Up-Regulated *Scr* Expression in the Drosophila Prothoracic Leg is Regulated by Two Cis-Regulatory Elements

BY

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This work is decated to my parents,
Ricky and Diane McCallough.
Without your help and support, this would not have been possible.

Table of Contents

LIST OF FIGURES

List of Tables

LIST OF ABREVIATIONS

A/P Anterior / Posterior
abd-A abdominal-A
Abd-B Abdominal-B
ac achaete
al aristaless
Antp Antennapedia
ap apterous
arm armadillo
Armact activated Arm
arr arrow
B Bar
bab bric-a-brac
BAC Bacterial Artificial Chromosome
bcd bicoid
bHLH basic Helix Loop Helix
bowl borther of odd with entrails limited
ci cubitus interruptus
CiA Activator form of Cubitus interruptus
CiR Repressor form of Cubitus interruptus
CRM cis-regulatory module
D/V Dorsal / Ventral
dac dachshund
Dfd Deformed
D-*h* dorsal *hairy*
Dl Delta
Dl/N Delta/ Notch
Dll Distalless
Dpp decapentaplegic
drm drumstick
dsh disheveled
E(spl) Enhancer of split (Complex)
en engrailed
Esg Escargot
exd extradenticle
Eya Eyes absent
ftz fushi tarazu
GOF Gain of function
hAEL hours after egg laying
hAPF hours after puparian formation
hb hunchback
HD homeodomain
hh hedgehog
hth homothorax
kb kilobases

L1 1st instar larva
L2 2nd instar larva
L3 3rd instar larva
lab labial
LOF loss-of-function
L-row longitudinal row
MC Mechanosensory macrochaete
mC Mechanosensory microchaete
N Notch
N-intra Notch intracellular domain
odd odd skipped
omb optomotor-blind
pb proboscipedia
PcG Polycomb-group proteins
PCR Polymerase Chain Reaction
PNS peripheral nervous system
Pr/Di Proximal / Distal
pt pretarsus (claw)
Ptc Patched
rn rotund
sc scute
Scr Sex combs reduced
So Sine oculis
T1 1st thoracic segment, prothoracic
T2 2nd thoracic segment, mesothoracic
T3 3rd thoracic segment, metathoracic
ta1 1st tarsal segment (basitarsus)
TFs transcription factors
ti tibia
Tkv Thickveins
Tkvact activated Thickveins
T-row Transverse row
UAS upstream activation sequence
Ubx Ultrabithorax
w white
wg wingless
z zerknullt
z2 zerknullt-related

Abstract

The Drosophila Hox gene *Sex combs reduced* (*Scr*) functions during embryonic development to specify regional identity along the anterior/posterior (A/P) axis which is well-studied. *Scr* also functions to specify the morphology of the prothoracic leg specifically the bristle pattern of the leg which is not well studied. This work examines the genetic regulation of *Scr* and two cis-regulatory modules (CRMs) that direct the expression of *Scr*. The expression of up-regulate *Scr* is regulated along all three axis in the prothoracic leg. This study shows that the transcription factors *Dachshund* (*Dac)* and *Distalless* (*Dll)* activate *Scr* expression along the Proximal/Distal (Pr/Di) axis and *Bric-a-brac* (*Bab*) refines the distal edge. Decapentaplegic (Dpp), Wingless (Wg) and *Engralied* refine the expression of *Scr* along the A/P and Dorsal/Ventral (D/V) axes to the anterior-ventral region of the prothoracic leg. This up-regulated expression of *Scr* is regulated by two cis-regulatory elements (CRM), one intronic CRM and one CRM 5' to the *Scr* transcription start site. The intronic CRM directs expression in the transverse row (T-row) bristle primordium with the 5' CRM directing expression in the T-row and sex comb primordium. A smaller sub clone of the 5' CRM, E fragment, fully recapitulates the up-regulated *Scr* expression and contains punitive binding sites for Dpp, En and Bab.

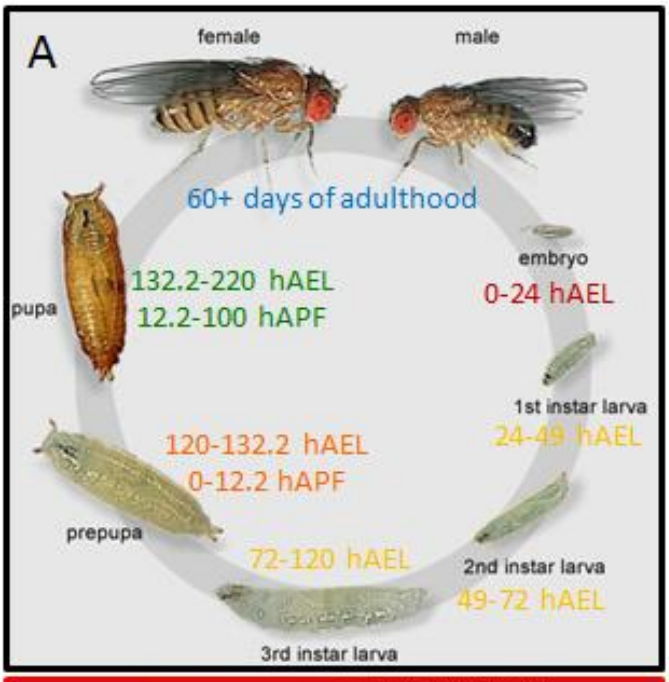
I. Introduction

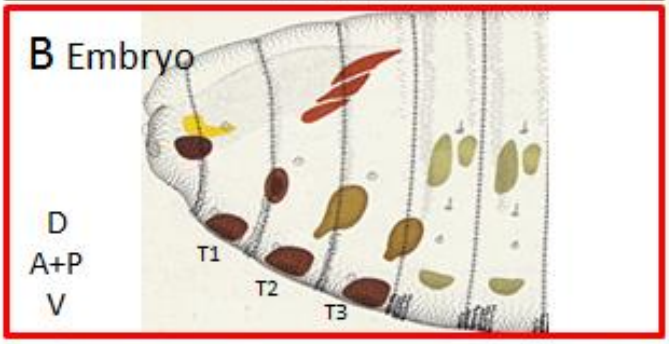
1.1 Pattern formation and serial homology

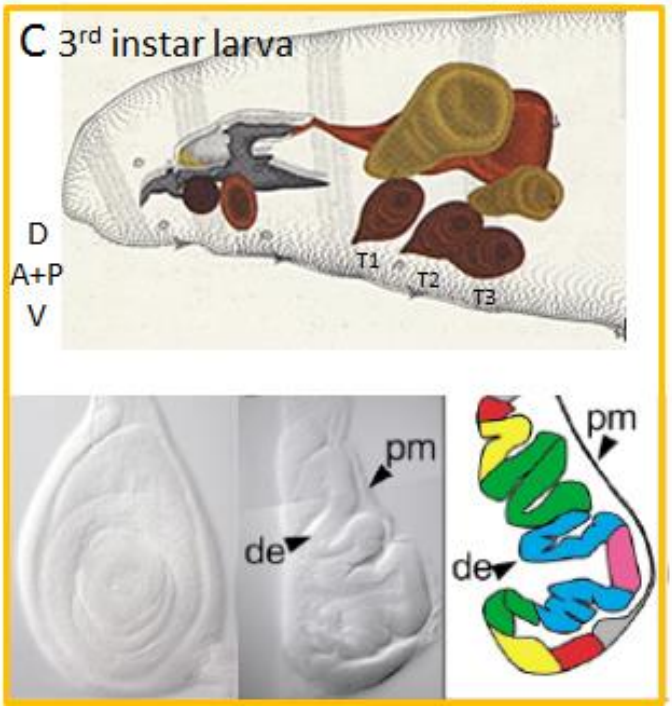
Developmental biology is the study of how a single cell divides and grows to produce a multicellular organism composed of trillions of cells that have undergone differentiation and morphogenesis. Pattern formation is a key process during development that gives rise to the animal's anatomical characteristics. Despite the vast morphological diversity amongst animals, the underlying developmental pathways are often highly conserved. For example, in limb development, the same patterning processes give rise to human arms, whale fins and bat wings. Therefore, defining the genetic and molecular mechanisms that support conserved developmental pathways, can not only inform our understanding of the major underlying principles but importantly, how regulation of these basic processes allow for divergence among anatomical structures within and between species. The events that produce three distinctly different leg pairs in *Drosophila melanogaster,* comprise an excellent model system in which to study the regulation of a highly conserved developmental pattern.

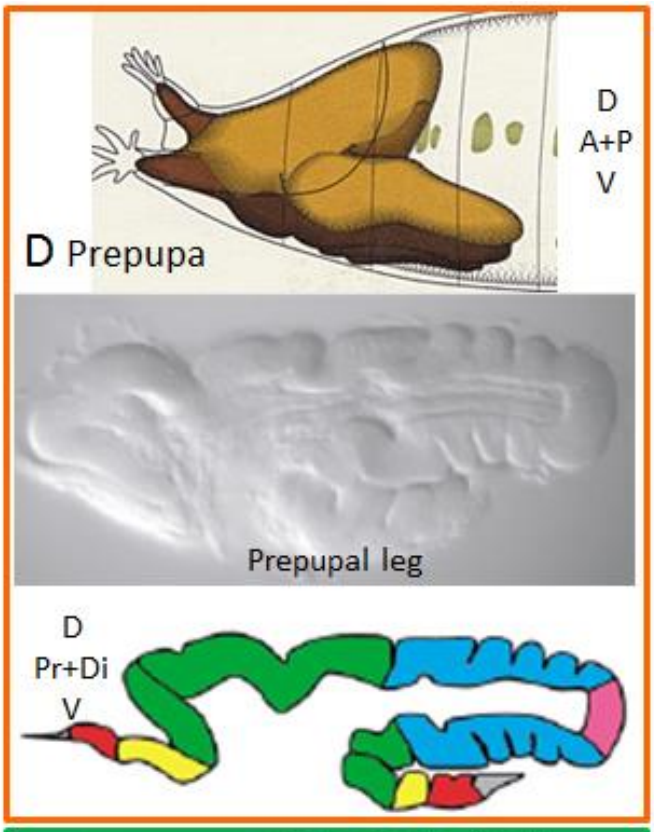
1.2 Drosophila leg development

The life cycle of Drosophila comprises four major stages; embryo, larval, pupal, and adult as shown in Figure 1A. The embryonic stage is further broken down into seventeen distinct stages that lasts 24 hours at 25^{o}C. In the early embryo, a series of synchronous nuclear divisions gives rise to a syncytium. After the 9th nuclear division, the nuclei migrate to the periphery of the egg, forming the syncytial blastoderm. After an additional four rounds of nuclear division during stage four of embryonic development,


A
female
male
60+ days of adulthood
embryo
0-24 hAEL
1st instar larva
24-49 hAEL
2nd instar larva
49-72 hAEL
3rd instar larva
72-120 hAEL
prepupa
120-132.2 hAEL
0-12.2 hAPF
pupa
132.2-220 hAEL
12.2-100 hAPF


B Embryo
D
A+P
V
T1
T2
T3


C 3rd instar larva
D
A+P
V
T1
T2
T3
pm
de
pm
de


D Prepupa
D
A+P
V
Prepupal leg
D
Pr+Di
V


E Pupa
D
A+P
V

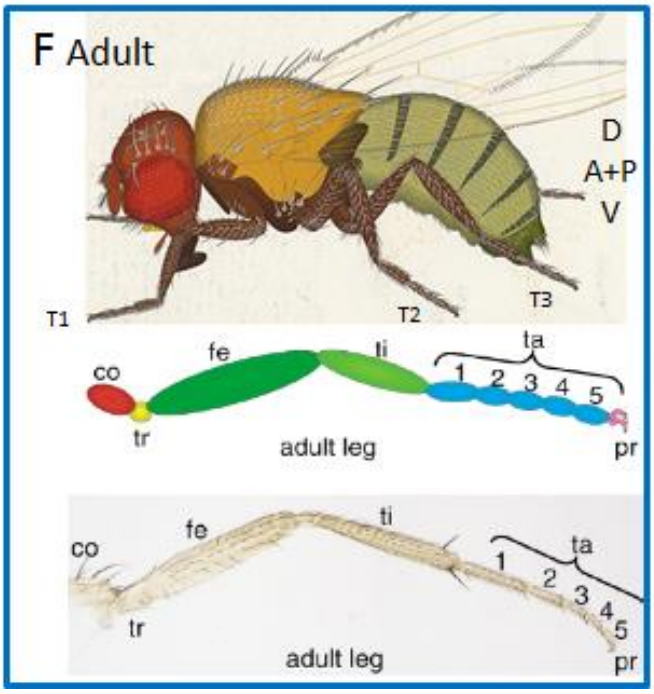

F Adult
D
A+P
V
T1
T2
T3
co
fe
ti
ta
1 2 3 4 5
tr
adult leg
pr
co
fe
ti
ta
1 2 3 4 5
tr
adult leg
pr

Figure 1 *Drosophila melanogaster* leg development

(A)The Drosophila life cycle consists of four main stages an egg, larval stage consisting of instar stages one through three, the pupal stage and adult. An average life cycle at 25^{o} C takes 220 hours as shown in the diagram. Each stage is timed from the moment of egg lay (hAEL) and can also be staged in hours after puparian formation (hAPF). Leg development of the Drosophila takes place during the first three stages of the life cycle. (B) Two patches of cells are selected from embryonic ectoderm in each of the thoracic segments T1, T2 and T3. The three brown patches one in each segment at stage 17 embryo represent the three of the future legs. (C) The primordial cells that were selected invaginate from the ectoderm during the L1 larval stage and are known as leg imaginal discs. The leg disks are depicted as brown patches in the 3rd instar larva diagram. A front and lateral view of a leg disk show a highly folded monolayer of cells with the disc epithelium (de) and peripodial membrane (pm) being discernable at the L3 stage. (D) In the prepupal stage all six imagianal discs telescope out to form a three dimensional leg. The cells in the center of the disc are the more distal structures of the leg and the periphery of each disc from proximal structures. (E) Each of the legs continues to evert in the pupal stage, depicted as the brown structures in the diagram. (F) Adult flies eclose with 6 fully formed legs comprised of ten segments along the Pr/Di axis: coxa (co), trochanter (tr), femur (fe), tibia (ti), tarsal segments 1-5 (ta1-5) and a pretarsus (pr). These images are adapted from Weigmann et al, 2003 (A), Hartenstein, 1993 (B-F) and Kojima, 2004 (C, D and F).

the embryo transitions to stage five, during which a cellular blastoderm is formed (Hartenstein, 1993). After the embryo is finished developing it emerges from the egg and progresses through three larval stages known as 1st, 2nd, and, 3rd instars (L1, L2, and L3) over a four-day period (Ashburner and Thompson, 1976; Bodenstein, 1950). When the 3rd instar larva is large enough, it stops feeding and forms a puparium, called the white prepupa. The white prepupal stages lasts an hour at 25°C and marks the beginning of the prepupal stage, lasting 12 hours leading to the inclosed pupa. The pupal stage last 3.5 days, during which developmental time points are counted in hours after puparium formation (hAPF) (Bainbridge and Bownes, 1981; Handler, 1982). Lastly, the adult fruit fly emerges from the puparium to breed and start the cycle over.

Drosophila leg development starts in embryogenesis and continues through the pupal stage. Two patches of 20 cells each, comprising the leg primordia, are selected from the embryonic ectoderm in each of the three thoracic segments, T1, T2, and T3 (Figure 1B). In the L1 stage, each leg primordium invaginates from the epidermis to form an imaginal disc that stays attached to the epidermis (Hartenstein. 1993). These imaginal discs continue to grow throughout the three larval stages, giving rise to a highly folded epithelial monolayer of cells (Figure 1C). When the larva forms a prepupa, each leg disc begins to telescope out from the center and continues to evert through the pupal stage, forming a three dimensional elongated cylindrical structure (Figure 1D, E). Each adult fly ecloses from the pupa with six legs, three homologous pairs, each leg containing ten segments: coxa (co), trochanter (tr), femur (fe), tibia (ti), five tarsal segments (ta1-5), and the pretarsus (pt) (Figure 1F) (Fristrom and Fristrom, 1993; Hartenstein, 1993; Kojima, 2004).

1.3 Patterning of the Drosophila leg

Development of the Drosophila leg requires patterning inputs along all three axes, anterior/posterior (A/P), dorsal/ventral (D/V), and proximal/distal (Pr/Di). Each of the three axes provides precise information to direct the growth and patterning of the leg. The A/P and D/V act to control the patterning along the circumference of the leg, and the Pr/Di patterning along the length of the leg.

The Pr/Di axis is established early during leg development, beginning with the expression of the transcription factor (TF) Distalless (Dll), induced by Decapentaplegic (Dpp) and Wingless (Wg) signaling in the center of each embryonic leg primordium (Cohen et al., 1993; Galindo et al., 2002; Simcox et al., 1989). At embryonic stage 14, Escargot (Esg) is expressed, surrounding Dll expressing cells in the leg primordium (Figure 2A) (Goto & Hayashi, 1999; Goto & Hayashi, 1997a). *esg* expression is repressed by Dll, thereby maintaining proximal and distal fates (Kubota et al., 2003). The proximal cells also express Homothorax (Hth), which recruits its cofactor, Extradenticle (Exd), into the nucleus. Together, Hth and Exd will specify proximal leg and body fates (Abu-Shaar & Mann, 1998; Cohen et al., 1989; Kojima, 2004). Hth and Exd also inhibit the response to Dpp and Wg signaling, preventing specification of distal leg fates (Abu-Shaar & Mann, 1998; González-Crespo et al., 1998).

Differentiation continues in the 2nd instar leg primordium with expression of *dachshund* (*dac*) between Hth and Dll expressing cells (Mardon et al., 1994). At the end of the 3rd instar stage, the expression patters of Hth, Dac and Dll create 5 domains in the leg imaginal disc. First, cells expressing only *hth* form the coxa and proximal trochanter. Next, the cells expressing *hth, dac,* and *Dll* form the distal trochanter and proximal femur.

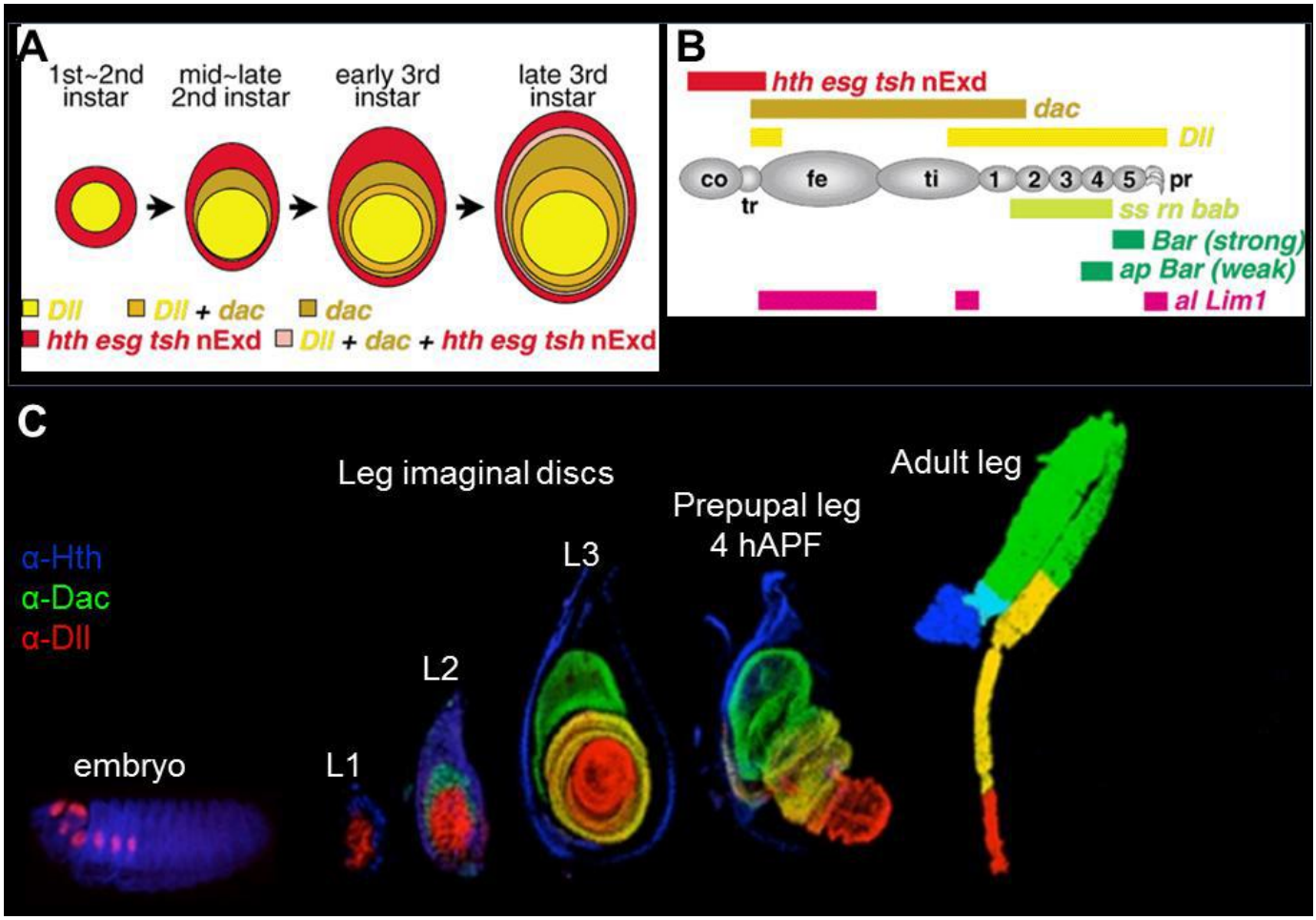


Figure 2 Proximal/distal patterning of the *Drosophila melanogaster* leg

(A) Drosophila leg imaginal discs are shown at the three larval stages of development. Patterning along the Pr/Di axes starts in the 1st instar stage with the expression of hth, esg, tsh and nExd in the proximal ring and Dll in the center of the disc. During development the disc continues developing and five distinct domains develop consisting of the overlapping expression of the genes hth, dac and Dll. (B) During the prepupal stage of development a further division of the tarsal segments takes place when the genes *ss*, *rn*, *bab*, *B*, *ap*, and, *Lim1* are expressed. (C) Drosophila leg development from embryo to adult is depicted with Hth, Dac and Dll expression domains shown at each time point. The expression domains of Dac and Dll overlap in the medial region of the adult leg which corresponds to the upregulated Scr expression seen in the T1 leg. These images are adapted from Kojima, 2004 (A, B) and Mann, 2014 (C).

The following distal segments express only *dac* giving rise to the femur and proximal tibia. The fourth expression domain of *dac* and *Dll* give rise to the distal tibia and ta segments one and two. Finally, the most distal domain is marked by the expression of Dll in the ta3-5 (Figure 2A, C). The overlap of *dac* and *Dll* expression in the tibia and ta1, seen in the 3rd instar disc (Dong et al., 2001) and the prepupal leg (Mann, 2016), corresponds to a medial leg fate (Figure 2C).

The distal tarsal segments are further patterned by epidermal growth factor (EGF) signaling which causes the expression of Pr/Di genes *spineless* (*ss*), *rotund* (*rn*), *bric-a-brac* (*bab*), *Bar (B)*, *apterous (ap),* aristaless (al), and *Lim1* (Figure 3B) (Cohen et al.,1992; Couderc et al., 2002; Duncan et al., 1998; Estella et al., 2012; Higashijima et al., 1992; Kojima et al., 1991; Pueyo et al., 2000; Schneitz et al., 1993; St Pierre et al., 2002). All of the joints between the leg segments are formed during the pupal stage, a process that that requires Notch (N) signaling (Mirth & Akam, 2002; Rauskolb & Irvine, 1999) and a group of joint patterning gene.

The Drosophila leg is also patterned along the circumference of the leg, which includes the A/P and D/V axes. The A/P boundary is established during embryogenesis and maintained throughout leg development. The cells in the anterior compartment do not mix with posterior compartment cells. While A/P cells do not mix they do signal across the compartmental boundary throughout development (Blair, 1995; Garcia-Bellido et al., 1976; Irvine & Rauskolb, 2001; Lawrence & Struhl, 1996). All posterior compartment cells express the selector gene, *engrailed* (*en*) (Crick and Lawerance, 1975), which specifies a posterior cell fate and is required to maintain the A/P compartment boundary. Hedgehog

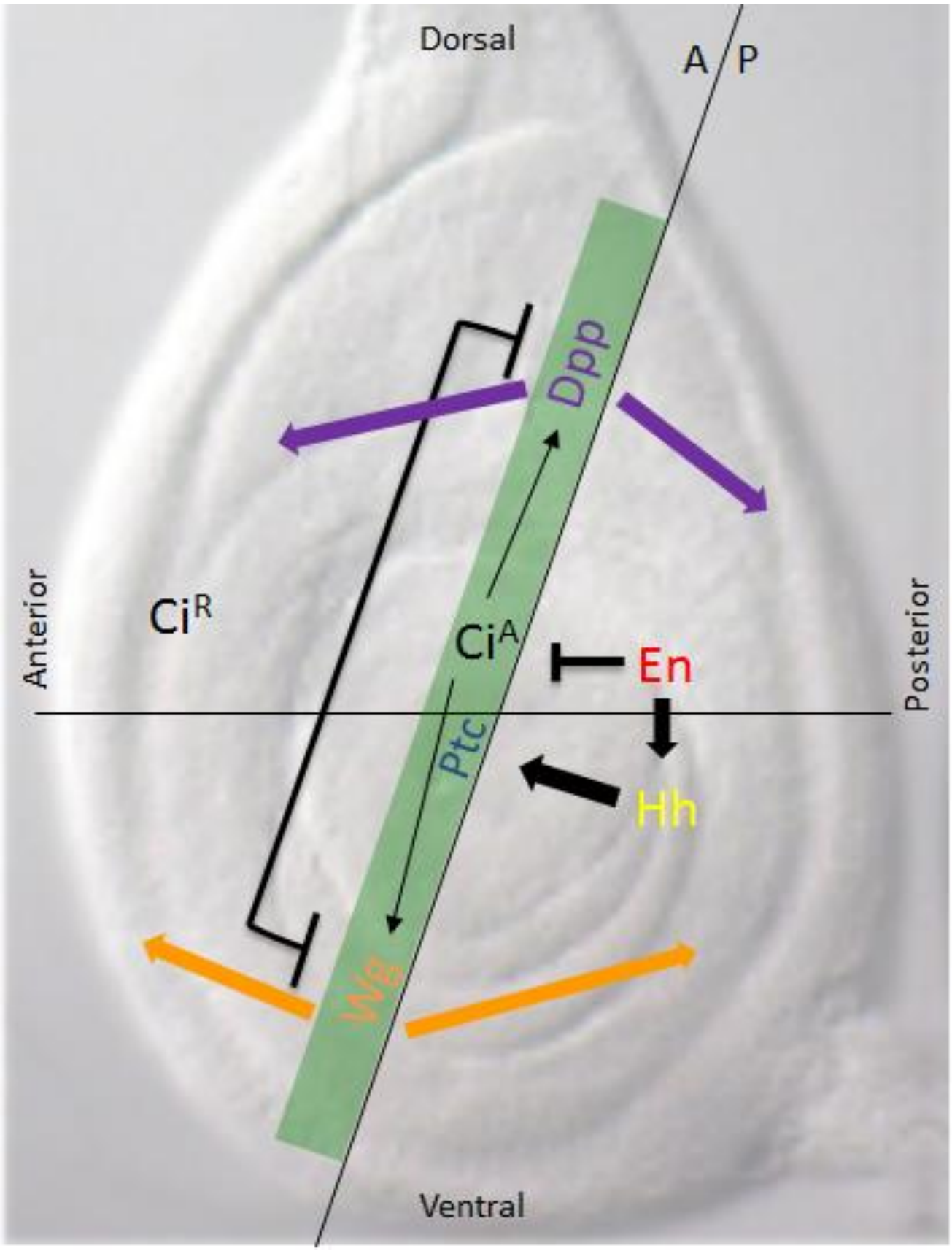

Dorsal
A
P
Dpp
Anterior
Ci^R
Ci^A
En
Posterior
Ptc
Hh
Wg
Ventral

Figure 3 Circumferential patterning along the *Drosophila melanogaster* leg

The circumferential regulators are depicted on a 3rd instar leg disc to visualize the regulatory interactions. The posterior compartment of the leg is specified by the transcription factor En that represses Ci, confining it to the anterior compartment. Hh requires En expression that then signals to its receptor Ptc across the compartment boundary. The narrow green strip when Hh signaling is high cause Ci to be cleaved into its activated form (Ci^A) which activates Dpp in the dorsal region and Wg in the ventral region. Dpp and Wg act as long-range morphogens patterning the D/V axis in a concentration dependent manner. These two morphogens are mutually antagonistic that repress the other resulting in either a Dorsal or ventral fate to develop. The combined action of these regulators on the A/P and D/V axes gives rise to circumferential patterning.

