 1996; Papadopoulos et al. 2010), the other conserved motifs and CTD also constitute 18 % of SCR sequence and have also been maintained by purifying selection. These sequences may have been maintained for their role in suppressing ectopic proboscis formation; therefore, conserved sequence motifs outside the HD and YPWM motif in other HOX proteins may also serve similar roles in suppression of activity (Tour et al. 2005; Papadopoulos et al. 2011; Merabet et al. 2011).

The deletion and fusion analysis suggests that the CTD is important and partially sufficient for induction of ectopic sex combs. The Scr^{6} hypomorphic allele supports the importance of the CTD and the hypomorphic Scr^{5}, Scr^{8} and Scr^{15} alleles suggest sequences outside to the CTD are also important for sex comb formation (Sivanantharajah and Percival-Smith 2009). Although no ectopic sex comb bristles were observed with expression of HOXA5 using the *UAS* promoter, ectopic sex comb bristles were observed with expression of HOXA5 from a heat-shock promoter and may suggest that the partial sufficiency of the CTD may represent an accentuation of a conserved sex comb function of HOXA5 (Zhao et al. 1993). Just like the sequences required for suppression, the sequences

required for sex comb formation are dispersed throughout the SCR protein sequence; therefore, with the exception of the HD, SCR is not composed of a set of modular individual domains that are sufficient for a particular function, but a set of sequences that make small and differential contributions to overall SCR activity, a phenomena termed differential pleiotrophy that is also observed in UBX and ABD-A (Tour et al. 2005; Hittinger et al. 2005; Merabet et al. 2011). Although these sequences may be binding sites for a signal, they also may be required indirectly to maintain the structure of another functional portion of the protein.

Analysis of induction of ectopic proboscises suggests that the LASCY, DYTQL, NANGE motifs, and CTD have a role in mediating negative regulation of SCR activity, but combining mutations to strengthen the ectopic proboscis phenotype was not successful at inducing a full proboscis transformation suggesting that, in addition to mediating negative regulation, these motifs may also be required for promoting labial palp identity. The hypomorphic alleles Scr^{15} and Scr^{6} that delete the DYTQL motif and change residues in the CTD, respectively, form fewer pseudotracheal rows supporting a role for these sequences in promoting labial palp identity (Sivanantharajah and Percival-Smith 2009).

The PB SCR interaction

The overlap of expression and interaction of PB and SCR may provide an insect example of what occurs when HOX protein expression domain overlap in other phylogenetic groups. The model that PB and SCR interact in a protein complex to determine labial identity is likely incorrect (Percival-Smith et al. 1997). The model nicely explains how when PB and SCR are co-expressed that neither maxillary palp nor tarsus identity is determined, due to both proteins being pulled into a complex with novel activity thereby inhibiting induction of maxillary palp and tarsus identity, respectively. The inability to detect a stable protein complex containing both PB and SCR and the lack of specificity of the inhibition of SCR by PB are not well accommodated by the model (Percival-Smith et al. 2005). Although the lack of co-immunoprecipitation does not rule out the existence of a PB SCR containing protein complex, it suggests that an interaction between the two is not