(hh) is a short range signaling molecule, expressed in the posterior compartment that signals to the anterior compartment and requires En for its expression (Figure 3).

Hh signals to its receptor Patched (Ptc), which prevents cleavage of the zinc-finger transcription factor, Cubitus interruptus (Ci), into its repressive form (Aza-Blanc et al., 1997). The full-length, activator form of Ci, which accumulates in response to HH signaling, then activates expression of Decapentaplegic (Dpp) dorsally and Wingless (Wg) ventrally in narrow stripes along the A/P boundary as shown in Figure. 3 (Basler and Struhl, 1994; Jiang & Struhl, 1996). Dpp and Wg are long range morphogens acting in a concentration-dependent manner in the dorsal and ventral regions respectively (González et al., 1991; Jackson & Hoffmann, 1994). The two morphogens are also mutually antagonistic as loss of Dpp results in expression of *wg* in the dorsal region and the reverse for the ventral region, i.e. loss of Wg results in ventral *dpp* expression (Campbell and Tomlinson, 1999). En, Hh, Ci, Dpp and Wg expression along the A/P and D/V axes is essential for patterning of the leg imaginal disc along the entire leg circumference (Figure 3), eventually resulting in development of pattern elements such as sensory organs, termed cheatae or bristles that cover the entire Drosophila body.

1.4 Patterning the peripheral nervous system of Drosophila

Drosophila sensory organs are components of the adult peripheral nervous system (PNS) and include multiple bristle types, such as large mechanosensory bristles or macrochaete (MC), small mechanosensory bristles or microchaete (mC), and chemosensory microchaete (Figure 4) (Held and Heup, 1996). The precise patterning of these bristles provides an excellent model for studying the molecular mechanisms of pattern formation.

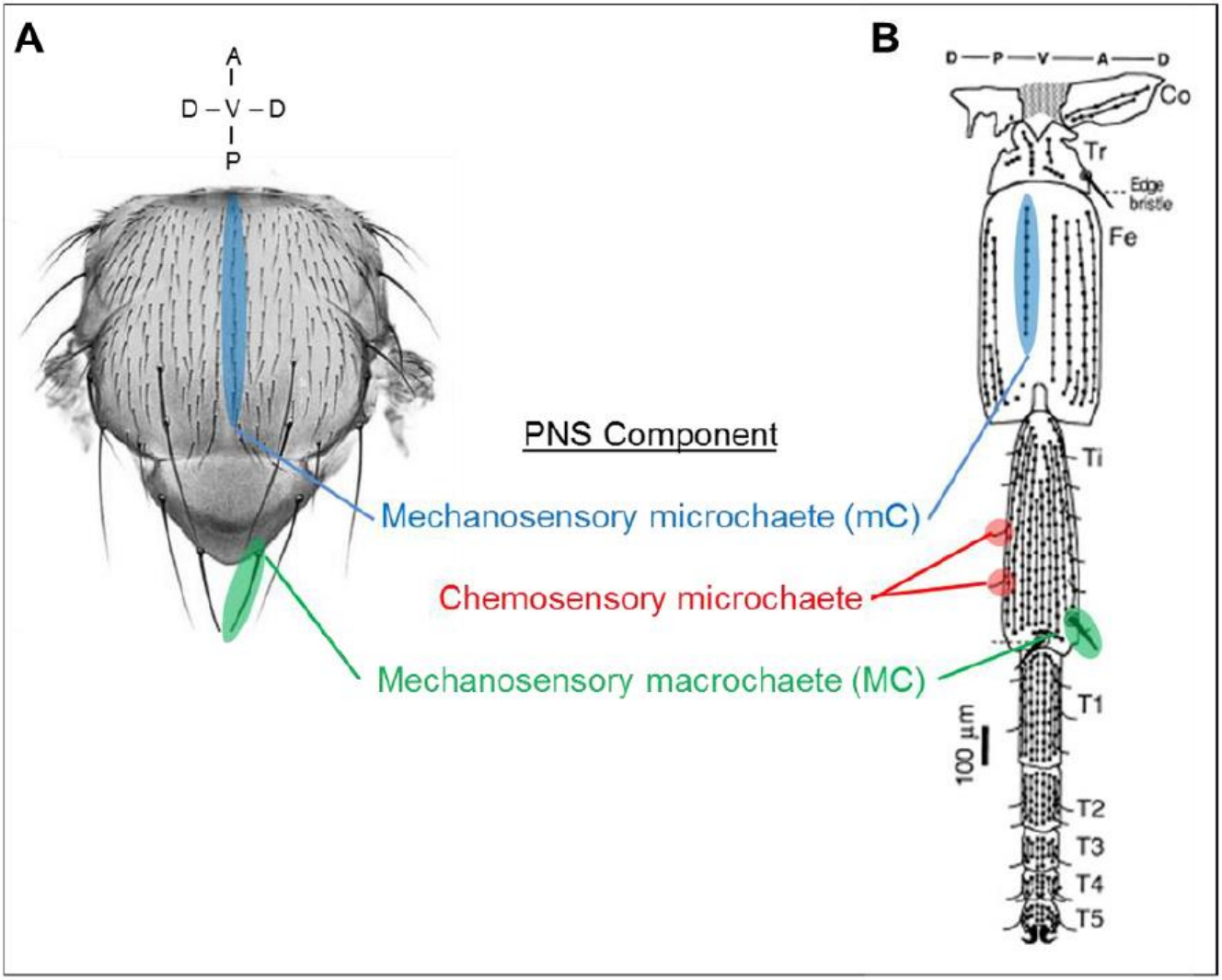


Figure 4 Drosophila peripheral nervous system bristles

(A) PNS bristles are arranged in precise patterns on the Drosophila notum. Each hemi-notum has eleven MC, in green, for a total of 22 and multiple L-rows of mC in blue. (B) The legs of Drosophila have precise rows of arrangements of MC and mC but also have a third bristle type the chemosensory bristles, in red, that appear at precise location. These images are adapted from Calleja et al., 2002 (A), and Held and Heup, 1996 (B).

The chemosensory microchaete and MCs are specified during the L3 stage of development, while the mCs are specified later in the prepupal stage (Bulanin, unpublished; Calleja et al., 2002; Lee, unpublished; Simpson & Marcellini, 2006). Chaete development in Drosophila happens in an elucidated multistep process that starts with the selection of neural precursors from epidermal cells. First, *achaete* (*ac*) and *scute* (*sc*), two redundant proneural genes that encode basic-helix-loop-helix (bHLH) transcription factors (TF), are expressed in spatially defined patterns (Villares & Cabrera, 1987) This initial proneural gene expression defines proneural clusters or fields from which sensory organ precursors (SOPs) will be selected (Artavanis-Tsakonas & Simpson, 1991) (Figure 5A).

Selection of the SOPs occurs through a process called lateral inhibition, which is mediated by Delta (Dl)/N signaling and during which Dl is upregulated in a presumptive SOP. That high level of Dl signals to surrounding cells, activating expression of Enhancer of split complex [E(spl)] repressors that inhibit *ac* and *sc* expression (Cabrera, 1990; Hartenstein & Campos-Ortega, 1984, 1986; Skeath & Carroll, 1992, 1994; Wodarz & Huttner, 2003). Cells that do not express proneural proteins after this step will give rise to epidermis, and the SOPs gives rise to the sensory organ. Several rounds of asymmetric cell divisions t gives rise to the sense organ, which is composed of a socket cell, shaft cell, sheath cell, neuron, as well as a glial cell that later undergoes apoptosis (Figure 5 B,C) (Fichelson et al., 2005; Held, 1995; Reddy & Rodrigues, 1999). The asymmetric division of SOPs is mediated by Dl/N signaling and the expression of *numb* (Figure 5B, C) (Hartenstein & Posakony, 1989; Lai, 2004).

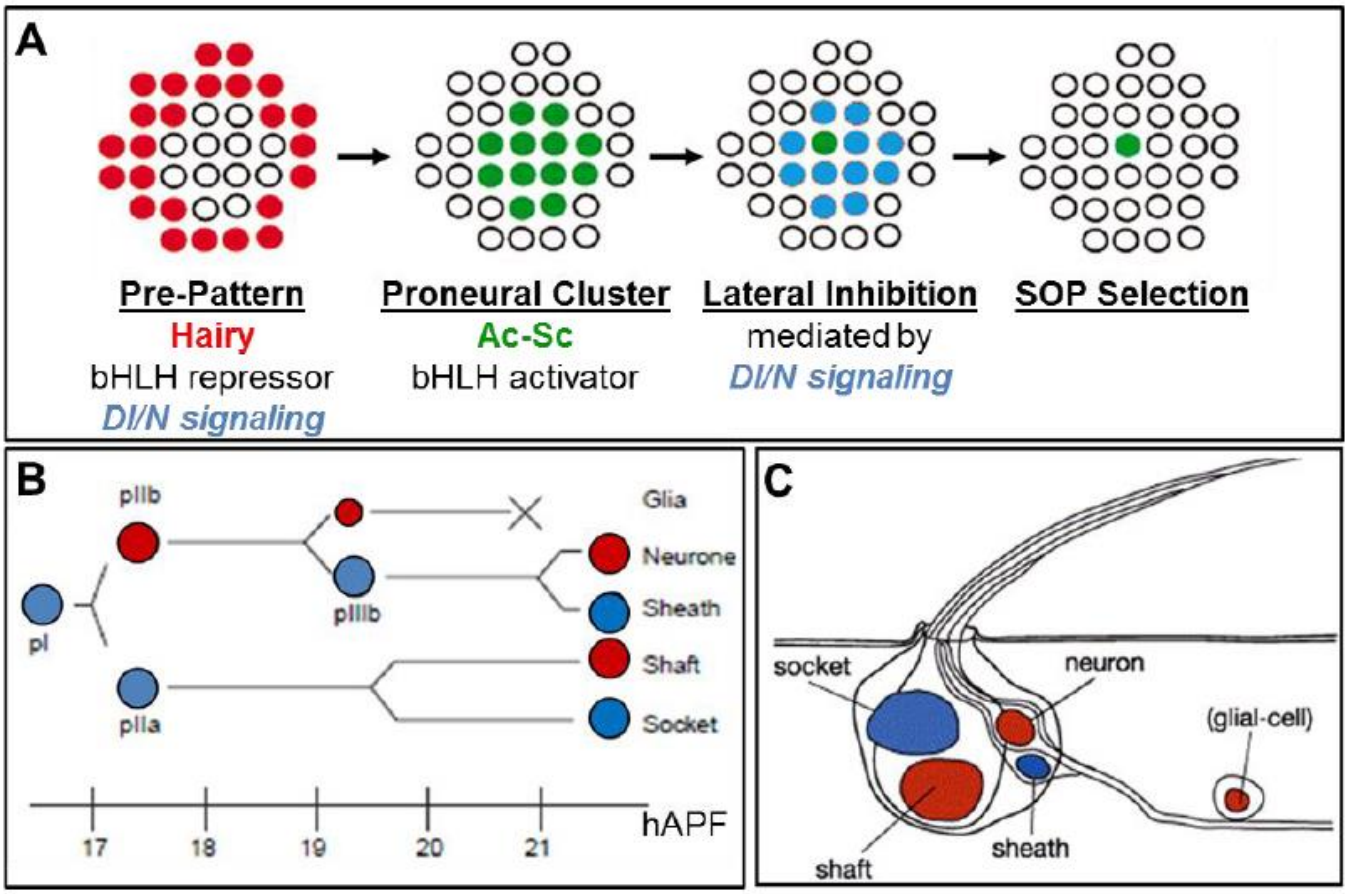


Figure 5 Drosophila peripheral nervous system development

(A) A pre-pattern is established by the TF Hairy and Dl/N signaling in specific fields of epidermal cells that negatively regulate *ac-sc* expression. In the absence of repressors ac-sc is expressed forming a proneural cluster, green cells. Dl/N signaling is activated a second time with in the cluster in a process called lateral inhibition. Once cell that expressed Dl at a higher level than the rest of the cluster then activates N signaling in the surrounding cells, blue cells, which represses Ac-Sc in the rest of the cluster. The single cell expressing Ac-Sc is selected as the SOP, green cell. (B) The SOP asymmetrically divides giving rise to different cells within the mechanosensory microchaete bristle. The divisions are mediated by Dl/N in in the blue cells and Numb in the red cells. The pIIa cell divides to give rise to the socket and the shaft cells. The pIIb divides into the glial cell which under goes apoptosis and pIIIb that then divides one more time giving rise to the neuron and sheath cells. (C) The cross section of mechanosensory organ depicts the structures in the organ. The shaft and socket are external structures on the fly body with the neuron and sheath cells are located internally. These images are adapted from Fisher & Caudy, 1998 (A), Fichelson et al., 2005 (B), and Lai and Orgogozo, 2004 (C).

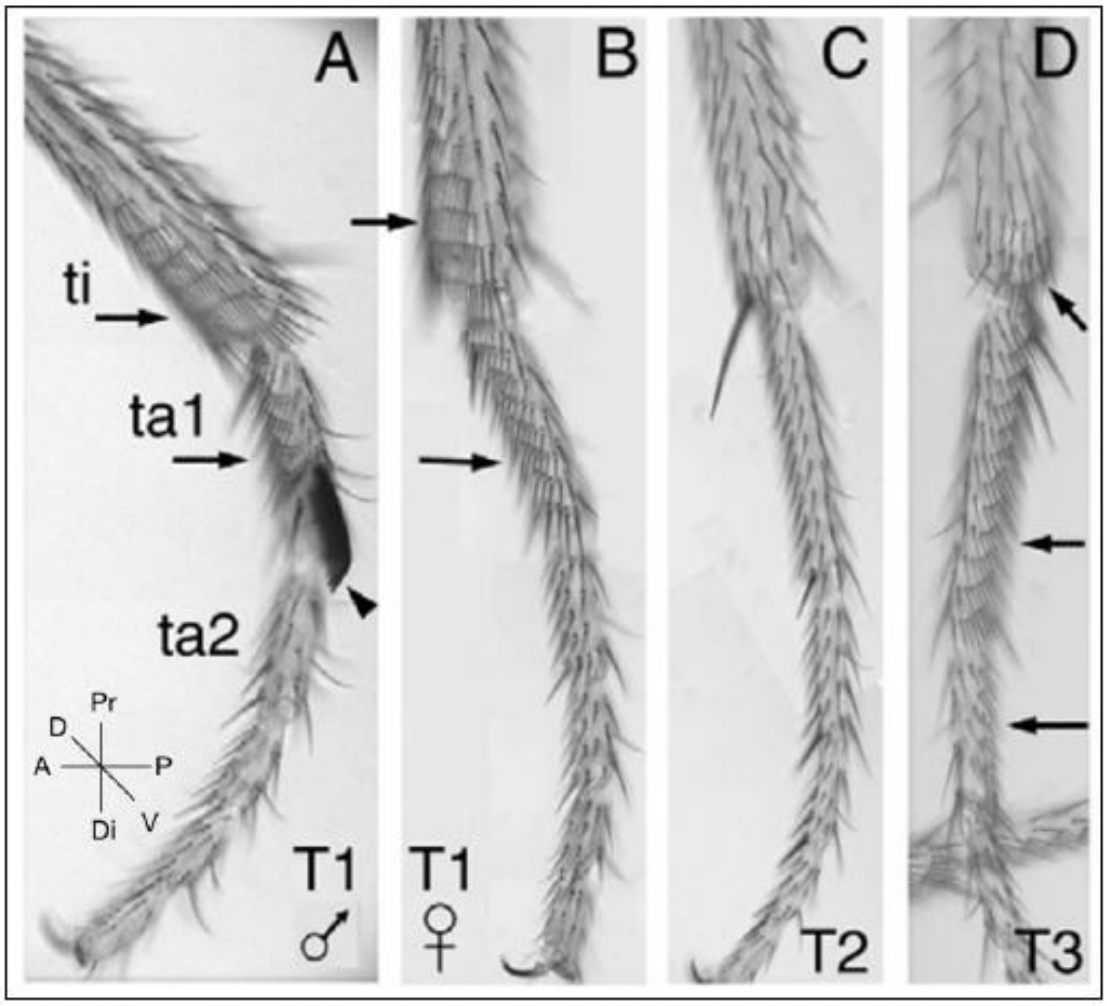
While previous studies have elucidated the general mechanisms of notum MC bristle patterning in Drosophila, here the focus is on leg bristle patterning, specifically the mCs.

1.4a **Peripheral nervous system patterning in Drosophila legs**

The leg pairs from different thoracic segments are homologous but display unique features, such as mC patterns (Figure 6). The T2 leg mC are arranged in longitudinal rows (L-rows) that run parallel to the Pr/Di axis (Figure 6C). Legs on the T1 and T3 have L-rows but also have a second arrangement of mC bristles, called transverse rows (T-rows), at specific locations on each leg (Hannah-Alava, 1958; Held, 1995). T-rows are used by flies for grooming of the eyes and wings (Vandervorst & Ghysen, 1980).

On the T1 leg, the T-rows are located on the antero-ventral surface of the distal tibia and ta1 (Figure 6 A, B) (Held, 1990). The male's most distal T-row in the T1 leg is a specialized densely-packed, short, thick, blunt, and darkly-pigmented set of bristles collectively called the sex comb that is rotated 90 degrees relative to the other T-rows (Figure 6A) (Held, 1990; Tokunaga, 1962). The T3 leg T-rows are located on the postero-ventral surface of the distal tibia, ta1, and ta2, (Figure 6D) (Held, 1990).

L-rows are present on all three pairs of legs, but the T2 leg lack T-rows, only possessing the L-rows, which is thought to represent the most primitive configuration (Lewis, 1978). The common L-row patterning pathway has been previously determined (Figure 7). The development of the L-row mC bristles requires the proneural TFs, *ac* and *sc*. Both genes are expressed in narrow stripes in prepupal legs at 6 hAPF. These *ac* and *sc* expressing stripes mark L-row proneural fields on each of the three pairs of legs


A
B
C
D
ti
ta1
ta2
Pr
D
A
P
Di
V
T1
♂
T1
♀
T2
T3

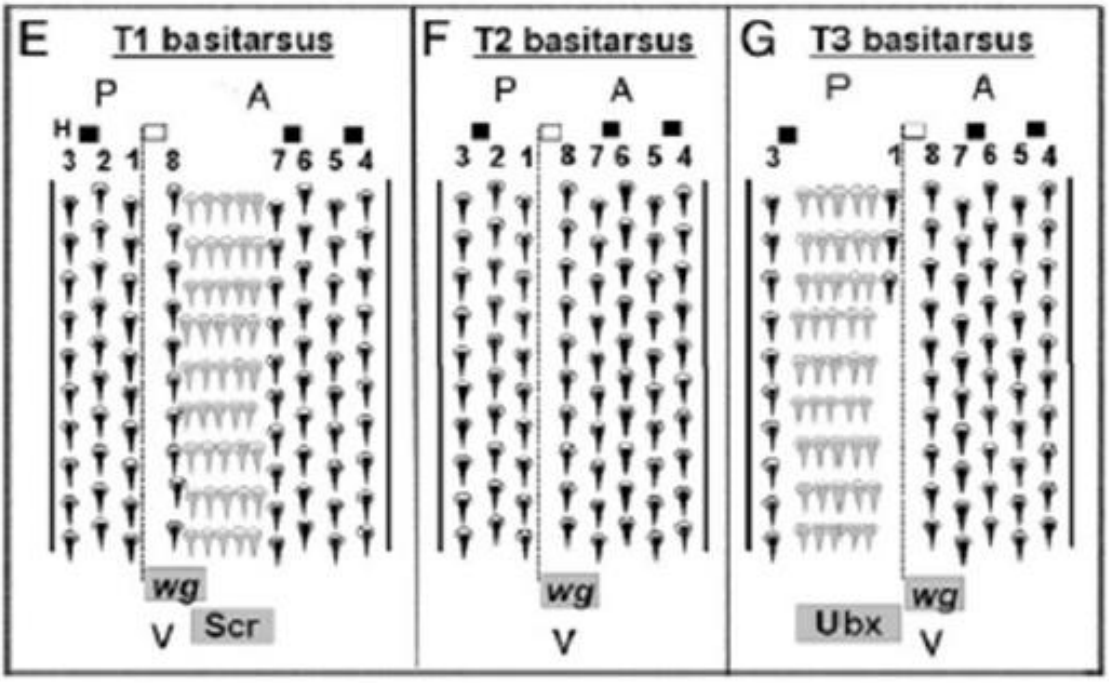

E T1 basitarsus
P
A
H
3 2 1 8 7 6 5 4
wg
V
Scr
F T2 basitarsus
P
A
3 2 1 8 7 6 5 4
wg
V
G T3 basitarsus
P
A
3 1 8 7 6 5 4
wg
Ubx
V

Figure 6 Microchaete on the Drosophila legs are organized in two distinct patterns

(C) Drosophila T2 legs display eight L-rows of mC that are parallel to the Pr/Di axis. (A, B) T1 legs possess L-rows but also display T-rows on the antero-ventral surface of the distal tibia (arrows). (A) Drosophila males possess a third bristle type called the sex comb (arrowhead) that is derived from the distal most T-row that is rotated 90^{o} that females (B) do not have. (D) The T3 legs have both L-rows and T-rows like the T1 leg. The T-rows are on the postero-ventral surface of the distal tibia, basitarsus and second tarsal segment (arrows). (E) The diagram depicts the mC of the basitarsus on the T1 (E), T2 (F), and T3 (G). L-rows have sockets that are darkly pigmented and to not directly touch other mC in contrast to T-row bristles that are lightly pigmented, tightly packed, and touching. This image is adapted from Shroff et al., 2007

(Orenic et al., 1993). SOPs selected from these proneural fields will give rise to the mCs in L-rows (Joshi et al., 2006). Hairy and Dl/N signaling establishes a prepattern of repression along the leg circumference, prior to the activation of *ac/sc* expression at 6 hAPF, which gives rise to the expression of *ac* in the L-row primordium (Joshi et al., 2006; Orenic et al., 1993). Together Hairy and Dl/N establish eight spatially defined stripes of *ac* expression and eight interstripes where *ac* is repressed. Hairy is a bHLH repressor (Ohsako et al., 1994; Van Doren et al., 1994) that is expressed in four of the eight interstripes, called "*hairy*-On" interstripes (Orenic et al., 1993). The other four interstripes are established by Dl, which is expressed in the eight stripes that express *ac*, signaling to N in the four interstripes not expressing *Hairy*, "*hairy*-Off" interstripes, to repress *ac* expression there. Dl/N signaling to the *hairy*-Off stripes activates genes in the E(spl) complex, which express bHLH repressors similar to Hairy. In the absence of the prepattern of repression *ac* would be activated uniformly along the leg circumference (Joshi et al., 2006; Lee, unpublished).

T1 and T3 legs have L-rows just like T2 legs but also have T-rows. Our lab has previously shown that the L-row pathway is modified to produce T-rows. The Hox genes *Sex combs reduced* (*Scr*) in the T1 legs and *Ultrabithorax* (*Ubx*) in the T3 legs function to modify the L-row proneural pattern pathway causing the formation of T-rows (Figure 8) (Shroff et al., 2007). L-rows on the T2 legs correspond to the stripes of *ac* in the leg primordia but in T1 and T3 legs a broad domain of *ac* expression marks the T-row primordia. (Orenic et al., 1993; Shroff et al., 2007). This different pattern of *ac* expression in the T1 and T3 legs is due to differential expression of *Dl* within the T-row primordia

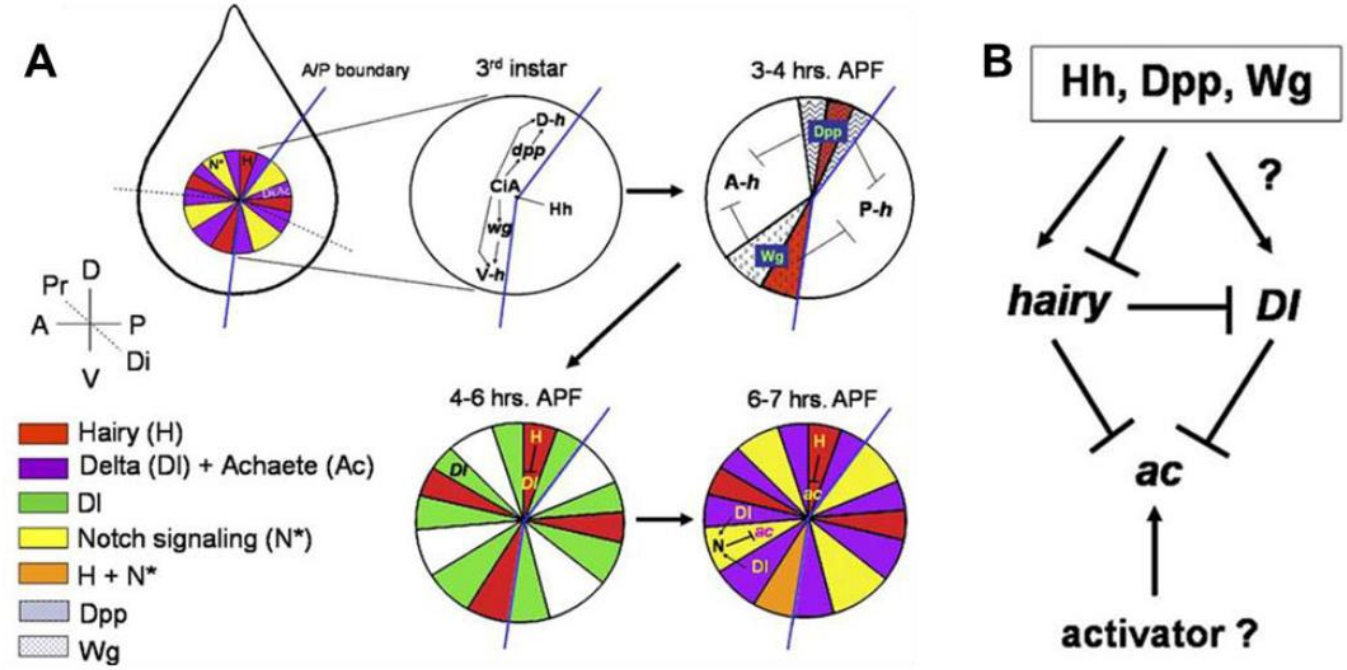


Figure 7 Longitudinal-row pattering is established by a prepattern of repression from Hairy and Delta

(A) *ac* expression is defined by a prepattern of repression that takes place in stages. The process is depicted on a 3rd instar for simplicity. In the 3rd instar stage Hh, Dpp, and Wg activate the Dorsal and ventral stripes of Hairy. Stripes of Hairy in the anterior and posterior compartments are established at three to four hAPF and are repressed dorsally by Dpp with Wg repressing ventrally. Dl expression is activated at four to six hAPF in the mC primordia and signals to N in the adjacent interstripes. After the pattern of prerepression is established by Hairy and D/N signaling ac is broadly activated. (B) The model for establishing L-rows is depicted here. The global regulators Hh, Dpp and Wg establish *hairy* and presumably *Dl* expression in spatially defined stripes on the Pr/Di axes in each leg. The periodic expression of the two genes creates the prepattern of repression for *ac*. This image is adapted from Joshi et al., 2006

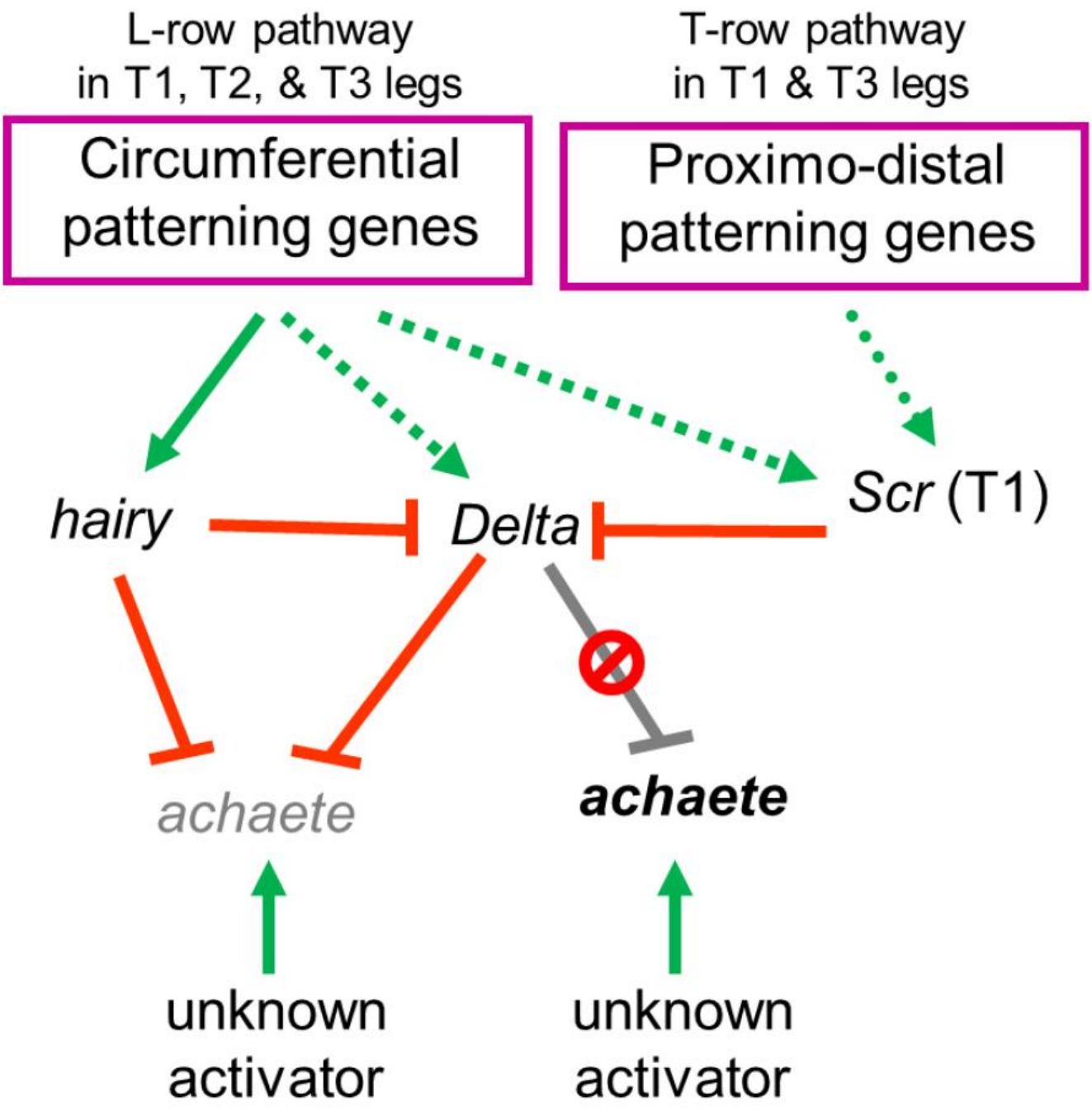


Figure 8 The longitudinal-row pathway is modified by the homeotic genes Sex combs reduced and Ultrabithorax to produce transverse-rows

Transverse rows (T-rows) are produced by modifying the L-row pathway in specific fields that allows for broad domains of ac expression that gives rise to T-rows. The two Hox genes Scr and Ubx in the T1 and T3 legs respectively repress Dl expression with in the T-row primoridia. The upregulated expression of both genes is presumed to be regulated by the global regulating genes. This model is adapted from Shroff et al., 2007.

(Shroff et al., 2007). The change in *Dl* expression is due to Scr and Ubx, in the T1 and T3 legs respectively, repressing *Dl* in the T-row primordia.

1.5 Homeotic genes and serial homology

The Drosophila body plan consists of 14 segments. Eight homeotic genes (Hox genes) are involved in an early stage of embryogenesis in establishing positional identity along the A/P axis (Figure 9) (Gellon & McGinnis, 1998; Hughes & Kaufman, 2002; E. B. Lewis, 1978). The Hox genes are split into two complexes, the Antennapedia complex which includes: *labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr),* and *Antennapedia (Antp),* and the Bithorax complex containing *Ultrabithorax* (*Ubx*), *abdominal-A (abd-A), and abdominal-B (abd-B). Scr, Antp*, and *Ubx* are expressed in the T1, T2, and T3 segments, respectively. Each thoracic segment contains a pair of legs that is serially homologous to legs from the other thoracic segments but exhibit differences. The expression and function of the Hox genes, *Scr* and *Ubx* in the developing leg primordia give rise to these morphological differences, such as the bristle patterns on the different leg pairs. Antp also serves another function, specification of a leg vs antennal fate. Early in leg development *Ant* is expressed in each of the leg primordia. Without this expression, T2 legs will develop into antenna instead of legs (Emerald and Cohen, 2004; Struhl, 1981). Therefore, how these Hox genes are regulated both spatially and temporally, is critical to understanding patterning.

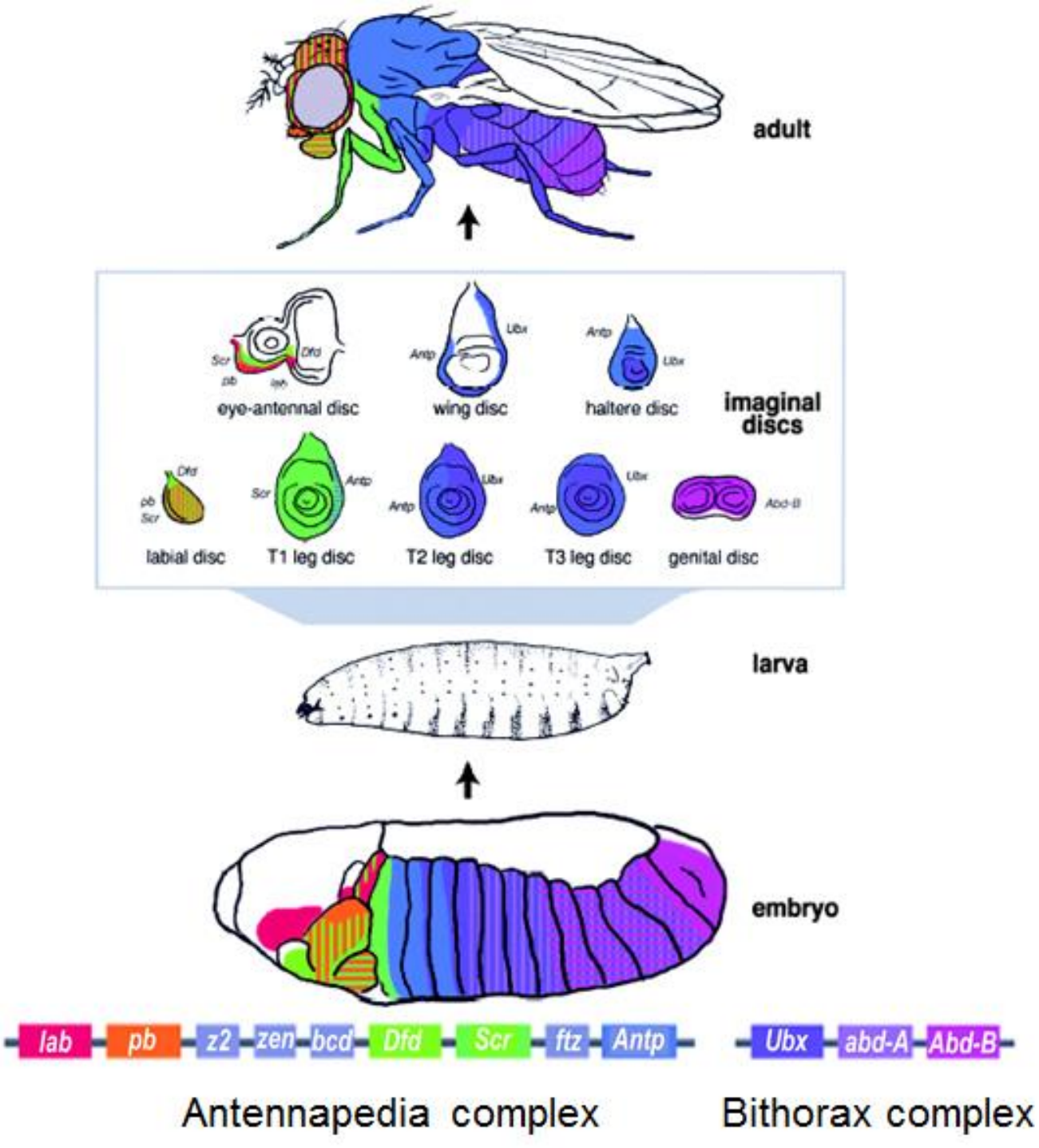

adult
imaginal discs
eye-antennal disc
wing disc
haltere disc
labial disc
T1 leg disc
T2 leg disc
T3 leg disc
genital disc
larva
embryo
lab
pb
z2
zen
bcd
Dfd
Scr
ftz
Antp
Ubx
abd-A
Abd-B
Antennapedia complex
Bithorax complex

Figure 9 Comparative expression of homeotic genes throughout Drosophila life cycle

The homeotic complex in Drosophila is split into the Antennapedia complex and the Bithorax complex on chromosome 3R. The Antennapedia Complex contains *labial (lab), proboscipedia (pb), zerknullt-related (z2), zerknullt (zen), bicoid (bcd), Deformed (Dfd), Sex combs reduced (Scr), fushi tarazu (ftz), and Antennapedia (Antp),* with the Bithorax Complex including *Ultrabithorax (Ubx), abdominal-A (abd-A), and Abdominal-B (abd-B).* Each homeotic gene is expressed along the A/P axis in the embryo starting with lab in the most anterior ending with abd-B in the posterior. Imaginal discs in each segment continue to express the corresponding homeotic gene that carries on into the adult stage. The four gene in gray do not function as homeotic genes and their expression is not shown here. This image is adapted from Hughes and Kaufman, 2002.

1.6 Regulation of homeotic gene expression

The regulation of Ubx provides an example that illustrates the importance of Hox gene regulation during Drosophila development. The *bx* region intronic cis-regulatory module (CRM), 30 kilobases (kb) downstream of the Ubx promoter (Qian et al., 1991) directs expression of *Ubx* in the early embryo within parasegments 6,8,10 and 12. At the same time this *bx* CRM represses expression in the anterior half of the embryo through the gap gene, Hunchback (hb), which binds the *Ubx* CRM preventing expression anteriorly (Qian et al., 1991). This example of Hox gene regulation illustrates the complexity of the spatial and temporal regulation needed for proper patterning.

Similarly, the concentration of Hox gene products is tightly regulated in mammals. For example the gene products from Hox A and Hox D complexes are required for the development of vertebrate limbs (Zakany and Duboule, 1999; Zakany et al., 1997). The length of the forelimb is directly related to the dose of HoxD-11, 12, 13 and HoxA-13. Losing gene products from these four Hox genes results in severe abnormalities in digit length as well as digit number, loss of all four producing stunted malformed digits (Zakany et al., 1997).

These examples of Hox gene regulation illustrate the complexity of the spatial and temporal regulation needed for proper patterning and subsequent consequences of misregulation. The critical importance of this regulation is further underscored when considering the role of homeotic genes in various human development processes.

1.7 Significance of Homeotic genes and their regulators in human disease and development

The Hox genes play a major role in development by regulating cell proliferation and differentiation. Consequently, in various forms of cancer, tumor growth has been linked to the misregulation of the human Hox cluster. Specifically, all but six of the thirty-nine human Hox genes have been shown to be mis-expressed in breast, prostate, colon, and lung cancers, whereas Hox11 has been shown to be mis-regulated in acute lymphoblastic leukemia in both children and adults (Bhatlekar et al., 2014; Alharbi et al., 2012).

Signaling pathways that regulate the Hox gene *Scr*, which is the focus of this thesis, have also been linked to human diseases. For example, Notch signaling has been implicated in lung, skin, and cervical cancers, oncogenic virus proliferation, Alagille syndrome, CADASIL disease as well as T-cell acute lymphoblastic leukemia (Westhoff et al., 2009; Talora et al., 2002; McDaniell et al., 2006; Joutel et al., 1996; Weng et al., 2004). The *Scr* regulator, Dpp/Bmp is linked to defects that arise during embryogenesis in multiple organ systems, and is associated with osteoarthritis as well as cancer (Kumarasinghe et al., 2011; Caestecker et al., 2000). Finally, Wg/β-catenin signaling has been implicated in diabetes, osteoporosis, osteoarthritis and multiple forms of cancers (Prestwich and MacDougald, 2007; Kumarasinghe et al., 2011; Morin et al., 2007). Whether these diseases are directly correlated with downstream effects on Scr remain to be determined.

Developmental defects during human growth and development are common and have a wide variety of phenotypes ranging from extra digits on hands and feet to missing

entire limbs. Polydactyly is one of these congenital defects in which vertebrates have a supernumerary number of fingers or toes, where as in other instances fewer digits (oligodactyly) arise. There are three forms of polydactyly: postaxial, preaxial, and central. The loss of HoxA complex genes results in oligodactyly and the HoxD loss gives rise to the opposite polydactyly phenotype. (Zakany et al., 1997).

Given the significance of Hox gene regulation in both human health and developmental patterning, understanding the genetic and molecular basis for this regulation is of critical importance. Here we focus on the regulation of the Hox gene *Scr* in the genetic model organism Drosophila, using the defined Scr role in leg mC patterning to identify genetic regulators and the molecular basis for their action.

II. Materials and Methods

2.1 Drosophila strains used

Drosophila melanogaster strains used in this study include: OregonR, *dpp-lacZ/CyO* (Spradling et al., 1999), *CyO-wg-lacZ* (Kassis et al., 1992), *dac-lacZ/CyO* (Spradling et al., 1999), *w;dac4FRT40A/CyO* (Mardon et al., 1994), *w;Ubi-GFP,FRT40A/CyO* (FlyBase Reports, 2000), *ywhsFLP;CyO/Sco* (Chou and Perrimon, 1996; Golic & Lindquist, 1989) *w; FRT42DDll^{SA1}/SM6,eve-lacZ* gift from G. Boekhoff-Falk (Dong et al., 2000); *yhsFLP;FRT42DUbi-GFP/* (Dong et al., 2000), *w;UAS-dac/TM3* (Dong et al, 2001), *ywhsFLP;UAS-Dll/CyO* (Dong et al., 2000), *ywAyGAL4,UAS-GFP* (FlyBase Reports, 1996, 1998), w; tubP-GAL80ts; TM2/TM6B (McGuire et al., 2003), w; UAS-mCD8.ChRFP (Bloomington Drosophila Stock Center Schnorrer, 2009.5.11), *w, UAS-bab1/cyo* (Bardot et al., 2002), yw; UAS-*Lim1*/CyO (Bloomington Drosophila Stock Center, Luo, L. 2011.9.21) w*hsFLP;tkv^{a12}FRT40A/CyO* (Szidonya and Reuter, 1988), *UAS-tkv^{QD}* (Neul and Ferguson, 1998), *wdsh^{3}FRT19A/FM7a* (Geer et al., 1983), *ywGFPhsFLPFRT19A/FM7a* (Clarkson and Saint, 1999), *FRT42Dpwn,arr^{2}* (Giorgianni and Mann 2011), *w;Ubi-GFP,FRT42D/CyO* (Lawrence et al., 2002), *Mad$^{1.2}$wg^{cx4}FRT40A/CyO* (Hays et al., 1999) (Blair and Ralston 1997) (Kim et al., 1997) *UASarm^{S10}yw* (Morel and Arias, 2004), *UAS-en* (Yoffe et al., 1995), *y;UAS-enRNAi* (Ni et al., 2008), *w1118;UAS-GFP$_{nls}$;rn-Gal4* (Bloomington Drosophila Stock Center, (2000.8.17) (St Pierre et al., 2002), y1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP}ZH-51D (Bischof et al. 2007), yv P{y[+t7.7]=*nos*-phiC31\int.NLS}X; P{y[+t7.7]=CaryP}attP40 (Bischof et al. 2007) (Groth et al. 2004) (Szabad et al. 2012), yscv P{y[+t7.7]=*nos*-phiC31\int.NLS}X; P{y[+t7.7]=CaryP}attP2*(Bischof et al. 2007) (Groth et al. 2004)

(Szabad et al. 2012), yw; AyGAL4,UAS-CD2/CyO, y+ (Bloomington Drosophila Stock Center,K. 1995.12.23), yw; *drm*^{3} FRT40A/CyO (Green et al., 2002), yw; *drm*^{3} *bowl*^{1} FRT40A/CyO (Hao et al. 2003), yw; UAS-*drm* (Lengyel, J. (2003.5.27), yv; lines^{RNAi} (Ni et al., 2011), w; Df/Cyo$^{vg\text{-}lacZ}$; UAS-N^{lCD}/Tm6(Hu), gift from Katzen lab, w; UAS-odd (Hao et al. 2003)

2.2 UAS-Gal4-Gal80 system

The UAS/Gal4 system was adapted from yeast by Andrea Brand and Norbert Perrimon (Brand and Perromon, 1993). This system makes it possible to ectopically express a gene of interest in an experiment. There are two components: a Gal4 driver and a target gene under control of an upstream activating sequence (UAS).Gal4 is downstream of a genomic enhancer, which will drive its expression in a tissue-specific pattern within the fly.. Many Gal4 lines are enhancer traps but they can also be specifically made using a CRM. The UAS sequence is upstream of a target gene making the UAS line. When these two fly lines are crossed, Gal4 binds to the UAS sequence inducing expression of the gene that is downstream of the UAS sequence. The target gene will be expressed in the tissue-specific pattern. (Figure 10) (Brand and Perromon, 1993)

Gal80 allows additional control of the expression of the target genes in this system. The protein Gal80, which is temperature sensitive, prevents Gal4 from binding the UAS sequence. At the permissive temperature of 18°C, Gal80 binds Gal4 but at the non-permissive temperature of 29°C the target gene is expressed because Gal80 does

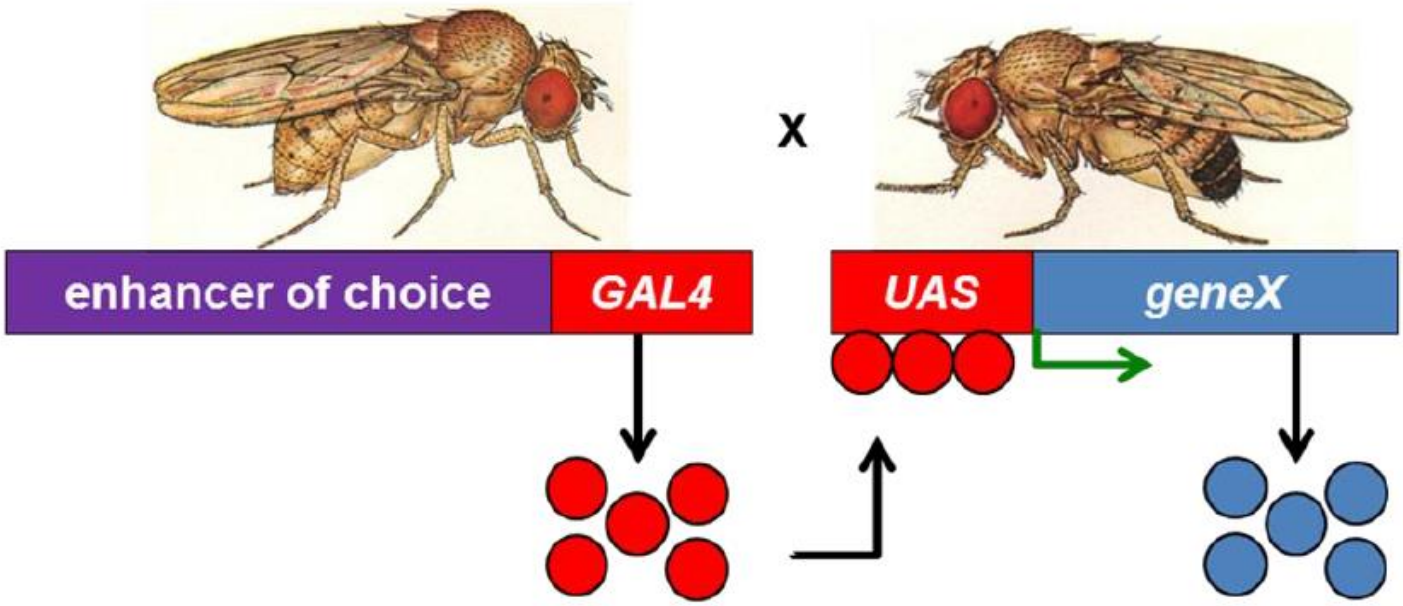


Figure 10 UAS/Gal4 system in *D.melanogaster*

The UAS/Gal4 system is used to ectopically express genes in the fruit fly. A GAL4 line expresses the Gal4 protein in a specific spatial and temporal pattern. The second line has the UAS sequence upstream of the gene to be ectopically express. Progeny from the mating of these two fly lines will express the target gene when the GAL4 binds the UAS sequence. Used with permission from Emily Wyskiel

not function at the higher temperature. (McGuire et al., 2003) The addition of Gal80 gives the ability to temporally control gene expression along with the spatial control that Gal4 line provides.

2.3 The FLP-FRT system for generating clones

The FLP-FRT system is used to create a genetically mosaic animal. This allows experiments to examine mutant alleles that would normally kill the animal if the animal was homozygous for the mutation. Kent Golic and Susan Lindquist adapted FLP-FRT from yeast to *D. melanogaster* in 1989 (Golic & Lindquist, 1989; Golic, 1991). Flipase, also called FLP, is an enzyme that induces recombination between two homologous sequences. The FRT is the sequence of DNA that is recognized by the FLP. FRT insertions near the centromere of all four chromosomes are available; mutations of interest can be recombined onto these chromosomes. Both the inserted FLP and FRT-mutant are in one fly line. The second fly line has the identical FRT with the marker GFP (Lee and Luo, 1999; Xu and Rubin, 1993). The F1 larva from this cross is heat- shocked giving a random chance of generating clones. The clone that is homozygous mutant is marked by the loss of GFP expression. The other two types of tissue possible are homozygous wild type, which is marked by two copies of GFP, also called the twin spot, and heterozygous tissue with one copy of GFP. (Figure 11)

This method was used to generate Loss of function (LOF) clones in this work. Larva (48-96 AEL) were heat shocked at 37˚C for 30 to 60 minutes. *Dll* LOF clones were made in larvae of the genotype *yhsFLP;FRT42DDll*^{SA1} and *Ubi-GFP,FRT42D* fly lines. The SA1 mutation is a null mutation (Dong et al., 2000). *dac* LOF clones were generated by crossing y*hsFLP;dac*^{4}*FRT40A* and *FRT40AUbi-GFP* flies. *dac*^{4} is a null

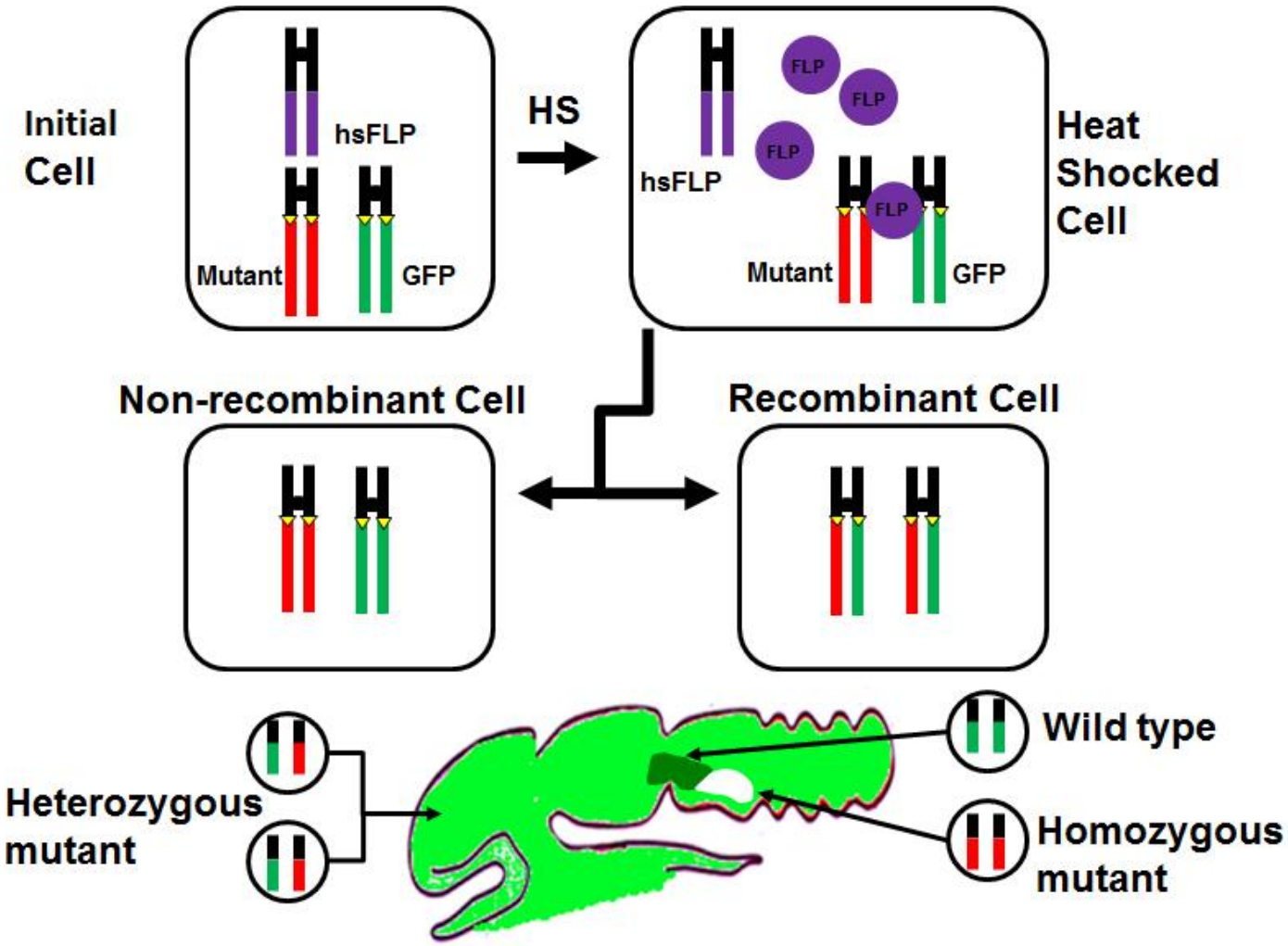


Figure 11 FLP/FRT System used in generating loss-of-function clones

The initial cell is heterozygous for the mutation of interest. Heat shocking the cell causes the production of FLP recombinase which can cause a recombination event between two FRT's, yellow triangles. Cells in the fly continue to undergo mitosis. There are three possible genotypes that can be seen after the heat shock in the mosaic tissue of the animal: the original heterozygote cells that do not undergo recombination, recombinant cells that are homozygous for the mutation or cell that are homozygous for the wild type allele. Homozygous mutants are identified by the loss of the GFP marker and the wild type having two copies of GFP. Recombinant calls are called clones because they are derived from a single cell that had undergone recombination.

allele (Mardon et al., 1994). LOF clones unable to respond to Dpp signals were made by expressing the null allele *tkv*^{a12}, a co-receptor, in the cross *Ubi-GFPFRT40A and yhsFLP;FRT40Atkv*^{a12} (Nellen et al., 1994, 1996). In the Wg pathway the intracellular mediator dsh LOF, dsh^{3} is a null allele, was generated by the cross [*wdsh3FRT19A/FM7a and ywGFPhsFLPFRT19A/FM7a*] (Geer et al., 1983). A second LOF experiment for Wg was conducted by knocking out arr a co-receptor, arr2 is a null allele with this cross *[w;Ubi-GFPFRT42D/CyO and FRT42Dpwn,arr*^{2}*]* (Giorgianni & Mann, 2011; Lawrence et al., 2002). To generate clones compromised in response to both Wg and Dpp signaling, *Mad*$^{1.2}$*wg*^{cx4}*FRT40A/CyO* was crossed to *w;Ubi-GFP,FRT40A/CyO.* Mad$^{1.2}$ is a strong hypomorphic allele and *wg*^{cx4} is a null allele (Lecuit et al., 1996; Wiersdorff et al., 1996) (Baker, 1988). LOF *drm* clones were made by crossing yw;*drm*^{3} FRT40A/CyO and *w;Ubi-GFPFRT40A/CyO.* The *drm*^{3} mutation is a strong hypomorphic allele (Green et al., 2002). Double loss of function clones for drm and bowl were created by crossing yw;*drm*^{3}*bowl*^{1}FRT40A/CyO and *w;Ubi-GFP FRT40A/Cyo.* The bowl^{1} mutation is a null allele (Wang and Coulter, 1996).