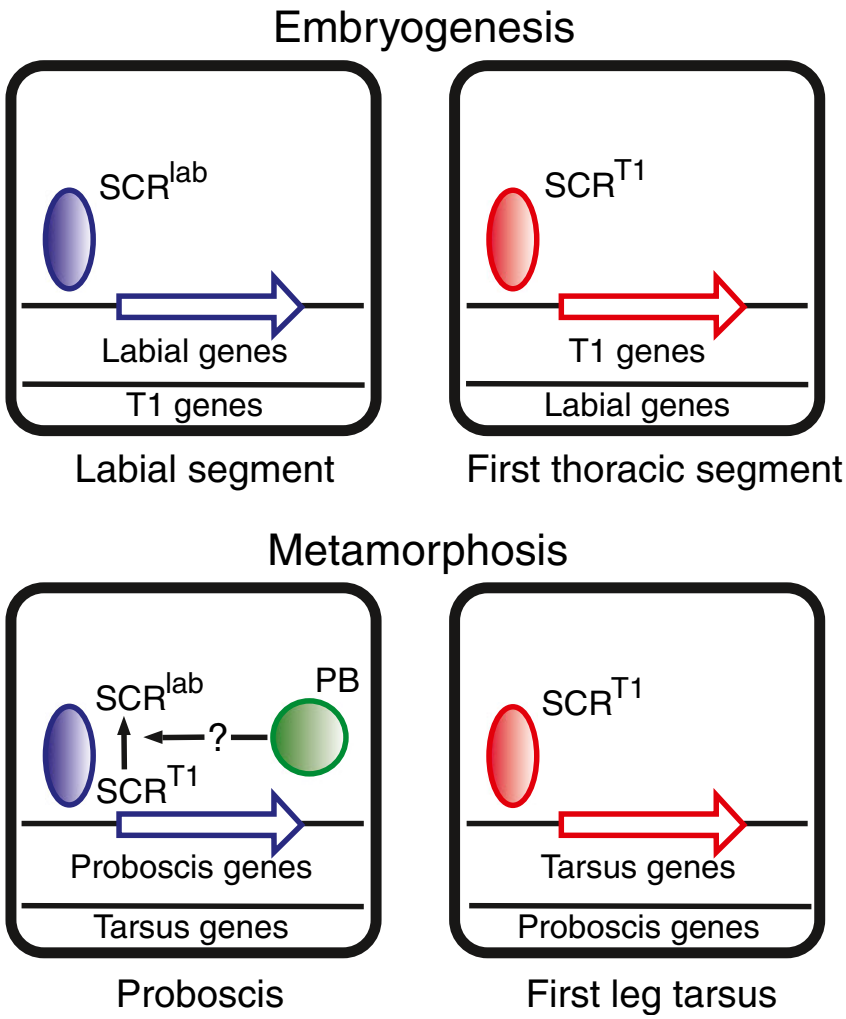


Fig. 7 Model for the roles of PB and SCR in determination of larval and adult labial and T1 segmental identity. The *rounded boxes* indicate cell nuclei, SCR^{T1} is indicated as a *red ellipse* bound to DNA, SCR^{lab} is indicated as a *blue ellipse* bound to DNA, and the *arrows* indicate some form of regulation of gene transcription (negative and/or positive) associated with determination of labial or T1 identity; no arrow, no effect on transcription. PB is indicated as a *green circle*

stable. The most important problem for the model is the weak PB-independent labial transformations observed with some SCR derivatives suggesting that SCR is the factor required for determining labial identity. This sole requirement of SCR during metamorphosis would explain why, although PB is expressed in the labial and maxillary segment during embryogenesis, determination of larval labial identity requires only SCR (Pultz et al. 1988; Pederson et al. 1996). Our present working model for adult proboscis determination is that proboscis primordial cells provide an environment devoid of most or all mechanisms that suppress the labial SCR activity (SCR^{lab}) and that PB, rather than being an essential partner with SCR for proboscis determination, is a competence factor that switches SCR from determining tarsus identity (SCR^{T1} activity) to determining proboscis identity (SCR^{lab} activity) (Fig. 7). PB could function by promoting the switch from SCR^{T1} to SCR^{lab} activity, or by overcoming a negative regulatory mechanism that suppresses SCR^{lab} activity. The proposal of multiple SCR activities, SCR^{T1} and SCR^{lab}, would explain the multiple differential pleiotrophy observed with hypomorphic *Scr* alleles (Sivanantharajah and Percival-Smith 2009).

Evolution of PB expression in insects

The conservation of co-linear expression is a striking characteristic of *Hox* genes (Carroll 1995). But, there is an interesting exception in insects; PB (HOX2) expression in the epidermis is shifted posteriorly to overlap with DFD (HOX4) and SCR (HOX5) expression domains (Abzhanov and Kaufman 1999; Hughes and Kaufman 2002). This pattern of PB expression is conserved throughout insect orders, and the requirement for PB and SCR to determine labial identity is conserved in *Drosophila, Tribolium*, and *Oncopeltus* (Percival-Smith et al. 1997; Beeman et al. 1989; Hughes and Kaufman 2000). Interestingly, murine HOXA2 in *Drosophila* is able to weakly inhibit SCR activity in tarsus determination, resulting in a proboscis to arista transformation similar to hypomorphic *pb* alleles (Percival-Smith and Laing Bondy 1999; Tayyab et al. 2004). We propose three events occurred during the evolution of insects: (1) SCR acquired the negative regulatory DYTQL motif and CTD; (2) SCR acquired the activity to determine labial identity (SCR^{lab}); and (3) PB expression shifts posteriorly to assist in the switch from the bilaterian conserved SCR^{T1} activity to the insect specific SCR^{lab} activity.

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