2.4 Generation of gain of function clones

To generate a clone that ectopically expresses a gene of interest, the UAS-Gal4 and FLP/FRT systems were combined into one experiment (Blair, 2003; Struhl and Basler, 1993). The basic components of this system include: a transcriptional terminator flanked by two FRT sites, FLP recombinase under control of heat-shock, the Gal4 transcription unit downstream of the FRTs, a UAS sequence attached to the gene of interest, and a marker under the control of a UAS sequence. (Figure 12) The FRTs flank the TT. In a non-recombinant cell the TT prevents the production of Gal4 and the

subsequent expression of the target gene under UAS control along with the marker GFP. The FLP is heat-shock inducible and when expressed randomly causes a recombination even in the cells it is expressed in. The Gal4 line used for this experiment is ubiquitously expressed and not an enhancer trap.

GOF clones were generated for this study using the system just described. Second and third instar larva were heat shocked for 30-60 minutes at 37°C. *dac* clones were made in larvae of the genotype *ywhsFLP;AyGal4,UAS-GFP;UAS-dac*. Clones expressing *Dll* were in larvae with genotype *ywhsFLP;AyGal4, UAS-GFP,UAS-Dll*. *Bab1* clones were made in larva *ywhsFLP;AyGal4,UAS-GFP/UAS-Bab-1*. *Lim1* clones were induced in *ywhsFLP;AyGal4, UAS-GFP,UAS-Lim-1* larva. Constitutively active Tkv clones were made in larvae *ywhsFLP;AyGal4, UAS-GFP,UAS-Tkv^{QD}*. The double GOF clones for *Arm* and *Tkv* were made in larvae of the genotype *ywhsFLP/UAS-arm^{S10}; UAS-tkv^{QD}/AyGal4, UAS-GFP*. Clones for *en^{RNAi}* were made in larva with the genotype *ywhsFLP;AyGal4, UAS-GFP,UAS-en^{RNAi}.* Clones for *en* were induced in *ywhsFLP;AyGal4, UAS-GFP;UAS-en* larvae. To test the CRM for response to *Tkv,* clones were induced in *ywhsflp*; *AyGAL4, UAS-CD2/ UAS-tkv^{QD};* CRM E-GFP larva. To test the CRM for response to *En,* clones were induced in *ywhsflp*; *AyGAL4, UAS-CD2; UAS-UAS-en*/CRM E-GFP larva. To test the CRM for response to *Bab,* clones were induced in *ywhsflp*; *AyGAL4, UAS-CD2/UAS-Bab1;* CRM E-GFP larva. *Drm* clones were made in larvae *ywhsFLP;AyGal4, UAS-GFP;UAS-drm*. Clones for *lines^{RNAI}* were induced in *ywhsFLP;AyGal4, UAS-GFP;UAS-*lines^{RNAi} larva. Clones for *Notch^{intra}* were induced in *ywhsFLP;AyGal4, UAS-GFP;UAS-N^{ICD}*. *Odd* clones were made in larva of the genotype *ywhsflp; AyGAL4, UAS-GFP/ UAS-odd.*

2.5 Immunohistochemistry

For antibody staining, 3rd instar imaginal discs and white prepupae aged until 4–6h APF were dissected at room temperature in 1XPBS. Both imaginal discs and prepupal legs were then fixed for one hour, on ice, in a solution of 1 volume of 37% formaldehyde to 9 volumes of fix buffer comprising Pipes [0.1M] pH6.9, EGTA [1mM] pH 6.9, 1% Triton X-100, and MgSO4 [2mM]. Tissue was then transferred for one hour to a blocking solution containing TRIS [50mM] pH6.9, NaCl [150mM], 0.5% NP40, and BSA [5mg/ml]. This and all subsequent steps were conducted at 4°C. After one hour the prepupal legs/discs were put into the antibody solution, wash solution plus the antibody of interest at the correct concentration, overnight for 12-24hours. The next day the discs/legs were washed 4 times, 30 minutes each, in a solution of TRIS [50mM] pH6.9, NaCl [150mM], 0.5% NP40, and BSA [1mg/ml]. Next, the discs/legs were incubated for 2-12 hours in secondary antibody. The discs/legs were then washed 4 times for 15 minutes. Lastly, the tissue was incubated overnight in mount solution, TRIS [50mM] pH 8.8 and 10% glycerol. The next day, discs/legs were mounted on slides in a working PDA solution. The stock PDA solution is 60% Glycerol and PDA [3mg/ml]. The working solution is made in the ratio of 1 volume PDA stock solution to 1 volume TRIS [0.5M] pH 8.8 to 4 volumes dH2O, making the final concentration of PDA [0.5mg/ml] (Carroll and Whyte, 1989).

Primary antibodies used include: mouse-α-Scr [1 :25] (DSHB, 6H4.1-s) / (Glicksman and Brower, 1988), Rat- α-Ci [1 :1] (a gift from R. Holmgren, Motzny and Holmgren, 1995), Rabbit-α-βgal [1 :500] (MP Biomedicals, previously Cappel, polyclonal

serum 1:500)**,** Mouse-α-Dac [1 :5 (DHSB, mAbdac1-1-s) (mAbdac2-3-s) / (Mardon et al., 1994),

2.6 Generation of DNA constructs

CRM fragments A, 5.4kb intronic and the Scr promoter were initially cloned by Stuti Shroff (unpublished) from the RP98-32J3 Bacterial Artificial Chromosome (BAC) (Shroff unpublished). The *Scr* promoter has the NheI at the 5' and BamHI at the 3' end, both of which are in the primers used to clone the DNA fragment. The intronic CRM use the same restriction site on the 5' and 3' ends, KpnI and is located in both of the primers. The A fragment has the KpnI site at the 5' end and NheI on the 3' end. The primers for the A fragment contain the restriction sites used to clone the fragment into the vector. Smaller sub clones of the A fragment also included the restriction sites within the primers. The primers used are in Appendix A. The previously cloned fragments and smaller subclones generated in this study were cloned into the S3aG vector (pS3aG was a gift from Thomas Williams (Addgene plasmid # 31171)) (these features are listed below). Fragments were TOPO cloned into the pCR™2.1-TOPO® TA Vector (Invitrogen). Transgenic animals with site-specific insertions of reporter genes were made via the PhiC31 system (see below). The mini-white gene is used to select transformants after microinjection. An orange eye color indicates transformation. All microinjections were performed by Rainbow Transgenic Flies, Inc.

2.7 Generation of transgenic flies using the PhiC31 system

All transgenic animals created in this work used the PhiC31system (Bateman et al., 2006; Bischof et al., 2007; Groth et al., 2004; Horn and Handler.2005; Oberstein et al., 2005; Thorpe et al., 2000; Venken et al., 2006). The newer PhiC31 system has four distinct advantages over the P-element system used in previous studies. First, DNA integration into the genome is site specific and not random like P-element insertions. Second, the construct inserted is considered permanent as opposed to a P-element which can be moved with the addition of Δ2-3 transposase. Third, it has a higher transformation efficiency than P-elements. Lastly, because all constructs are inserted at the same location, differences in gene expression due to position effects are avoided.

The PhiC31 system has two components: The landing site and the plasmid that will be integrated into the genome. A landing site contains two loxP sites, an RFP marker with a 3x3P promoter and an attP landing site. All the lines that were injected for this study contained a germline specific source of phiC31 intergrase. The S3aG plasmid used in this study has a multiple cloning site (MCS), eGFP reporter with a nuclear localization sequence (GFP is a reporter in this vector not a marker), loxP site, and attB site. This plasmid is injected to the posterior region of a Drosophila embryo. The germline integrase induces a recombination between the attP and attB sites in some animals (Figure 13). The surviving animals are then crossed to *w*⁻ flies and the progeny are screened for transformants. A transformant will have an orange to red eye color.

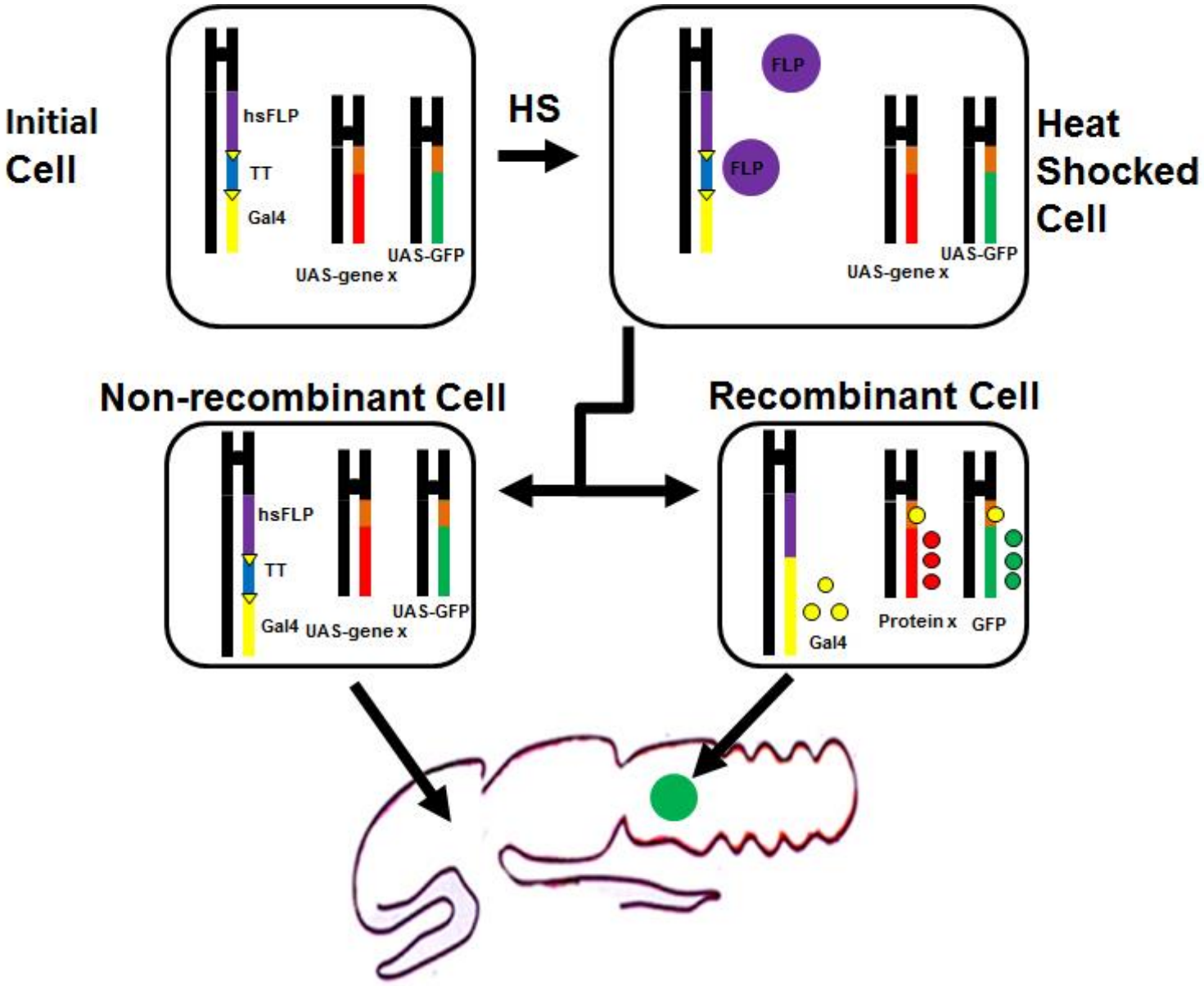


Figure 12 Gain of Function clones generated by the combination of UAS/GAL4 and FLP/FRT systems

The original cell has a transcriptional terminator (TT) preventing the transcription of Gal4 along with the target gene of interest and marker under UAS control. The organism is heat shocked and produces FLP that can cause a recombination event between two FRT sites (yellow triangles) that flank TT. If a recombination event occurs, the TT will be flipped out. These recombinant cells now produce Gal4 that in turn binds the UAS sequence upstream of the gene X and GFP. The GOF clones expressing gene x are marked by the expression of GFP.

2.8 Quantification of GFP reporter constructs

Reporter expression was examined in the 3rd instar leg discs using the ImageJ software. Each disc was analyzed in ImageJ by combining all z-stacks into one image. The GFP intensity was then measured in two separate boxes of equal size on the disc: one in the anterior compartment where there is reporter expression and the second in the posterior region where there is no reporter expression. The mean for the anterior box is divided by the mean for the posterior box to give the relative GFP expression for each leg disc.

2.9 Microscopy

Images were collected on a Zeiss Axiovert 200M equipped with ApoTome or Axio Observer Z1 with Apotome.2 and a digital camera. Fluorescent images were collected as apotomized Z-stacks.

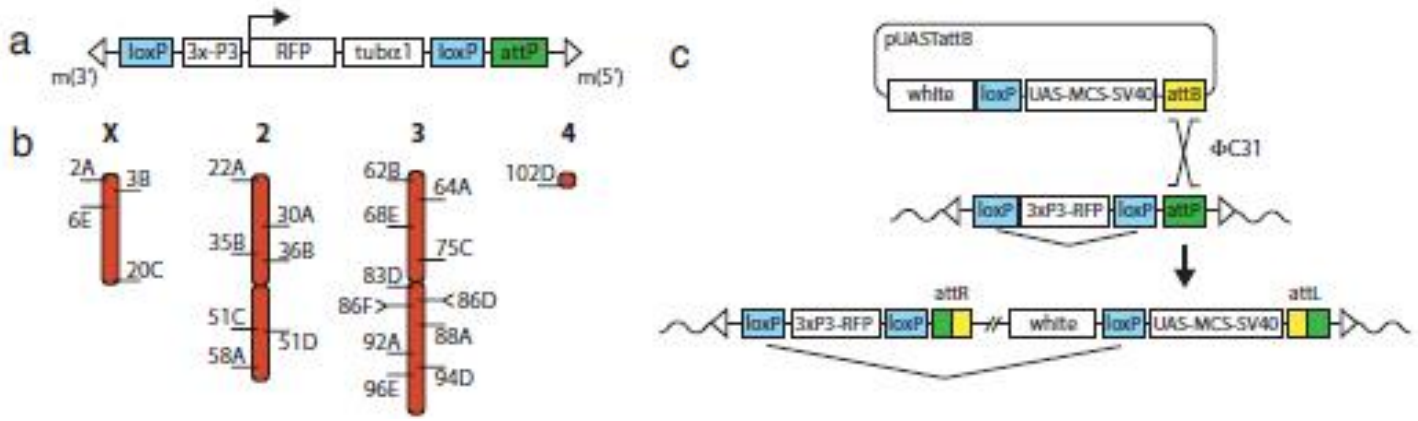


Figure 13 ΦC31 system in Drosophila

The ΦC31 systems is used to make transgenic flies. Landing sites in the fly genome consists of two loxP sites, 3x-P3 promoter, RFP tagged tubα1 and attP site. (A) The fly genome has these landing sites inserted on all four chromosomes that can be identified by the RFP marker in the fly eye. One of these 23 lines is used as the injection line. (B) The plasmid construct contains a mini-white gene, loxP site, an MCS site and attB site. (C) After the plasmid is injected in an embryo the endogenous source of ΦC31 integrase causes a recombination event between the attB and attP sites causing the construct to be integrated into the fly genome. Adapted from Bischof et al., 2007 with permission. Copyright (2007) National Academy of Sciences, U.S.A.

III. *Sex combs reduced* expression in the transverse bristle row primordium of prothoracic legs is established in response to patterning inputs in three dimensions

3.1 Introduction

There is a vast literature that demonstrates conservation of certain signaling pathways and transcription factors responsible for the development of animal appendages. To understand the genetic and molecular mechanisms of pattern formation in these body structures, we use the genetic model organism *Drosophila melanogaster*. The Drosophila adult possesses three pairs of serially homologous legs, one pair on each thoracic segment (T1, T2, T3), which appear similar in size and structure. However, each leg pair ultimately develops unique features, including the patterning of sensory bristles, which are components of the peripheral nervous system (PNS). Several bristle types are observed on the legs, including chemosensory and mechanosensory bristles, which protrude from the legs to sense physical stimuli in the environment. The mechanosensory bristles can be either small or large and are respectively known as microchaetae (mC) or macrochaetae (MC). Our focus is on the generation of distinct mC patterns among legs derived from different thoracic segments with the goal of gaining insight into the mechanisms that generate morphological diversity among the serially homologous legs.

The legs are segmented along the proximal-distal (P/D) axis, consisting of, from distal to proximal, five tarsal segments, the tibia, femur, trochanter and coxa (Fristrom and Chihara, 1978). Variations in mC patterns among the T1-T3 legs are observed in the tarsal segments and the tibia. The middle pair of legs (T2) display what is thought to be

a primitive mC bristle pattern (Held, 2002), consisting of a series of longitudinal rows (L-rows) that are situated at regular intervals around the circumference of the leg. Within the L-rows, which extend along the P/D axis, the bristles are regularly spaced (Figure 6). In contrast, on T1 and T3 legs, a subset of mC are organized in a series of transverse rows (T-rows) that are orthogonal to the L-rows and within which the bristles are positioned directly adjacent to each other. The T-rows are regularly spaced along the P/D axis of the tibia and proximal tarsal segments of T1 and T3 legs, forming brush-like arrangements. On the tibia and the first (basitarsus or ta1) and second tarsal (ta2) segments of T3 legs, T-rows are found in the posterior compartment, replacing one L-row and flanked by two others. On T1 legs, the field of T-rows occurs between two L-rows in the anterior compartment of the basitarsus and tibia (Hannah-Alava, 1958; Held, 2002).

The fact that these bristle patterns are reproducible between flies provides a powerful system in which to perform forward genetic screens to identify the genes involved in sensory organ patterning. This approach has led to the identification of two redundant proneural genes, *acheate* (*ac*) and *scute* (*sc*), both of which encode basic-helix-loop-helix (bHLH) transcription factors (Villares and Cabrera, 1987; Rushlow et al., 1989) and are required for specification of sensory organ precursors (SOPs) (Stern, 1954; Garcia-Bellido, 1978; Garcia-Bellido, 1979). In the Drosophila adult limb primordia, the imaginal discs, *ac* and *sc* expression is required in groups of cells, called proneural clusters, from which SOPs are selected (Romani et al., 1989, Cubas et al., 1991, Skeath et al., 1992). Selection of SOPs from the proneural clusters is mediated in part by the Notch (N) ligand, Delta (Dl), which sends a lateral inhibitory signal from the SOPs to surrounding N-expressing cells (Hartenstein and Posakony, 1990; Simpson, 1990).

As compared to the MC and the chemosensory bristles, the mC are more numerous and are specified later in leg development (Hartenstein and Posakony, 1990; Held, 1990). It follows, then, that *ac* expression in the leg mC primordia is not observed until the mid-prepupal stage, six hours after puparium formation (APF), while expression of *ac* in the proneural fields of the other bristles takes place during larval and early prepupal stages (Orenic et al., 1993). In T2 mid-prepupal legs, *ac* is expressed in a series of longitudinal stripes, alternating with interstripes that lack *ac* expression, all along the leg circumference. This pattern of *ac* expression is established via broad activation of *ac*, which is refined into a periodic pattern via repression mediated by Hairy and Dl/N signaling (Orenic et al., 1993, Joshi et al., 2006). Hairy and Dl exhibit periodic expression that is established prior to *ac* expression in the L-row primordia. Hairy is a bHLH repressor (Ohsako et al., 1994; Van Doren et al., 1994) that is expressed within alternating *ac* interstripes, termed the *hairy*-ON interstripes, and prevents *ac* expression in these domains. The complementary set of *ac* interstripes that do not express Hairy, the *hairy*-OFF interstripes, are established by Dl, which is expressed in stripes that will eventually co-express *ac* and represses *ac* expression in adjoining interstripes by activating the N receptor (Joshi et al., 2006) (Figure 7). The expression of *ac* is later further restricted to the mC SOPs within the *ac*-positive stripes, likely by lateral inhibitory signaling via Dl/N signaling.

In contrast to the L-rows, the T-row proneural fields on T1 and T3 legs are marked by wide domains of ac expression, established in response to differential expression of *Dl* in these legs (Shroff et al., 2007). Thus, two distinct *ac* expressing cell patterns emerge from this developmental program; the eight longitudinal stripes that will give rise to L-rows

and broad domains that will give rise to T-rows. While *Dl* expression overlaps that of *ac* in the L-row proneural fields, in the T-row proneural fields, *Dl* expression is repressed by the Hox transcription factors, Sex combs reduced (Scr) in T1 legs and Ultrabithorax (Ubx) in T3 legs. Repression of *Dl* by Scr and Ubx in T1 and T3 legs, respectively, leads to altered *ac* expression in the ta1 and tibia (Shroff et al., 2007).,

Scr, the focus of this study, plays a critical role at several time points in Drosophila development. Along with several other homeotic genes, *Scr* is first involved in an early stage of embryogenesis in specifying segmental or positional identity along the anterior/posterior (A/P) axis (reviewed in Gellon & McGinnis, 1998; Hughes & Kaufman, 2002; E. B. Lewis, 1978). Hox genes also have a role in specifying positional identity of adult structures. For instance, Ubx specifies a haltere, a T3 limb, versus wing, a T2 limb, fate (Lewis, 1978; Weatherbee et al., 1998; White & Akam, 1985) by suppressing wing development in the haltere. Whereas Scr specifies the first thoracic segment, which includes the T1 pair of legs, Ubx specifies the T3 legs (Lewis et al., 1980; Struhl, 1982). In the case of *Scr*, there are two levels of expression; a low level of expression that presumably specifies the T1 leg fate and a second upregulated region of *Scr* expression that is coincident with the domains of Dl repression, which is responsible for T-row formation in the adult T1 legs (Glicksman and Brower, 1988; LaJeunesse and Shearn, 1995; Shroff et al, 2007). Coincidentally, in the absence of *Scr* function, the T1 leg morphology looks like the primitive pattern of L-rows in the T2 leg. The same morphological change takes place in the absence of *Ubx* in the T3 leg (Shroff et al., 2007).

Given the pivotal role that *Scr* plays in development of the T1 leg morphology, understanding how this highly conserved Hox gene is itself regulated poses an important

question. This study focuses on the regulation of *Scr* expression in the T-row primordia. Specifically, we investigated the genetic mechanisms by which *Scr* is regulated along all three axes of the prepupal leg. Candidate regulators of *Scr* expression were identified based on their coincident expression patterns relative to the upregulated domains of *Scr* expression. As summarized in Table 1, potential regulators along the P/D axis include the transcription factors, Distalless (Dll), Dachshund (Dac), the redundant Bric-a-brac (Bab) proteins, Bab1 and Bab2. Potential regulators of *Scr* expression along the A/P and D/V axes that lead to patterning along the leg circumference include the transcription faction Engrailed (En) as well as Decapentaplegic (Dpp) and Wingless (Wg) signaling pathways as exemplified in Figure 14. Confirmation and further characterization of the function of these potential *Scr* regulators was investigated through genetic analysis, as described below.

3.2 Results

3.2a *Sex combs reduced* expression in the prothoracic leg T-row primordium is activated and refined by proximal/distal leg patterning genes

Along the P/D axis of the T1 leg, upregulated *Scr* expression in the T-row primordia is confined to the distal half of the tibia and the basitarsus. Two candidate regulators of *Scr* expression are Dac and Dll, which specify distal and proximal leg fates, respectively (Cohen et al., 1993; Galindo et al., 2002; Simcox et al., 1989; Mardon et al., 1994). Expression of Dll, a homeodomain transcription factor (Cohen et al., 1989), extends from the distal tip of the leg disc to the mid-tibia, while Dac, a transcriptional co-factor, is

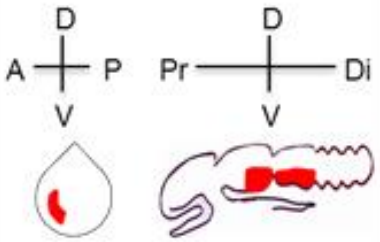
Potential Regulator	Expression in the prothoracic Drosophila leg	Diagramed at L3 & 6 hAPF
Sex combs reduced (Scr)	T1 L3 disc expression and adepithelial expression of T1-T3 L3 discs (Martinez-Arias et al., 1987); low expression throughout the entire T1 L3 disc, elevated expression in an antero-ventral crescent, and adepithelial expression in T1-T3 L3 discs (Glicksman & Brower, 1988); upregulated expression in the T1 prepupal leg in antero-ventral domains of the distal tibia and ta1 (Shroff et al., 2007).	
Distalless (Dll)	Imaginal limb primordia of embryonic T1-T3 segments (Simcox et al., 1991); medial and central L3 disc expression, corresponding to prepupal distal tibia through ta5 (Lecuit & Cohen, 1997; Mann, 2014).	
dachound (dac)	Proximal and central L3 disc expression, corresponding to prepupal trochanter through ta2 (Lecuit & Cohen, 1997; Mann, 2014).	
bric-a-brac (bab)	Distal L3 disc expression (Godt et al., 1993) and graded expression in prepupal legs, highest in ta4 and lowest in ta1 (Couderc et al., 2002).	
engrailed (en)	Specifies the posterior compartment of the discs and prepupal legs (Brower, 1986).	
decapentaplegic (dpp)	Strong dorsal and weak ventral expression along the A/P boundary (Brook & Cohen, 1996); specifies dorsal fate.	
wingless (wg)	Strong ventral expression along the A/P boundary (Baker, 1988); specifies ventral fate.	

Table 1 Comparison of upregulated Scr expression to its potential regulators in the prothoracic leg

Upregulated Scr in the tibia and basitarsus of the T1 when compared to other genes known to pattern the leg can provide foresight as to possible regulators of Scr. The six potential regulators shown here are based on the expression patterns established in previous literature in the third instar leg disk and a prepupal leg at 6 hAPF. Leg imaginal discs are depicted dorsal up, ventral down, anterior to the left, and posterior to the right. The prepupal legs are depicted proximal to the left, distal to the right, and dorsal up, ventral down. Adapted with permission from Emily Wyskiel.

expressed from the proximal leg through ta2 (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994; Gorfinkiel et al., 1997). Dll and dac expression overlap in the medial leg coinciding almost precisely with upregulated *Scr* expression (Dong et al., 2001; Mann, 2014). This suggests these two transcription factors might be involved in *Scr* regulation. To explore this possibility, *Scr* expression was assayed in mutant clones that lacked either *dac* or *Dll* function in prepupal leg discs between 4-6 hrs. APF (unless otherwise stated, all mutant clones in this study were analyzed in prepupal legs). Loss of *dac* or *Dll* function in the T-row primordia resulted in compromised upregulated Scr expression (Figure 15 A-B'') (Shroff and Orenic, unpublished). This demonstrates that both Dac and Dll are required for *Scr* expression. Based on these data, we suggest that Dac and Dll function combinatorially to activate upregulated *Scr* expression.

If co-expression and function of *d*ac and *Dll* is required to activate *Scr,* this implies that co-expression of the two genes at ectopic sites along the P/D axis should result in ectopic upregulated *Scr* expression. This was tested by generating gain-of-function (GOF) clones for each gene. GOF clones expressing *dac* in the distal tarsal segments (ta3-5), where only *Dll* is normally expressed, leads to ectopic *Scr* expression (Figure 16 A-A''). Whereas, clones expressing *dac* in the posterior compartment of the basitarsus and ta2 segments do not show ectopic *Scr* expression. This is likely due to repression by Engrailed (En) in the posterior compartment and will be discussed in section 3.2d. Furthermore, overexpression of *Dll* in the second tarsal segment (ta2), where the *dac* and *Dll* genes are normally co-expressed but *Scr* is not activated, leads to ectopic *Scr* expression (Figure 16 B-C''). We postulate that overexpression of Dll in ta2 may be outcompeting Bab, which normally represses *Scr* expression in ta2 (discussed below).

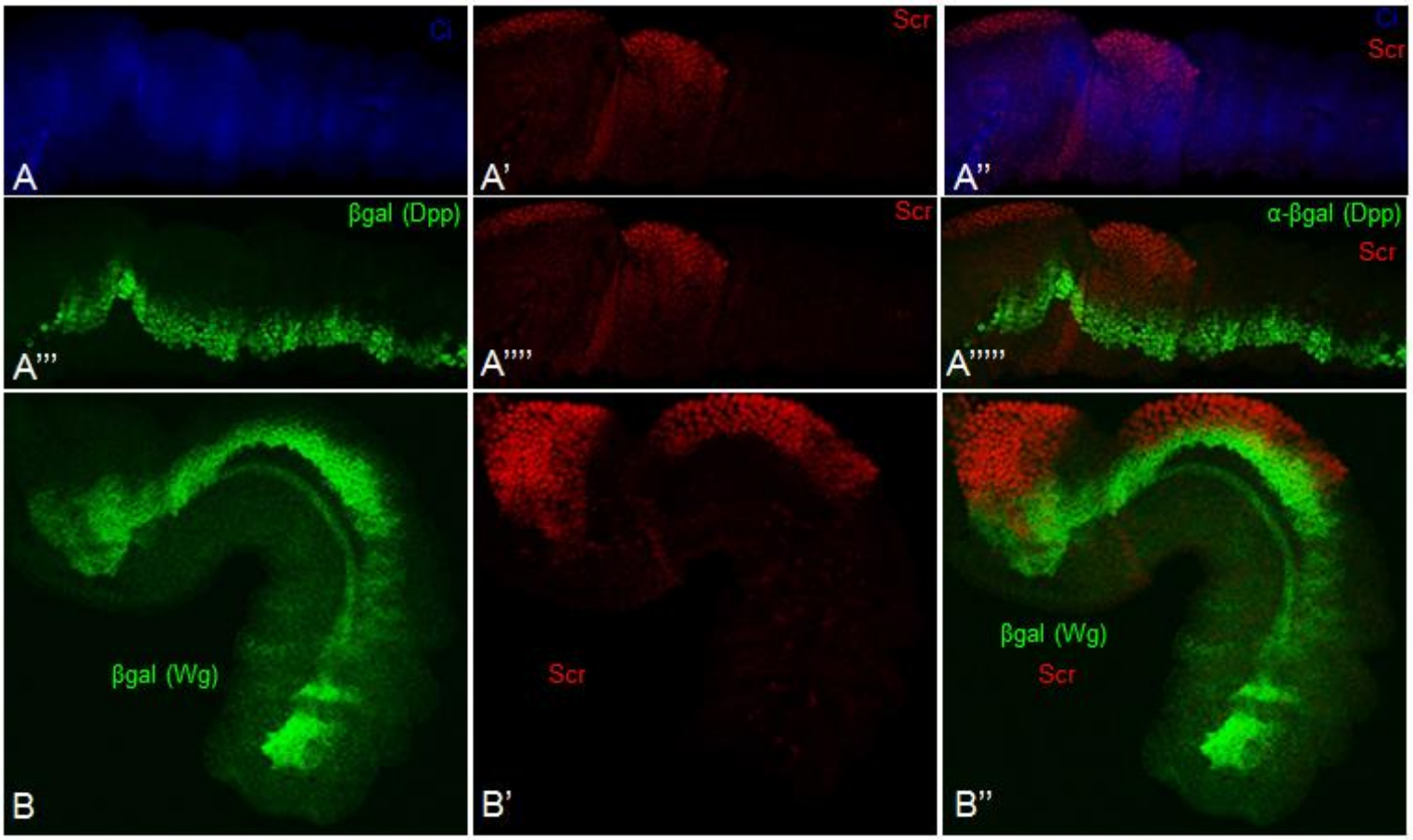


Figure 14 Potential regulatory interactions are suggested by relative expression patterns of upregulated *Scr* expression in prothoracic legs and various regulators of leg development

Prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left and distal is to the right in all panels. (A-A''''') Dorsal aspect of a leg labeled with anti-Scr, anti-Ci and anti-ß-gal to detect *dpp-lacZ* expression is shown in panels A-B''. Scr is expressed in the anterior compartment, marked with anti-Ci (A-A''). *dpp-lacZ* expression marks the dorsal-most leg cells. Note that the Scr expression in the tibia extends less dorsally than does the basitarsal expression (A''-A'''''). (B-B'') Ventral aspect of a leg labeled with anti-Scr and anti-ß-gal to detect *wg-lacZ* expression. The domains of upregulated Scr expression are positioned directly adjacent to *wg-lacZ* expressing cells, implicating Wg signaling in regulation of Scr expression in these regions.

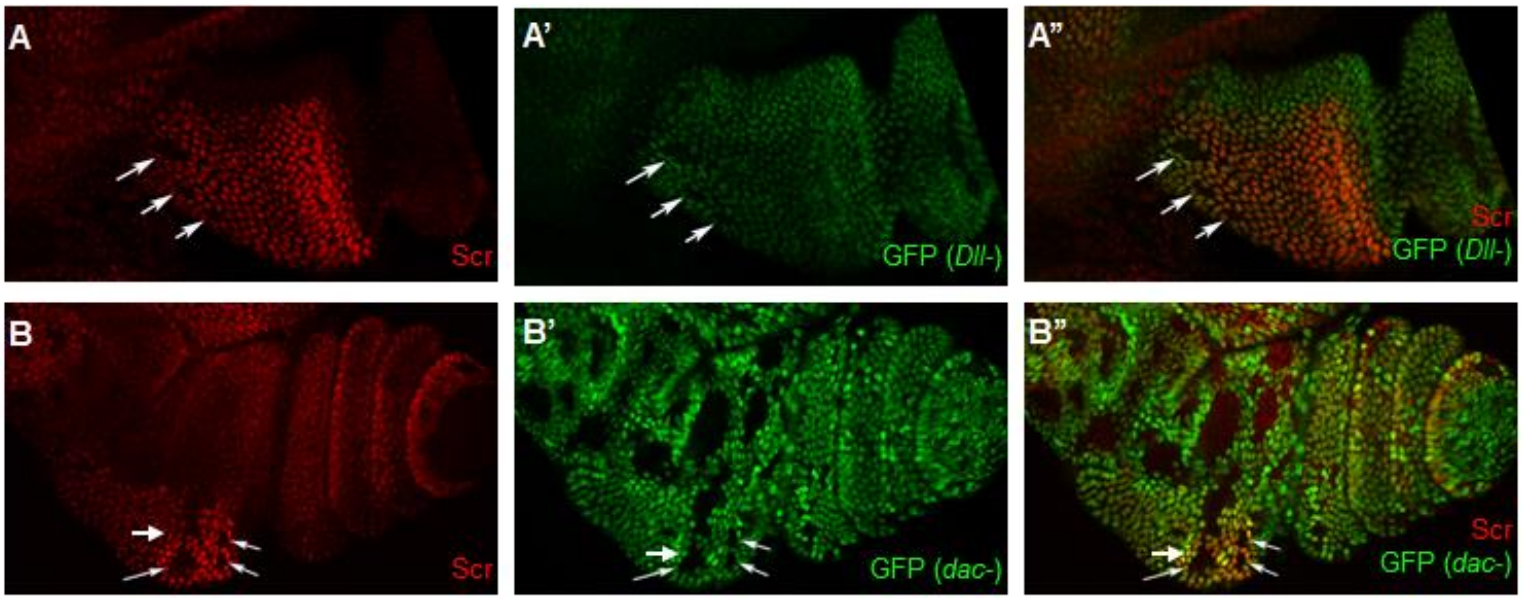


Figure 15 *dac* and *Dll* are necessary for *Scr* expression in the T-row primordium

In all panels, prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left; distal is to the right; dorsal is on top and ventral is on the bottom. LOF clones are marked by the absence of GFP expression. *Dll* clones, marked by arrows, in the basitarsus show loss of Scr expression, visualized with anti-Scr. (A-A'') *dac*$^{-}$ clones, marked by arrows, in the basitarsus show loss of Scr expression. (B-B'') Images courtesy of Stuti Shroff, unpublished.

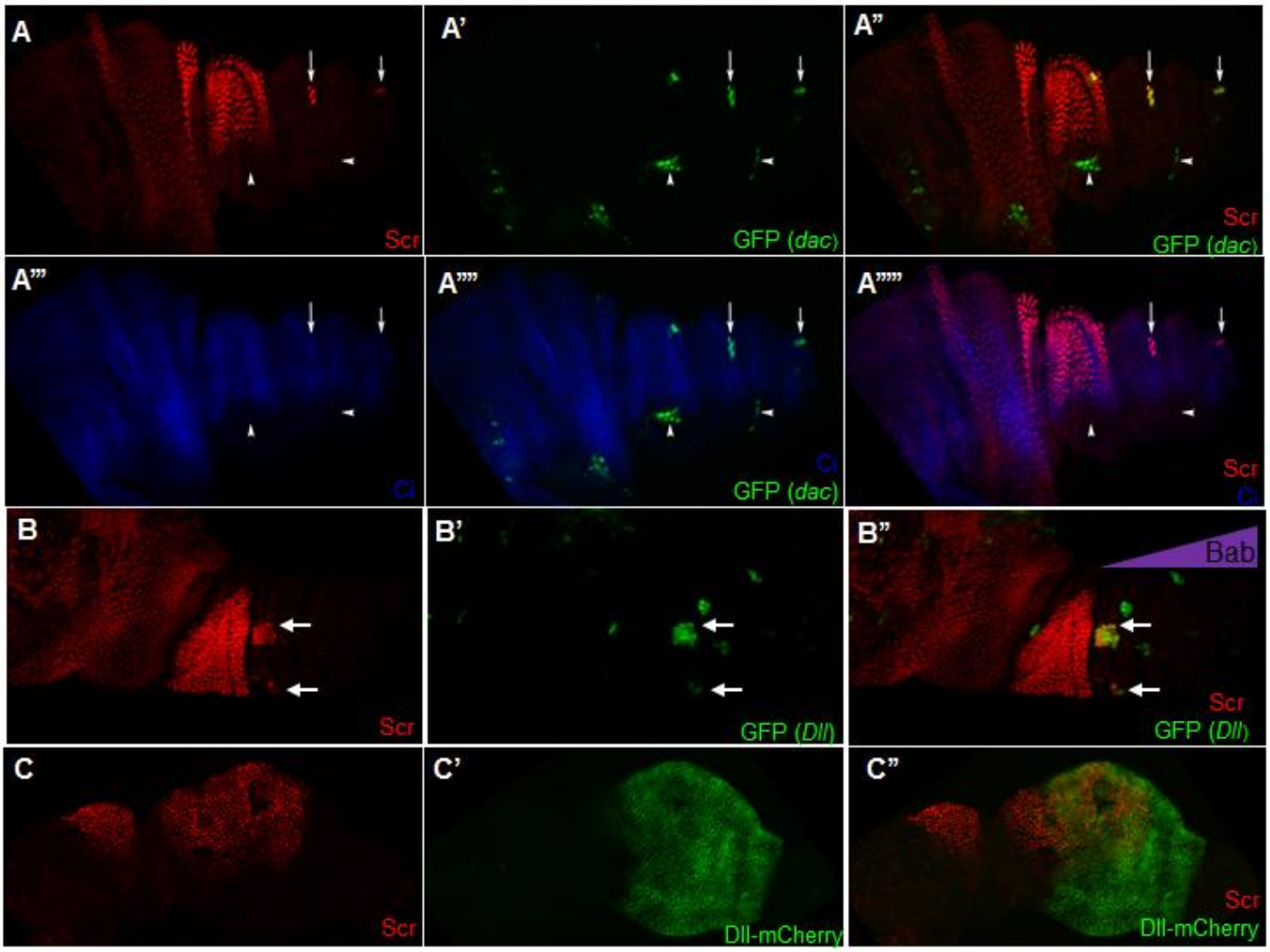


Figure 16 Dac and Dll combinatorialy activate Scr expression

In all panels, prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left; distal to the right, and a ventral aspect is shown. GOF clones are marked by GFP expression. Expression of *dac* in the ta3 and 5, marked by arrows, results in ectopic upregulated *Scr* expression. *dac* clones in the basitarsus and ta3, marked by arrowheads, and in the posterior compartment do not exhibit ectopic *Scr* expression. (A-A''''') Clones expressing *Dll* in the femur and ta2, marked by arrows, express ectopic *Scr*. (B-C'') Dll overexpression, directed by *rotund* (*rn*)-Gal4, in ta4 through distal ta1, marked by mCherry, results expanded expression to the ta2 segment but not into more distal segments. (D-D'')

These results support the hypothesis that *dac* and *Dll* co-expression are required for upregulated *Scr* expression in the T-row primordium.

We also asked whether the redundant *bab-1 and bab-2* genes function in regulation of upregulated *Scr* expression along the P/D axis. *bab1* and *bab2* encode related BTB/POZ transcriptional repressors (Zollman et al., 1994) that are expressed in a gradient that extends from and is highest in the distal ta4 segment up to, but not including, the basitarsus (Godt et al., 1993). We hypothesize that Bab1 and Bab2 refine the distal border of *Scr* expression in the T1 legs. Consistent with this hypothesis, it has previously been shown that *Scr* expression expands into distal segments of the T1 leg disc in a Bab mutant background (Randsholt and Santamaria, 2008). However, this analysis was done with a deficiency that deletes multiple genes, in addition to *bab1* and *bab2*, and it is, therefore, possible that deletion of genes other than *bab1* or *bab2* is responsible for the observed phenotype. Therefore, we asked whether clones expressing ectopic Bab1 in the basitarsus turns off *Scr* expression and found that they did so. The same ectopic *Bab1* expression in the tibia does not inhibit *Scr* expression. (Figure 17). These results suggest that, while the effect of Bab1 depends on the position at which it is expressed within the leg, at least in the basitarsus, Bab1 is a repressor of *Scr* expression but additional examples are needed to confirm this result.

On the proximal boundary, Lim1 expression borders that of *Scr*. The lim1 gene encodes a LIM-homeobox that is expressed in the coxa, femur, distal tip of the tibia and ta5 (Pueyo et al., 2000). Since the expression domains of these two genes meet but do not overlap in the proximal tibia, Lim1 is a good candidate for refining the proximal edge

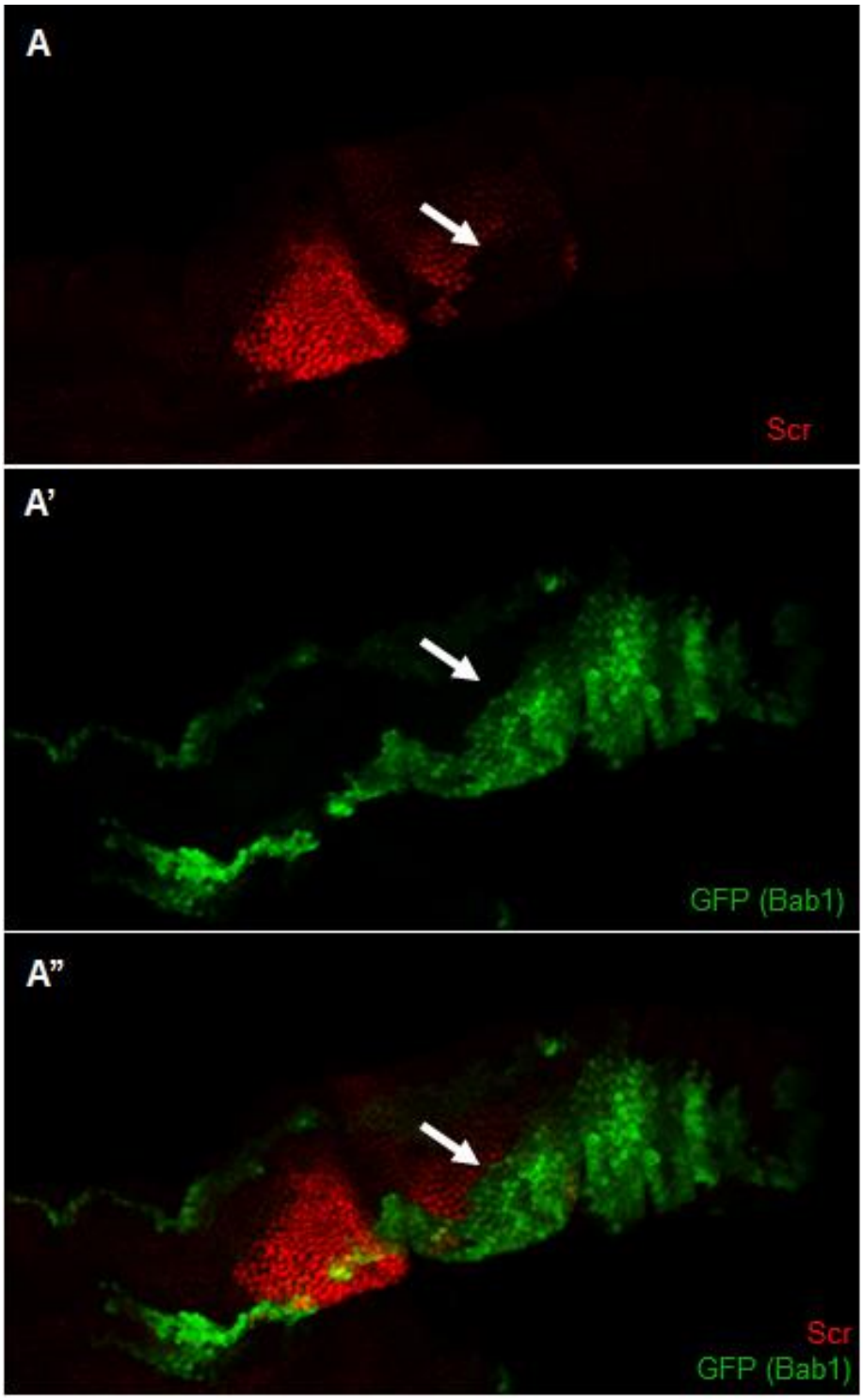


Figure 17 *Bab* represses *Scr* expression in the basitarsus

In all panels, prepupal legs are oriented such that proximal is to the left; distal is to the right; dorsal is on top, and ventral is on the bottom. GOF clones marked by GFP. *Bab1* ectopically expressed, marked by the arrow, in the basitarsus represses *Scr* expression. The same clone extends into the tibia but does not repress *Scr* expression. (A-A'')

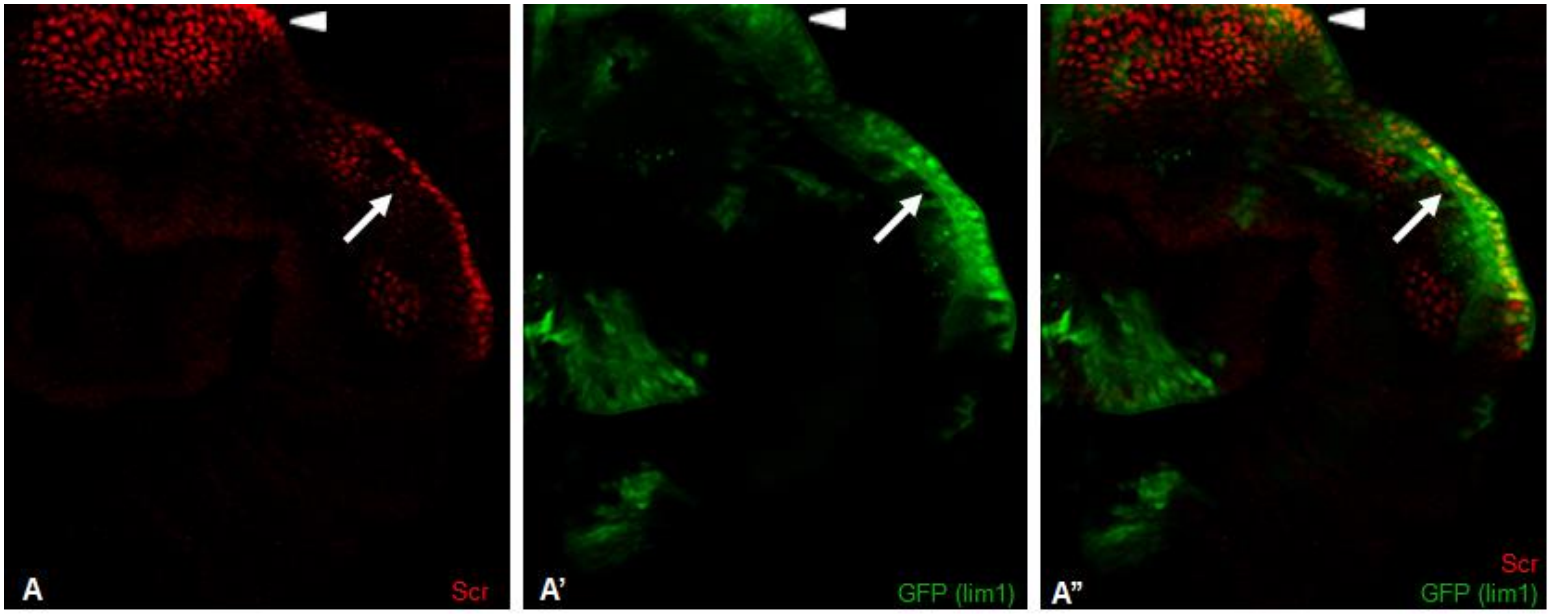


Figure 18 Ectopic lim1 expression represses *Scr* expression in the basitarsus

Prepupal legs are oriented such that proximal is to the left; distal is to the right; and a ventral aspect is shown. GOF clones marked by GFP. *Lim1* ectopically expressed, marked by the arrow, in the basitarsus represses *Scr* expression. (A-A'')

of *Scr* expression. If Lim1 defines the most proximal edge of *Scr* expression in the T1 leg, we would expect ectopic expression of *lim1* to turn off *Scr* expression. To test this prediction a GOF clone expressing *lim1* in the tibia and basitarsus was generated. Under these conditions, Lim1 turns off *Scr* expression in the basitarsus but not the tibia. (Figure 18) These data are consistent with the notion that Lim1 plays a role in defining the proximal boundary of *Scr* expression but additional examples are needed to confirm this result.

3.2b Decapentaplegic refines the dorsal boundary of Sex combs reduced via repression

In prepupal legs, upregulated expression of *Scr* along the T1 leg circumference is restricted to an antero-ventral region. Given that the activators of *Scr* expression, Dll and Dac, are expressed along the entire leg circumference, it is not clear how *Scr* expression is restricted along the D/V axis. A candidate regulator is regulator is the secreted morphogen Dpp, which is expressed in a stripe just anterior to the A/P compartment boundary that is highest in the dorsal leg and lower in the ventral leg. Dpp functions to specify dorsal fates in leg discs via a concentration dependent mechanism (Basler and Struhl, 1994; Diaz-Benjumea et al., 1994; Campbell and Tomlinson, 1995) and could potentially act to restrict *Scr* expression to the ventral leg.

To test the hypothesis that Dpp inhibits dorsal expression of *Scr*, we generated LOF clones that were homozygous mutant for *thickveins* (*tkv*), which encodes a co-receptor for Dpp (Brummel et al., 1994; Ruberte et al., 1995). Loss of Dpp signaling should result in ectopic *Scr* expression in the dorsal T1 leg. Consistent with this prediction,

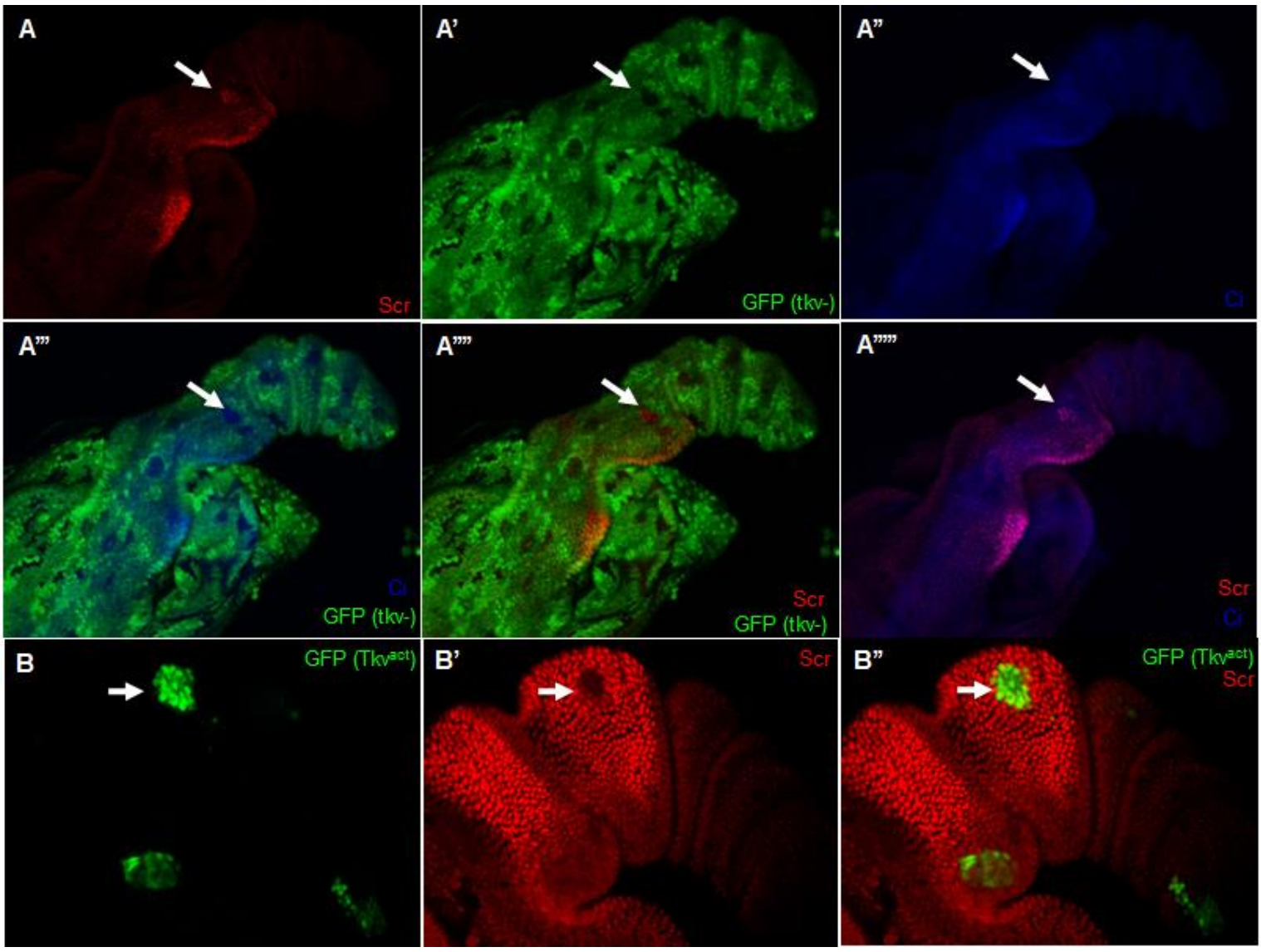


Figure 19 Dpp signaling defines the dorsal boundary of Scr expression in the T-row primordium

In all panels, prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left; distal to the right, and a ventral aspect is shown. LOF Tkv clones are marked by the loss of GFP. The clone in the basitarsus, marked by the arrow, shows ectopic Scr expression. (A-A''''') GOF clones expressing a constitutively active Tkv receptor are marked by GFP which repress Scr expression. GOF clone in the basitarsus, marked by the arrow, represses Scr expression. (B-B'')

tkv loss results in ectopic *Scr* expression in the dorsal basitarsus (Figure 19 A-A''''). This data shows Dpp signaling is required for the repression of *Scr* in the T1 leg dorsal region.

To determine whether Dpp signaling through Tkv is sufficient to repress *Scr*, we generated GOF clones expressing a constitutively active form of the Tkv receptor (Tkv-act) (Casanueva & Ferguson, 2004). Ectopic Tkv signaling in the endogenous *Scr* expression domain should inhibit *Scr*. As shown in Figure 19 B-B'', the clone expressing Tkv-act autonomously represses Scr in the basitarsus. These results confirm that Dpp signaling is necessary and sufficient for the repression of upregulated *Scr* expression in the dorsal leg.

3.2c Wingless signaling is not required for *Sex combs reduced* expression

In addition to Dpp-mediated repression, it is conceivable that establishment of ventrally restricted upregulated *Scr* expression along the D/V axis involves ventral leg specific activation. A candidate factor that might function in concert with Dll/Dac to induce upregulated *Scr* expression is the Wg morphogen, a member of the Wnt family of signaling proteins. Wg is expressed in a ventral stripe adjacent to the A/P compartment boundary (González et al., 1991), opposite to the dorsal Dpp stripe, and functions to specify ventral leg fates (Jackson and Hoffmann, 1994). Several observations are consistent with the hypothesis that Wg might function in activation or maintenance of *Scr* expression. The upregulated domain of *Scr* expression is adjacent to the source of Wg-secreting cells. Furthermore, a subset of clones expressing a constitutively active form of Armadillo (Arm-act), a transcriptional mediator of the Wg pathway, have been shown to

ectopically express *Scr* in the dorsal leg (Wyskiel and Orenic, unpublished and not shown). Finally, expression of Arm-act throughout the dorsal basitarsus results in dorsal expansion of T-rows and sex combs, formation of which requires *Scr* expression and function (Shroff and Orenic, unpublished). Therefore, to determine whether Wg signaling is required for upregulated *Scr* expression, we examined *Sc*r expression in LOF *disheveled* (*dsh*) mutant clones. Dsh is an intracellular mediator of the Wg pathway that is required for response to the Wg signal. Loss of *dsh* has no effect on *Scr* expression (Wyskiel and Orenic, unpublished). We next examined *Scr* expression in clones lacking function of the Wg co-receptor Arrow (Arr) (Giorgianni & Mann, 2011; Lawrence et al., 2002), and, consistent with our observations with *dsh* LOF clones, found that loss of *arr* function no effect on *Scr* expression. (Figure 20). These data indicate that, while ectopic Wg signaling can induce *Scr* expression and formation of T-rows and sex combs, Wg signaling is not required for activation or maintenance of ventral *Scr* expression.

3.2d Decapentaplegic repression of *Sex combs reduced* expression is independent of Wingless

During development, the leg disc is divided into dorsal and ventral territories, which are respectively patterned by Dpp and Wg and which are established and maintained through mutual antagonism between the two signaling pathways. Specifically, Dpp represses *wg* expression in the dorsal territory and vice versa. The mutually repressive interactions between Dpp and Wg raise the possibility that the ectopic expression of *Scr* in *tkv* LOF clones is a consequence of Wg expression in these clones, rather than Dpp-mediated repression. This notion is supported by the observation that Arm-act GOF

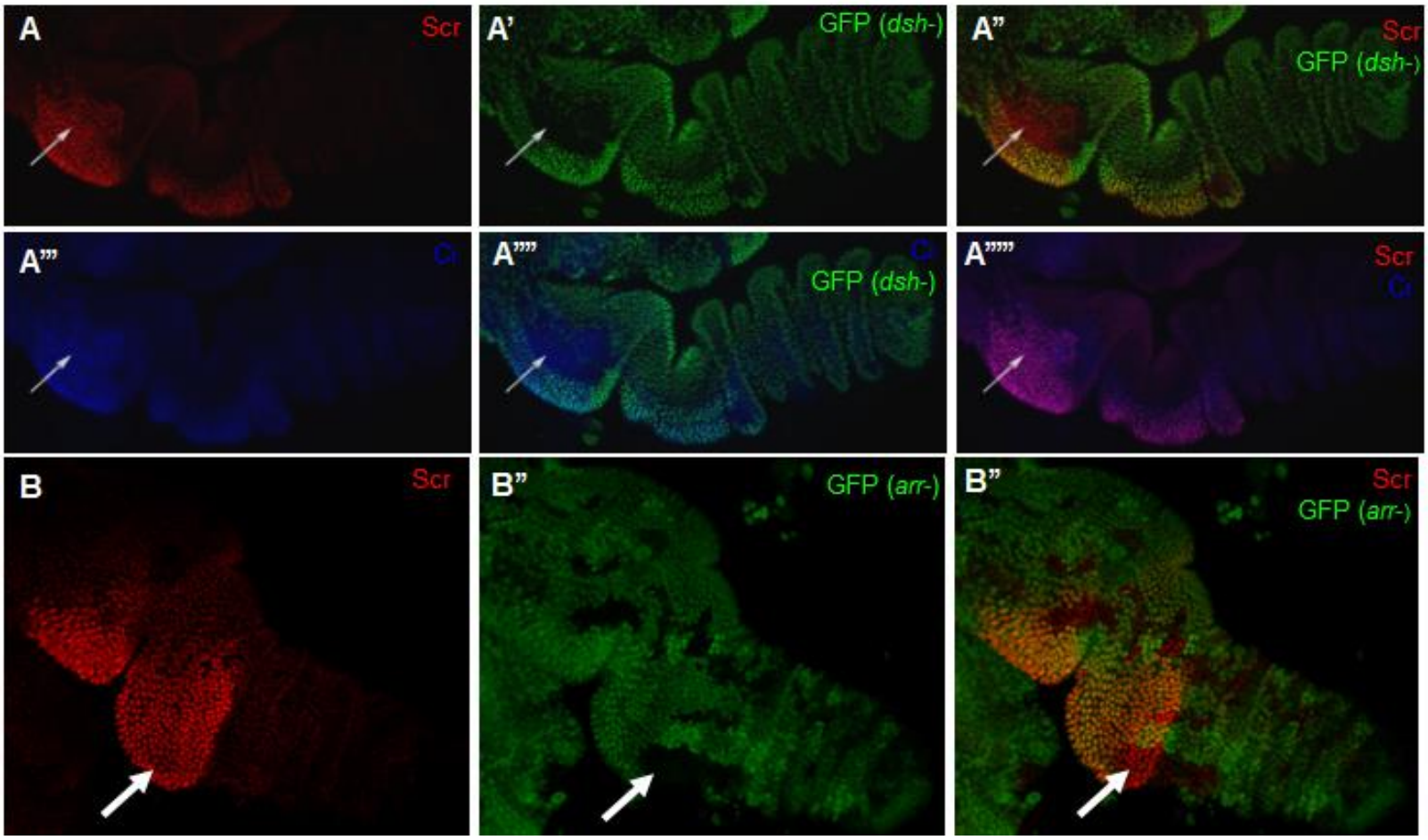


Figure 20 Wg signaling is not required for *Scr* expression

In all panels, prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left; distal is to the right; dorsal is on top and ventral is on the bottom. LOF dsh and arr clones are marked by the loss of GFP. The loss of the Wg intercellular mediary dsh does not result in the loss of Scr expression. (A-A'''') The loss of the Wg signaling, by the loss of one of its receptors arr, does not affect *Scr* expression. dsh images courtesy of Emily Wyskiel unpublished

clones in the dorsal leg can also express *Scr*. To determine whether *Scr* expression in clones that lack Dpp responsiveness is dependent on Wg, we assayed double LOF clones that were mutant for *wg* and *Mad*, which encodes a transcriptional mediator of the Dpp pathway. In the dorsal leg, these clones behaved like the LOF *tkv* clones and ectopically expressed *Scr* (Figure 21 A-A'') (Shroff and Orenic, unpublished). As expected, the converse experiment, in which double GOF clones expressing a constitutively activate form of Armadillo (Arm-act) (Morel and Arias, 2004), a transcriptional mediator of Wg signaling (Couso et al., 1994), together with Tkv-act turned off ventral Scr expression. (Figure 21 B-B''''). These observations suggest that Dpp regulation of *Scr* is independent of Wg and are consistent with our finding that Wg signaling is not required for activation or maintenance of *Scr* expression.

3.2e ***Engrailed*** **restricts** ***Sex combs reduced*** **expression to the anterior compartment**

Scr expression is confined to the anterior compartment of the T1 leg disc and does not expand into the posterior compartment where *en* is expressed. *en* functions as a posterior compartment selector gene, which encodes a homeodomain transcriptional repressor (Crick & Lawrence, 1975). If posterior compartment *Scr* expression is repressed by En, then we would expect ectopic *Scr* expression when *en* function is lost in the posterior compartment. On the contrary, upregulated *Scr* expression in the T-row primordium, should be lost when En is ectopically expressed. To determine whether En regulates *Scr* expression in the T-row primordia, we made LOF and GOF clones. RNAi knockdown of *en* in posterior compartment clones resulted in ectopic posterior *Scr*

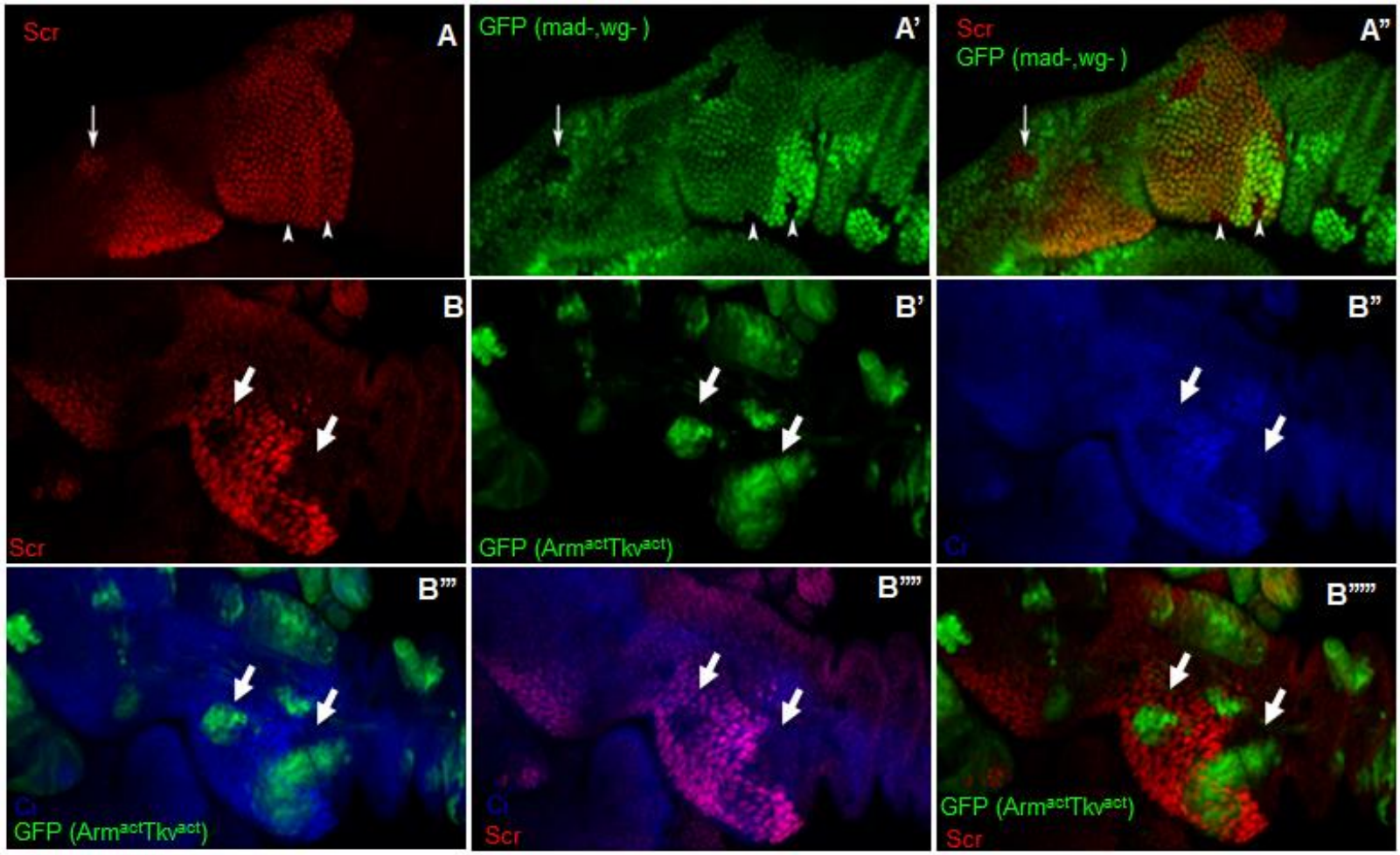


Figure 21 Dpp represses Scr expression in the dorsal region with Wg preventing repression by Dpp in the ventral region

In all panels, prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left; distal is to the right; dorsal is on top and ventral is on the bottom. LOF Mad and wg clones are marked by the loss of GFP. Double loss of function clones show ectopic Scr expression, marked by an arrow. It behaves like a tkv- clone suggesting Dpp is downstream of Wg in the Scr regulatory pathway. The loss of Wg in the double LOF clone indicates it is not required for *Scr* activation. (A-A'') GOF Arm^{act} and Tkv^{act} are marked by GFP and turn off Scr expression. Clones are marked by arrows. (B-B'') Double loss of function pictures courtesy of Stuti Shroff, unpublished

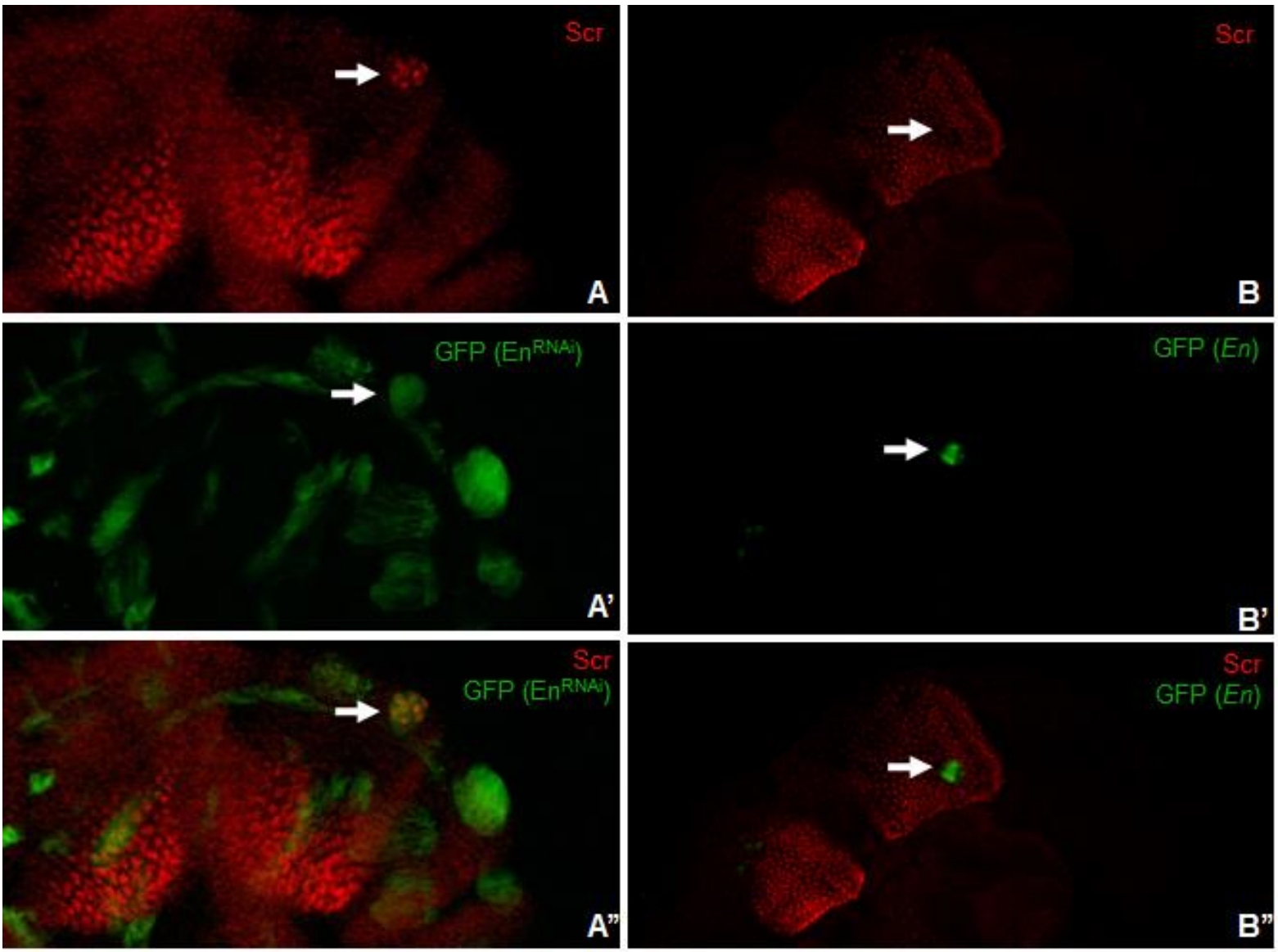


Figure 22 Engrailed restricts *Scr* expression to the anterior compartment

In all panels, prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left; distal is to the right; dorsal is on top and ventral is on the bottom. Both LOF and GOF En clones are marked by GFP. The clone, marked by the arrow, expressing RNAi for En shows ectopic Scr expression. (A-A'') Ectopically expressed En in the T-row primordium turns off Scr expression. The clone marked by the arrow. (B-B'') En^{RNAi} images courtesy of Emily Wyskiel, unpublished.

expression (Figure 22 A-A''). Whereas anterior compartment *En* GOF clones resulted in loss of *Scr* expression (Figure 22 B-B''). Together, these experiments show that En represses *Scr* expression in the posterior compartment of the T1 leg.

3.3 Discussion

3.3a Sex combs reduced is integrated into the leg patterning hierarchy to establish the segment- specific sense organ pattern

Intensive investigations over the last 30 years have elucidated the genetic and molecular mechanisms involved in regulation of Hox gene expression. Many of these studies have been done in the Drosophila embryo in which each of the eight Hox genes is expressed in a specific domain along the A/P axis, specifying parasegmental identity. The Hox genes are also expressed in Drosophila imaginal discs and function to generate distinctive morphologies among appendages derived from different segments. Analyses of the regulatory mechanisms involved in establishing proper spatial and temporal patterns of Hox gene expression have revealed several levels of regulation. In Drosophila embryos, the expression domains of the Hox genes are initially established in response to the maternal and segmentation genes that set up the segmental periodicity characteristic of the larva and adult (Nusslein-Volhard 1991; Lawerence 1992). Also important to defining the domains of Hox gene expression are cross-regulatory interactions among the Hox transcription factors, which involve repression of anteriorly expressed Hox genes by more posteriorly expressed Hox genes (Akam, 1987; Doboule and Morata, 1994; Hafen et al., 1984; Harding et al., 1985). Furthermore, proper

maintenance of Hox gene expression throughout development involves epigenetic regulation by the Polycomb group genes and Trithorax proteins.

An underappreciated aspect of Hox gene expression is that these genes are often differentially expressed within parasegments or organ fields, such as the imaginal discs or vertebrate limb buds. This modulated expression suggests that Hox genes might function in cell fate specification within parasegments or organ fields and, furthermore, that they are subject to regulation by transcription factors and signaling pathways that control patterning within these fields. For example, in the haltere and T3 leg discs, *Ubx* is expressed in all cells, but its expression is strongly elevated in a subset of posterior compartment cells. In T3 legs, this elevated domain of expression is required for development of a group of T3-specific sense organs (Brower, 1987; Pirrotta et al., 1995). Differential Hox gene expression is also observed along the P/D axis of vertebrate limb buds and is required for formation of specific structures along this axis (Zakany and Duboule, 1999; Zakany et al., 1997). The regulatory mechanisms underlying modulated Hox gene expression within organ fields are understudied but bear investigation to provide a comprehensive understanding of how these genes contribute to body plan patterning.

In this study, we addressed the genetic mechanisms involved in establishing differential *Scr* expression within T1 leg imaginal discs. During larval and prepupal stages, two levels of *Scr* expression are observed, low level expression throughout most of the discs and upregulated expression in specific domains that is essential for development of T-rows and sex combs. Here, we identify multiple genes that specifically control upregulated *Scr* expression, implying that expression of *Scr* in the T-row primordia

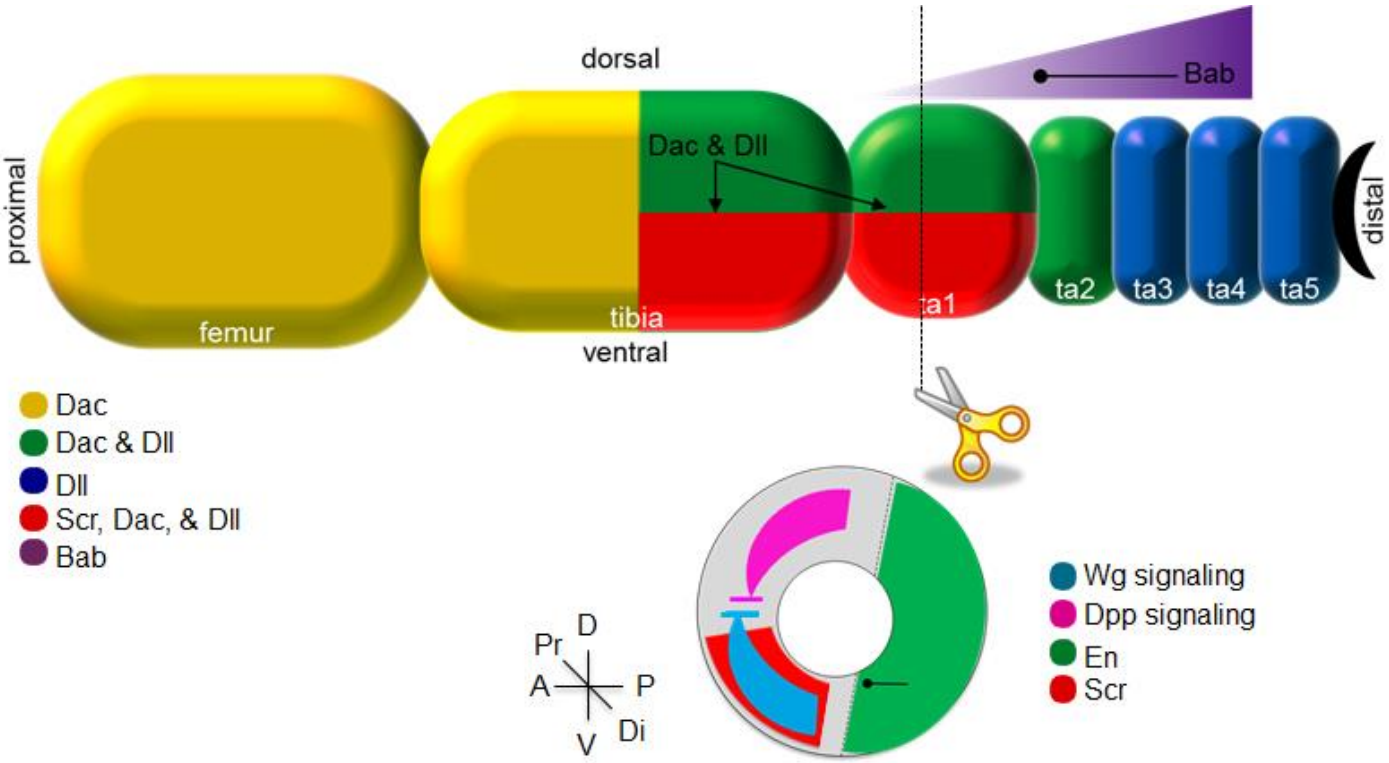


Figure 23 Model for the upregulation of *Scr* expression in the prothoracic leg of Drosophila

The model is oriented proximal to the left, distal to the right, and dorsal up, ventral down. A cross-section of the first tarsal segment at the dotted arrow is diagramed in the circle and is oriented anterior to the left, posterior to the right, and dorsal up, ventral down. Integration of patterning information along all three axes produces the upregulated *Scr* expression (Red) in the antero-ventral territory of the distal tibia and first tarsal segment. Two Pr/Di regulators *dac* (yellow) and *Dll* (dark blue) combinatorically activate *Scr* expression with in the region of overlap (green). Bab (purple) refines the distal expression in the leg by repressing expression in the ta2-5. Regulators along the circumference refine upregulated Scr expression to its antero-ventral domain. *dpp* (pink) directly represses *Scr* expression in the dorsal region with *en* (light green) repressing in the posterior compartment. *wg* (light blue) does not directly activate but prevents repression by *dpp* in the ventral region. Used with permission from Emily Wyskiel.

is regulated independently of the broad low level expression. Our studies show that *Scr* is integrated into the leg patterning hierarchy and is a target of global regulators of leg development, including the signaling pathways and transcription factors that control patterning along the legs' three axes (Figure 23). *Scr* acts as a link between these global and local regulators of sense organ development, including Dl/N and the proneural gene, *ac*. Within the T-row primordia, *Scr* modulates the expression of *Dl*, resulting in a pattern of *Dl* expression that is unique to the T1 leg, which allows expression of *ac* in broad domains that define the T-row proneural fields. Our findings provide a view into the connections between the function of global regulators of development and the formation of specific morphological features, such as the sense organ patterns in the Drosophila adult limbs.

3.3b Wingless signaling is not required for activation of Scr expression

Several observations implicate the Wg signaling pathway in activation of upregulated *Scr* expression in T1 legs. The domain of upregulated *Scr* expression is positioned directly adjacent and partially overlapping the stripe of wg expression in the ventral T1 leg. Furthermore, previous studies in our lab showed that ectopic activation of Wg signaling in the dorsal leg can induce upregulated S*cr* expression. Specifically, GOF clones generated by Emily Wyskiel expressing Arm^{act}, a transcriptional mediator of Wg signaling, showed ectopic upregulated *Scr* expression in the basitarsus. A second experiment, done by Stuti Shroff, in which Arm^{act} was expressed throughout the dorsal basitarsus in the T1 leg, resulted in dorsal expansion of T-rows and sex combs. Given that upregulated Scr expression is necessary for development of these structures, this

would imply that Scr expression was also expanded. These observations led to the hypothesis that Wg is required for upregulated *Scr* expression. However, the *wg* LOF experiments, presented here, establish that Wg signaling is not required for activation or maintenance of upregulated *Scr* expression. This is further confirmed the analyses of clones doubly mutant for Wg and Dpp pathway components.

With regard to regulation of upregulated *Scr* expression, how can the discrepancy between LOF and GOF analyses of Wg pathway components be explained? A likely explanation lies in the mutually repressive interactions between Dpp and Wg and that clonal analyses were almost exclusively used for our studies. Expression of both *dpp* and *wg* is activated near the A/P compartment boundary and requires the function of Hedgehog, which is secreted from the posterior compartment and signals at short-range to a stripe of cells near the A/P compartment boundary. Therefore, when Dpp signaling is compromised in the dorsal leg, ectopic *wg* expression is only observed in clones near the A/P boundary and vice versa. In the ventral T1 legs, we assayed *dsh* and *arr* mutant clones that overlap the upregulated *Scr* expression domain and that are too distant from the source of Hh signal to activate *dpp* expression. Therefore, any effect of these clones on *Scr* expression would be due to loss of Wg signaling, rather than to ectopic Dpp signaling. The observation that *Scr* expression was not affected in such clones indicates that Wg is not required to activate *Scr* expression. In a similar vein, only a subset of Arm-act clones, those near the A/P boundary, were able to induce *Scr* expression. It is likely that repression of *dpp* expression in these clones relieved Dpp-mediated repression of *Scr*. Our findings suggest that the only function of Wg in regulation of *Scr* is to prevent Dpp-medicated repression of upregulated *Scr* expression in the ventral leg. However, a

formal demonstration of this would require analysis of *Scr* expression in T1 legs that are wholly mutant for *wg*, in which we would expect loss of upregulated *Scr* expression due to ventral expansion of *dpp* expression.

3.3c Activation of *Sex combs reduced* expression

What then is responsible for the upregulation of Scr in the T-Row primordia? The overlapping expression patterns of two P/D patterning genes, Dac and Dll, with *Scr* in the medial region of the T1 leg made them potential candidates. The subsequent genetic data in this study indicates that these two genes are both necessary and sufficient for the upregulated expression of *Scr* in the antero-ventral region of the T1 leg.

While other Dll targets have been identified we believe this data is the first of its kind to identify *Scr* as a target gene of both Dac and Dll. While, Dac is a nuclear factor that is not well understood (Caubit et al., 1999; Mardon et al., 1994), Dll is known to be expressed early in leg development and is required for proper development. Dll has also previously been shown to act as a transcriptional activator. Specifically, Dll along with a second transcription factor, Rotund, bind to a cis-regulatory module of bab2, which directs expression in legs, to initiate transcription (Baanannou et al, 2013). This data is good evidence that Dll could act in the same manner to regulate *Scr* in the T-row primordia of the T1 leg.

Scr activation by Dac and Dll could happen by several potential mechanisms. The two proteins might function in a common complex to activate *Scr* in the T-row primordia. This is similar to a known function for Dac in the Drosophila retinal determination gene

network. In this process, Dac is part of a complex with the transcription factors Eyes absent (Eya) and Sine oculis (So). The Eya/Dac complex is known to be necessary for eye development but the role of Dac is not well characterized (Hanson, 2001). Alternatively, as Dac is known to function as a co-repressor, Dac might prevent repression of *Scr* in the basitartus, through its repression of Bab (Mardon et al., 1994).

3.3d. Spatially defined Sex combs reduced expression is defined by repressive inputs in three dimension.

Expression of *Scr* in the Drosophila leg is regulated along all three axes of the developing leg, the P/D. A/P and D/V axes. Although upregulated *Scr* along the P/D axis is activated by Dac and Dll, both of these transcription factors are expressed symmetrically along the leg circumference, which has two axes, A/P and D/V, suggesting that *Scr* expression must be further refined by repressive inputs along the A/P and D/V axes.

This study provides genetic evidence that En restricts *Scr* expression to the anterior compartment, by repressing expression in the posterior compartment. Specifically, clonal loss of En in the posterior compartment resulted in ectopic Scr upregulated expression. Conversely ectopic anterior expression of En repressed Scr expression. En, which is a known transcriptional repressor that specifies the posterior compartment in all three Drosophila leg pairs, is a homeodomain protein (Brower, 1986). Given that En and Dll bind to the same core homeodomain binding motif present in the *Scr* cis-regulatory module (Noyes et al., 2008), this raises the possibility that En may

repress posterior *Scr* expression by out competing Dll binding, which would otherwise activate *Scr* transcription.

The D/V P extent of *Scr* expression is regulated by Dpp-mediated repression. Dpp expression is expressed in the dorsal region along the A/P compartment boundary, and has been previously shown to act in a concentration dependent manner (Jackson and Hoffmann, 1994). *Scr* appears to show regional differences in response to the Dpp gradient, in that the *Scr* antero-ventral expression pattern is further from the A/P compartment boundary in the tibia than in the basitarsus (Figure 14). Alternatively, the Dpp gradient might be shaped differently in the tibia vs basitarsus, resulting in the observed differences in *Scr* expression.

Along the P/D axis, we have shown that *Scr* expression is limited by the transcription factor Bab on the distal edge of the basitarsus and is excluded from ta2, despite the coexpression of the Src activators, Dll/Dac. This was determined using a GOF Bab clone that repressed *Scr* expression in the basitarsus but not in the tibia. The repression of Scr by Bab is consistent with a previous study by Santamaria (Randsholt and Santamaria 2008) in which a heterozygous *bab1*; *bab2* double mutant leg exhibited expansion of *scr* expression into more distal tarsal segments. Bab is expressed in a concentration gradient from ta4 to ta2 that decreases in the proximal direction. Our data suggest that in ta2, overexpression of Dll can outcompete Bab resulting in ectopic ta2 *Scr* expression. These data imply that Bab represses *Scr* expression in a concentration-dependent manner, and that in ta2 Dll/Dac activation overcomes Bab-mediated repression. This mutually exclusive expression pattern between Dac and Bab is consistent with their functional relationship in another leg development process, in which

a LOF mutation in the gene, Lines downregulates Bab expression, resulting in Dac expression where Bab is usually expressed (Greenberg and Hatini, 2009).

In summary this study identifies regulators of *Scr*, suggesting a model for the upregulated expression pattern observed in the T1 leg (Figure 22). The upregulated *Scr* expression modulates the patterning of mCs on the T1 leg, as compared to T2 and T3 legs, resulting in formation of T-rows and sex combs in the antero-ventral T1 leg. This expression domain is established via activation by the P/D patterning genes, Dll and Dac and refined by Several known repressive inputs along all three axes, with the proximal edge defined by Lim1 and the distal edge by Bab. Dpp restricts *Scr* expression in the dorsal region and presumably, Wg prevents Dpp repression of *Scr* in the ventral region. The *Scr* expression is further restricted to the anterior compartment by En giving Scr a refined expression domain in the antero-ventral region of the T1 leg. As will be discussed in the next chapter, T-row expression of *Scr* is directed by two CRMs, one of which also directs expression in the sex comb.

3.3e Future studies

This genetic investigation of *Scr* raises additional questions, such as: What is the molecular mechanism through which Dll and Dac together activate *Scr* expression? Since Dac is known to function as a cofactor in transcriptional complexes (Tavsanli et al., 2004), the next experiment would be a co-immunoprecipitation to see if the two transcription factors are in the same transcriptional activation complex. In addition, DNA binding analyses, either via ChIP or EMSAs, combined with functional analyses of putative

binding sites could be performed to further understand the molecular mechanism through which Dac and Dll regulate *Scr*. If either or both bind the same cis-regulatory module(s), it would suggest direct regulation by each of these genes. Although Dac is known to function as a transcriptional co-repressor, many transcription factors can function in both activation and repression. Therefore, it would be of particular interest to determine if Dac acts as a direct activator of *Scr* or whether it acts indirectly via repression of Bab. Secondly, what, if any, other genes are involved in the differential regulation of *Sc*r in the tibia and basitarsus? Leg development is a complex process controlled many genes, and this study has only examined a handful.

IV. Identification of two cis-regulatory modules that direct *Sex comb reduced* expression in prothoracic legs

4.1 Introduction

The distinctive anterior/posterior (A/P) patterning of Drosophila morphology during development is achieved by the differential expression and function of eight homeobox containing genes called Hox genes. They are first expressed during embryogenesis where they confer region-specific identity along the A/P axis (Gellon and McGinnis, 1998; Hughes and Kaufman, 2002; E. B. Lewis, 1978). Expression of the Hox genes is then maintained in each segment of the developing animal and continues until adulthood directing development of organs, limbs and secondary characteristics such as bristle patterns (Lewis, 1978; Weatherbee et al., 1998; White and Akam, 1985). Although this developmental scheme is highly conserved across species, the spatio-temporal regulation of Hox gene expression remains to be full elucidated.

During early embryogenesis Scr and Ubx specify the first and third thoracic segments, respectively, of the developing fly embryo (Lewis et al., 1980; Struhl, 1982). These two genes also function on a local level during the postembryonic development of the T1 and T3 legs to promote specific bristle morphologies. This process requires the differential expression of these genes within the T1 and T3 leg primordia, the imaginal discs. Specifically, *Scr* and *Ubx* expression is elevated during larval and prepupal stages within the primordia of a subset of mechanosensory bristles, the T-row , and this upregulated expression is required for the specification of T-row bristles and,. In the case of *Scr*, there are three levels of expression during leg development: low-level expression throughout most of the disc, elevated expression in the T-row primordia in larvae and

prepuae, and super-elevated expression in the sex comb primordia in pupae. We have established that the refined upregulation specifically of *Scr* expression in the T1 leg disc occurs during the prepupal stage by the concerted actions of several transcription factors including Dac, Dll, Bab, and En as well as Mad, which acts downstream of Dpp signaling. The focus of the present study is to gain a further understanding of how this complex *Scr* regulation during prothoracic development is orchestrated at the level of *Scr* transcriptional control.

Gene expression is transcriptionally regulated through the activity of cis-regulatory modules (CRM) and their interactions with promoters. These CRMs can be relatively close by or 100's of kb away from the transcription start of a gene. In the case of the *Scr* locus, a previous study by Gindhart and Kaufman (1995) identified eighteen DNA fragments that exhibited *Scr* enhancer activity. Of these, three fragments are of interest because they direct *Scr* reporter activity in the prothoracic segment where we have shown *Scr* is upregulated to produce T-rows and the sex comb in male flies. These potential CRMs include a 5.4kb BamHI located in the second intron of the *Scr* gene, a 3.7kb HindIII fragment 20kb upstream of the transcription start for *Scr* and a 10kb fragment 40kb upstream of *Scr* (Figure 24). This study focuses on the 5.4kb and 10 kb fragments. The goal of the this study is to determine if either of these CRMs control the upregulated *Scr* expression that we have previously established is required for T-rows and sex comb formation.

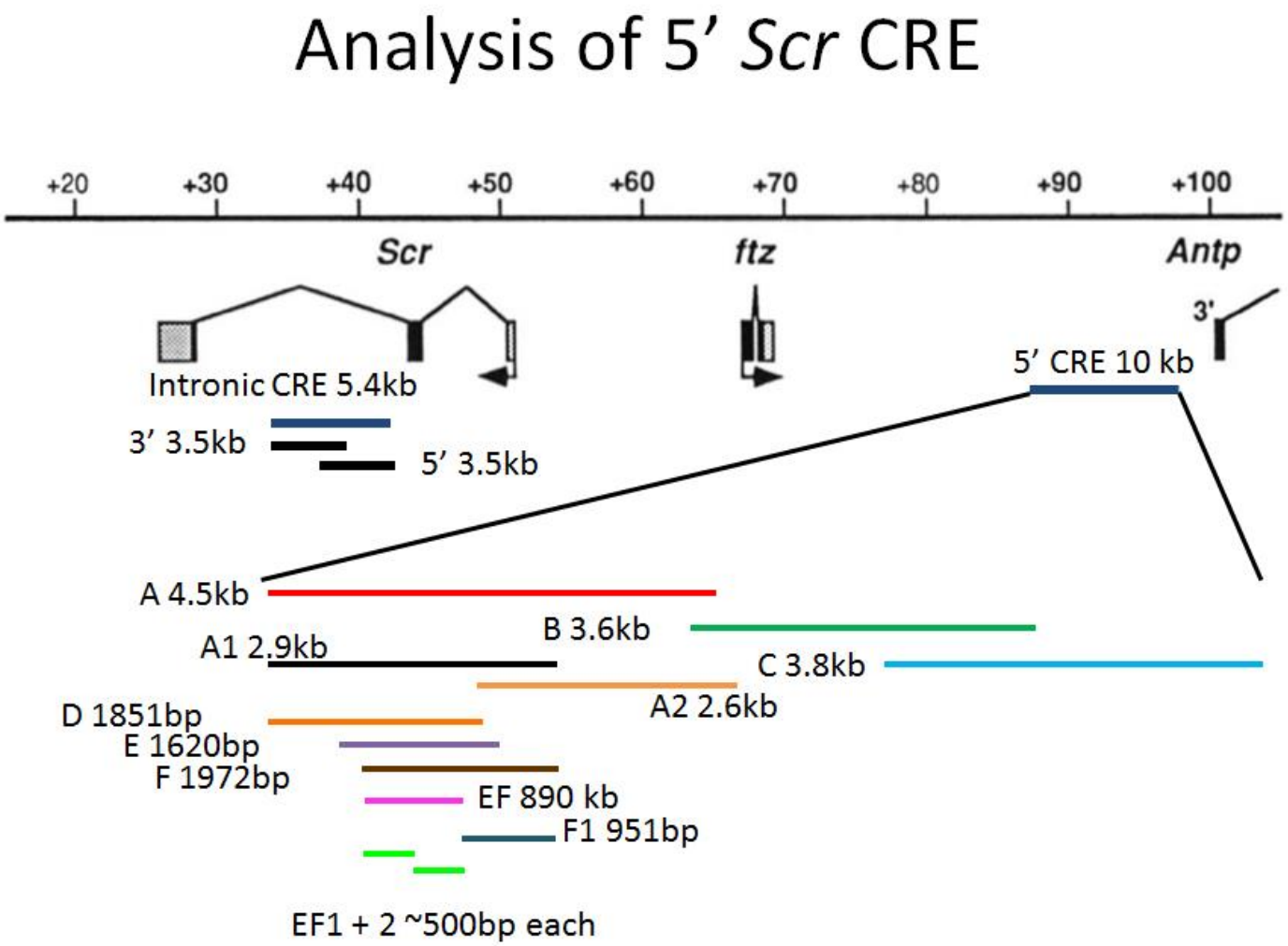


Figure 24 Map of the *Sex combs reduced* locus

The map (adapted from Gindhart et al., 1995) shows 85kb of the *Drosophila melanogaster* Antennapedia complex on chromosome 3R. *Scr* is on the left with three exons, denoted as rectangles, and two introns shown as lines connecting the exons. *ftz* is 15kb 5' of *Scr* and the 3' end of *Antp* is 50kb 5' of *Scr*. Two fragments were tested for enhancer activity. A 5.4kb intronic fragment that was divided into two 3.5kb overlapping fragments (Tanika and Kopp, unpublished). An upstream 10kb 5' fragment, located close to *Antp*), was divided into three smaller fragments the three fragments A, B and C (Shroff and Orenic, unpublished). The A fragment was further divided into smaller fragments. The E fragment is the smallest fragment that directs expression that fully recapitulates endogenous *Scr* expression.

4.2 Results

4.2a An intronic cis-regulatory module directs *Sex combs reduced* expression in the transverse row primordium

Initially, we focused on a sub-fragment from the putative5.4kb intronic CRM (5' 3.5kb), which was introduced into a reporter vector with RFP under control of the *hsp70* basal promoter and integrated into the fly genome via P-element mediated transformation (Barmina and Kopp unpublished). At 6h after puparium formation (APF), this 3.5kb fragment directed expression that only partially recapitulated the endogenous upregulated *Scr* expression in the T-row primordium. Specifically, we observed sparser RFP expression in the distal tibia and proximal basitarsus than would normally be expressed and additional ectopic expression in tarsal segments 2-5.

Since the 5' 3.5kb fragment did not fully recapitulate upregulated *Scr* expression, we next tested a construct containing the larger 5.4kb fragment, originally identified by Gindhart and Kaufman (1995). We opted to test the activity of this fragment in the S3aG vector (gift from Thomas Williams), which overcomes a limitation of P-element mediated transformation. A disadvantage of genomic integration mediated by P-elements mediated is that transgene gene integration is nearly random. This makes comparison of expression directed by different reporter genes difficult due to position effects, which result from influence on transgene expression by nearby chromatin. Therefore, we employed a site specific integration system, ΦC31 (Bischof et al., 2007), for analysis of the 5.4 kb fragment. The pS3aG carries a *gfp* reporter gene under control of the *hsp70* promoter and is compatible with the ΦC31 system. In the

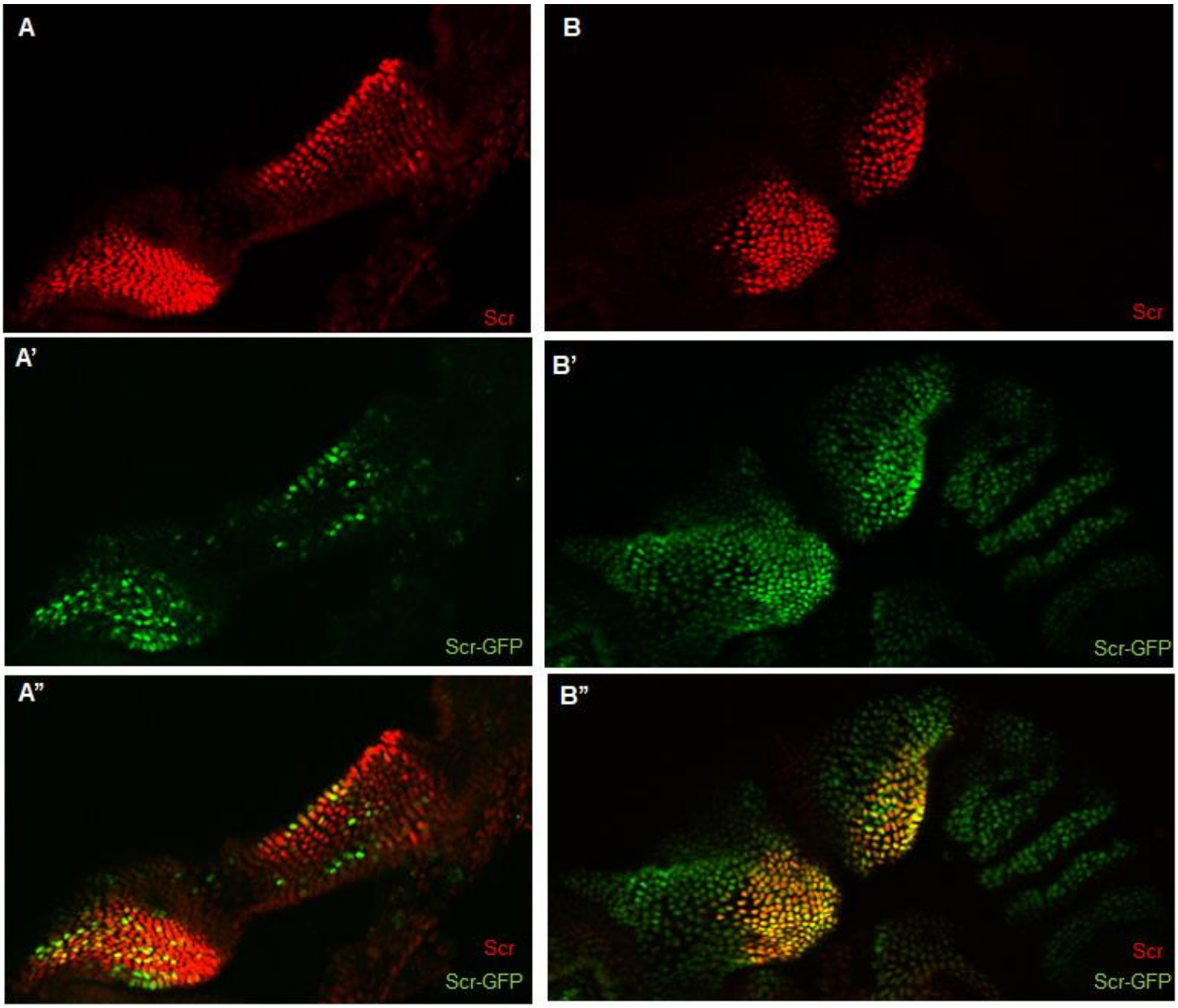


Figure 25 The 5.4kb intronic fragment more faithfully recapitulates endogenous *Scr* expression in the T-row primordia when in combination with the *Scr* vs the *hsp70* promoter

Prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left and distal is to the right in all panels. (A-A'') Ventral aspect of a prepual leg bearing a 5.4kb-inronic-*hsp70*p-*GFP* reporter gene and labelled with anti-Scr. Reporter gene driven GFP expression (green in A' and A'') poorly recapitulates endogenous *Scr* expression (red in A and A"). (B-B'') Ventral aspect of a leg bearing a 5.4kb-inronic-*Scr*p-*GFP* reporter gene and labeled with anti-Scr. Reporter gene driven GFP expression (green in B' and B'') recapitulates the endogenous *Scr* expression (red in B and B'') with ectopic expression along the leg circumference and in the ta2-5 segments.

pS3aG vector, the *gfp* gene is under control of the basal *hsp70* promoter. The 5.4 kb fragment also failed to direct expression that fully recapitulates the endogenous *Scr* pattern, exhibiting regions of weak and spotty GFP expression within the upregulated *Scr* domains of the tibia and basitarsus (Figure 25 A-A'').

Multiple studies have provided evidence suggesting specificity or preferences in interactions of enhancers with particular promoters (Merli et al., 1996; Butler and Kadonaga, 2001; van Arensbergen et al., 2014). . We, therefore, decided to use the endogenous *Scr* promoter in conjunction with the full-length 5.4kb intronic fragment to determine if this context, the 5.4 kb fragment would direct expression that more closely resembles that of endogenous *Scr*. We, therefore, further modified pS3aG (pS3aG-*Scr-p*) by replacing the *hsp70* promoter with the *Scr* promoter and cloned the 5.4kb fragment into this new vector. Under these conditions, the reporter gene expression better recapitulated the endogenous *Scr* expression in the T-row primordium, although, as observed with the 5' 3.5kb fragment, there was also ectopic expression in the ta2-5 segments (Figure 25 B-B''). In addition, we observed broader expression along the leg circumference. This may be a consequence of perdurance of GFP expressed during larval stages, when expression of *Scr* extends more dorsally.

The data presented above establish that the intronic 5.4kb fragment together with the *Scr* promoter recapitulate the endogenous *Scr* expression at six hours APF. With the 5.4kb fragment at the twenty four hour time point, the reporter gene recapitulated the *Scr* expression in the T-row primordium, but, interestingly, the super-elevated sex comb expression was not observed (figure 26 A-A'').

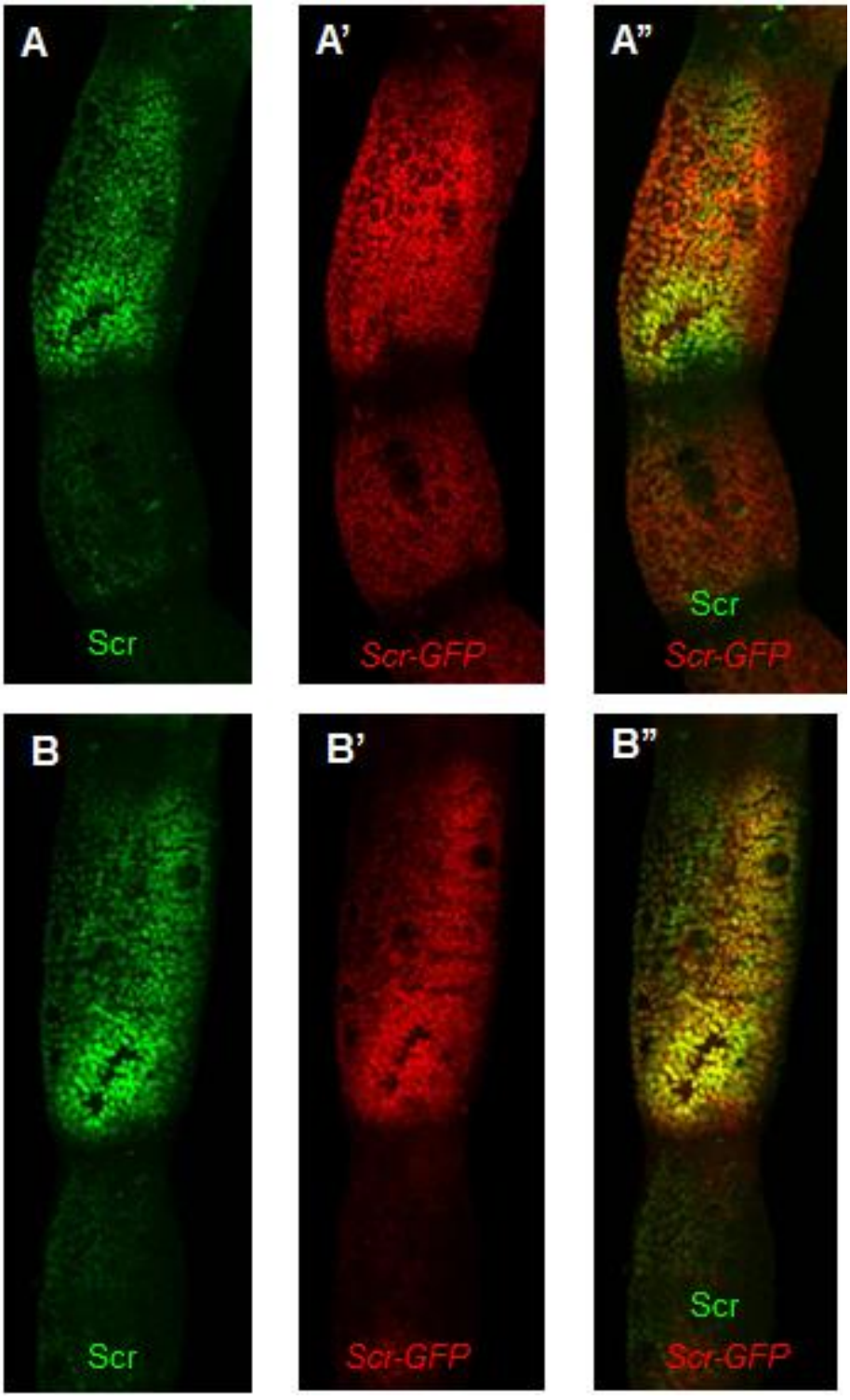

A
Scr
A'
Scr-GFP
A"
Scr
Scr-GFP
B
Scr
B'
Scr-GFP
B"
Scr
Scr-GFP

Figure 26 The 5' CRM A fragment directs expression that fully recapitulates endogenous *Scr* expression in the sex comb primordia at 24 APF, but 5.4kb intronic fragment does not

In all panels, prepupal legs (24 hrs. APF) are labeled with anti-Scr, and a ventral aspect is shown. All legs are oriented such that proximal is at the top and distal is at the bottom and both CREs were tested in in S3aG-*Scr-p-GFP.* (A-A'') The 5.4kb intronic CRE directs expression that recapitulates endogenous T-row expression but not the sex comb expression. (B-B'') The 5' CRE A fragment directs expression that recapitulates both early Scr expression in the T-row primordia and late expression in the sex comb primordia. Images courtesy of Artyom Kopp, unpublished.

These data suggest that the 5.4kb intronic fragment is capable of directing appropriate T-row *Scr* expression but is missing elements required for sex comb *Scr* expression. Since this fragment is not able to reproduce *Scr* expression at the later time point, a second CRM might be responsible for the correct sex comb expression at both time points.

4.2b An upstream *Sex combs reduced* cis-regulatory module directs both early expression in the transverse row primordium and late expression in the sex comb primordium

Upstream of the *Scr* gene is a second fragment, 10 kb in length, that was previously shown to direct expression in larval legs. To identify the minimal CRM domain within this 10kb fragment, previous lab members (Shroff and Wyskiel) subcloned tested three smaller overlapping fragments, named A (4.5kb), B (3.6kb) and C (3.8kb), to test for enhancer activity in leg discs (Figure 24). These fragments were cloned, in conjunction with the *Scr* promoter, into pStinger, a promoterless GFP reporter vector, and transgenic flies were made, using P-element-mediated transgenesis. Of the three fragments tested, only the A fragment directed GFP expression in the T1 leg in a pattern that closely matched endogenous *Scr* expression in the T-row primordia. We then cloned the A fragment into the S3aG-*Scr-p* vector (described above) above in order to generate a site-specific insertion. With both systems, the GFP expression pattern directed by fragment A was virtually identical to that of endogenous *Scr* expression in the T-row primordia of 6h APF (Figure 27 A-A'') legs. The S3aG-*Scr-p* vector was used for all subsequent experiments.

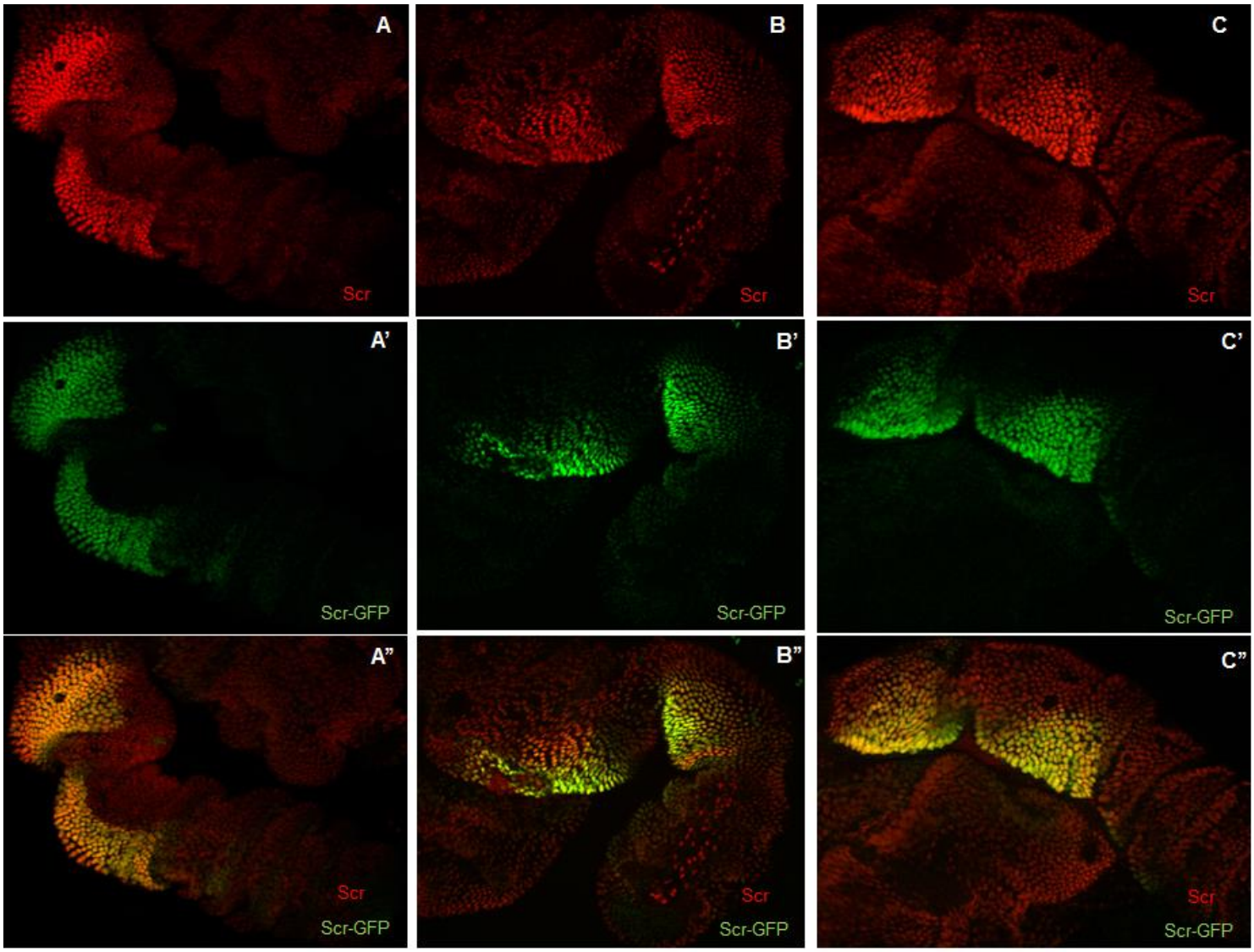


Figure 27 Three 5' CRM fragments direct expression that fully recapitulates endogenous expression

Prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left and distal is to the right, and a ventral aspect is shown. All CREs were tested in in S3aG-*Scr-p-GFP* and legs were labelled with anti-Scr. The A fragment in A-A'', A1 fragment in B-B'' and the E fragment in C-C'' all drive reporter expression that recapitulates endogenous Scr expression.

To identify the minimal *Scr* CRM within the A (4.5kb) fragment, further sub-cloning was performed, splitting the A fragment into A1 (2.9kb) and A2 (2.6kb) fragments (Fig. 24). Only the A1 fragment directed GFP expression, which appeared to be identical to that directed by the full-length the A fragment (Figure 27 B-B''). This A1 fragment was further subdivided to produce overlapping fragments D, E, and F, and unique fragments F1, EF, EF1 and EF2, as shown in Figure 23, each of which was tested for enhancer activity in the S3aG-*Scr-p* vector. The F1 and EF1 fragments failed to drive GFP expression, whereas fragments D, F and EF all produced diminished levels of GFP expression relative to endogenous *Scr* expression and lacked expression bordering the joints of both the tibia and basitarsus (Figure 28). Of the remaining two sub-clones (EF2 and E), E is the smallest fragment that fully recapitulates *Scr* expression in the T-row primordium (Figure 27 C-C''). The smaller fragment EF2 that is within E, produces a comparable T-row expression pattern but also drives ectopic expression in the dorsal tibia (Figure 29). Together these experiments define E as the minimal *Scr* CRM at 6h APF in the T-row primordium of the T1 leg.

In addition to spatially recapitulating the *Scr* expression pattern we examined whether the A fragment subclones of the CRM expressed GFP in larval leg imaginal discs during the wandering 3rd instar stage. Given the data above, CRM fragments E, EF, and EF2 were examined. As expected, fragments E and EF recapitulate the endogenous *Scr* expression pattern in the T1 leg imaginal disc at the 3rd instar stage and at 6H APF. EF2 was also chosen because despite the dorsal expansion into the tibia, it is the smallest fragment that directs reporter expression. To accurately determine the level of GFP expression all leg discs were fixed and analyzed at the same time and under the same

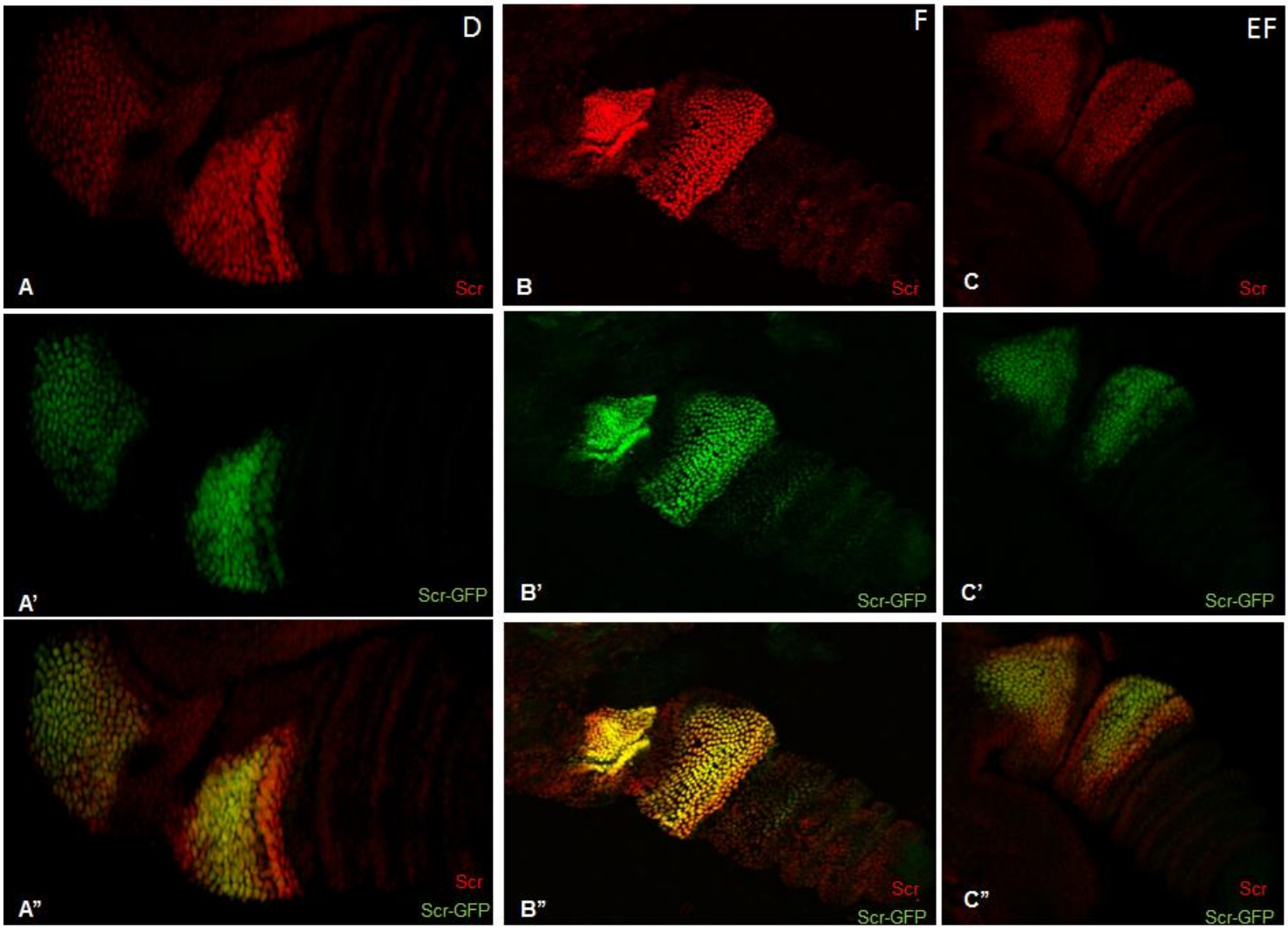


Figure 28 5' CRM fragments D, F and EF direct expression that partially recapitulates endogenous *Scr* expression

Prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left and distal is to the right, and a ventral aspect is shown. All CREs were tested in in S3aG-*Scr-p-GFP* and legs were labelled with anti-Scr. The D fragment in A-A'', F fragment in B-B'' and the EF fragment in C-C'' all drive reporter expression that partially recapitulates endogenous Scr expression but expression levels appear to be lower than that directed by the intact E fragment and lacked expression bordering the joints of both the tibia and basitarsus.

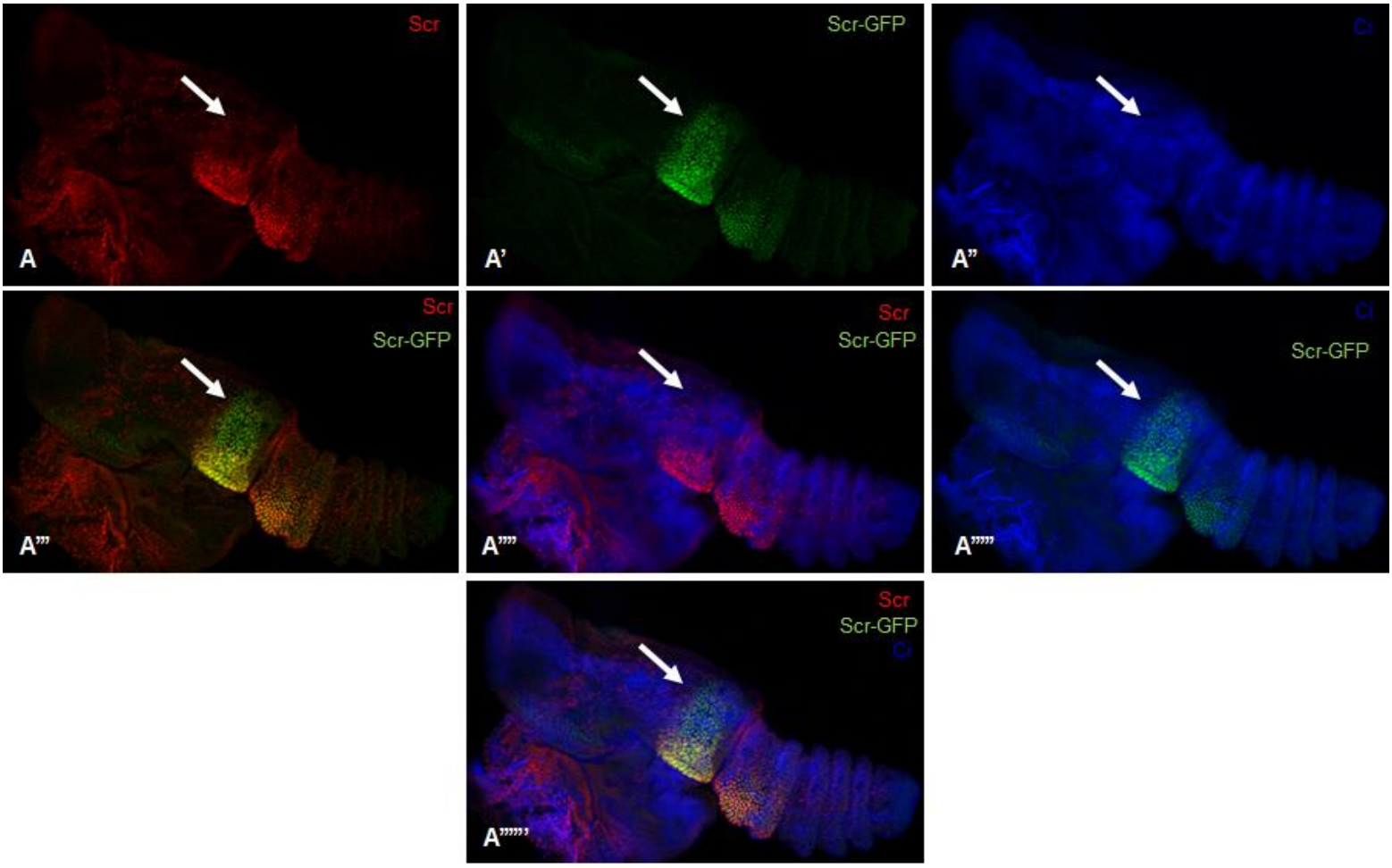


Figure 29 The EF2 fragment directs dorsally expanded reporter expression in the tibia

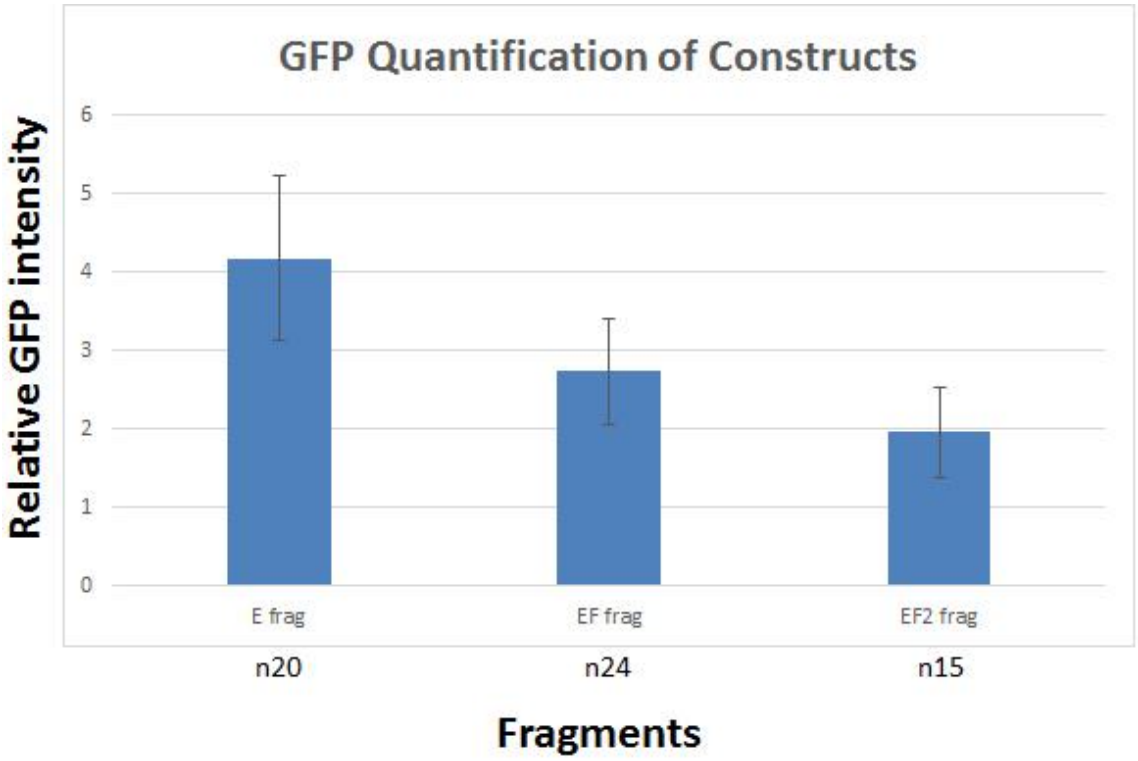
Prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left, distal is to the right, and a ventral aspect is shown. All CREs were tested in in S3aG-*Scr-p-GFP* and legs were labelled with anti-Scr. The EF2 fragment (A-A'''''') directs expression that recapitulates the endogenous *Scr* expression well except in the tibia where the reporter expression extends up into the dorsal region all the way up to the compartment boundary.

conditions. Relative expression of GFP by the E fragment was the highest at 4.18 with the two smaller CRMs expression levels lower at 2.74 for the EF fragment and 1.97 for the EF2 construct. These data indicate that while the information for spatial patterning is present in all the three constructs the smaller constructs lack some elements that control the intensity of expression.

To determine whether the A-fragment is also the CRM for super-elevated *Scr* expression in the sex comb primordia GFP expression was examined at 24 h APF (Barmina and Kopp, unpublished). At this time point, as expected, the A fragment fully recapitulates the expression of endogenous *Scr* in the T-row primordium (Barmina and Kopp, unpublished; Figure 26 B-B''). In addition, the A fragment also reproduces the expression pattern of the super-elevated sex comb expression. These data indicate that the E fragment of this CRM contains all the regulatory information needed to fully recapitulate spatial and temporal expression *Scr* expression in the T-row and sex comb primordia.

4.2c Functional analysis of two conserved domains of the upstream *Sex combs reduced* cis-regulatory module

In order to begin to identify sequences that are important for function of the upstream *Scr*-CRM, we compared the E fragment of *Drosophila melanogaster* to the other eleven fruit fly species that have been fully sequenced. Aligning the sequences from all 12 species for the 1.6kb E fragment (Kent WJ, 2002). Twelve blocks of conserved sequence (CS) were identified (CS1-12), of which 3 (CS6, 7, and 8) were highly conserved (Figure 30). To test the functional significance of these regions, CS6 and 8 were separately deleted from the E fragment using polymerase chain reaction (PCR) and



E fragment 4.18
EF fragment 2.74
EF2 fragment 1.97

Table 2 Quantification of GFP intensity for three 5' CRE fragments

A quantification of the reporter constructs E, EF and EF2 show varying levels of relative GFP expression. The E fragment expresses GFP at the highest intensity of the three at a level of 4.18 with the EF fragment expressing at a lower level of 2.74 and the EF2 fragment expressing at the lowest level of 1.97.

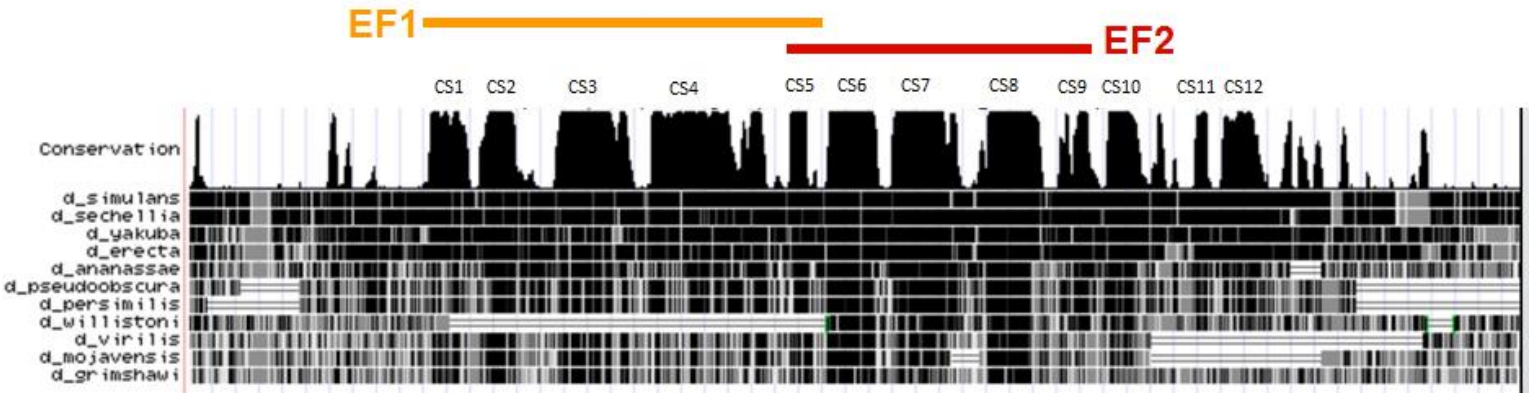


Figure 30 A conservation analysis of the E Fragment between 12 species of Drosophila

The E fragment sequenced was compared to eleven closely related and fully sequenced Drosophila species; *D.simulans, D.sechellia, D.yakuba, D.erecta, D.ananassae, D.pseudoobscura, D.persimilis, D.willistoni, D.virilis, D.mojavensis, D.geimshawi*. The Blat alignment of the all the species shows twelve conserved sequence (CS) blocks. Three of the twelve CS blocks, CS6, 7 and 8, show very high conservation across all species. The alignment was produced using the Blat tool on the UCSC genome browser at genome.ucsc.edu (Kent WJ, 2002).

these constructs were reintroduced using ΦC31 and examined at 6h APF. By comparing the GFP expression pattern of the CS6 deletion to that of Ci immunostaining, we determined that the GFP expression pattern extended into the posterior compartment of the T1 leg in both the tibia and basitarsus (Figure 31-32). In chapter 3, we demonstrated that Engrailed (En) represses *Scr* expression in the posterior compartment. Consistent with the notion that En requires CS6 to repress *Scr*, a putative conserved En binding site was discovered in CS6 region, suggesting that En might function directly through this CS to restrict *Scr* expression to the anterior compartment. CS8 shows a relatively lower reporter expression but the pattern coincides to endogenous *Scr* expression (Figure 33). Together these data suggest that CS6 and CS8 contain sequences that regulate *Scr* expression in the T-row primordium.

4.2d The response of the upstream *Sex combs reduced* cis-regulatory module to Bric-a-Brac, Decapentaplegic and *Engrailed* mimics that of endogenous *Sex combs reduced* expression

In chapter 3, our genetic analyses have shown that *Scr* is regulated by multiple genes along all three axes: Decapentaplegic (Dpp) signaling represses *Scr* along the Dorsal/Ventral (D/V) axis, En along the A/P axis and Bric-A-Brac (Bab) along the proximal/distal (Pr/Di) axis. Above, we describe the identification of a CRM that fully recapitulates the spatial and temporal expression of *Scr* in the T-row and sex comb primordia. In addition to expression analysis, another important test of CRM function is to determine whether it mimics the endogenous gene in its response to known regulatory inputs. Therefore, here we test response of the upstream CRM to Dpp, En and Bab.

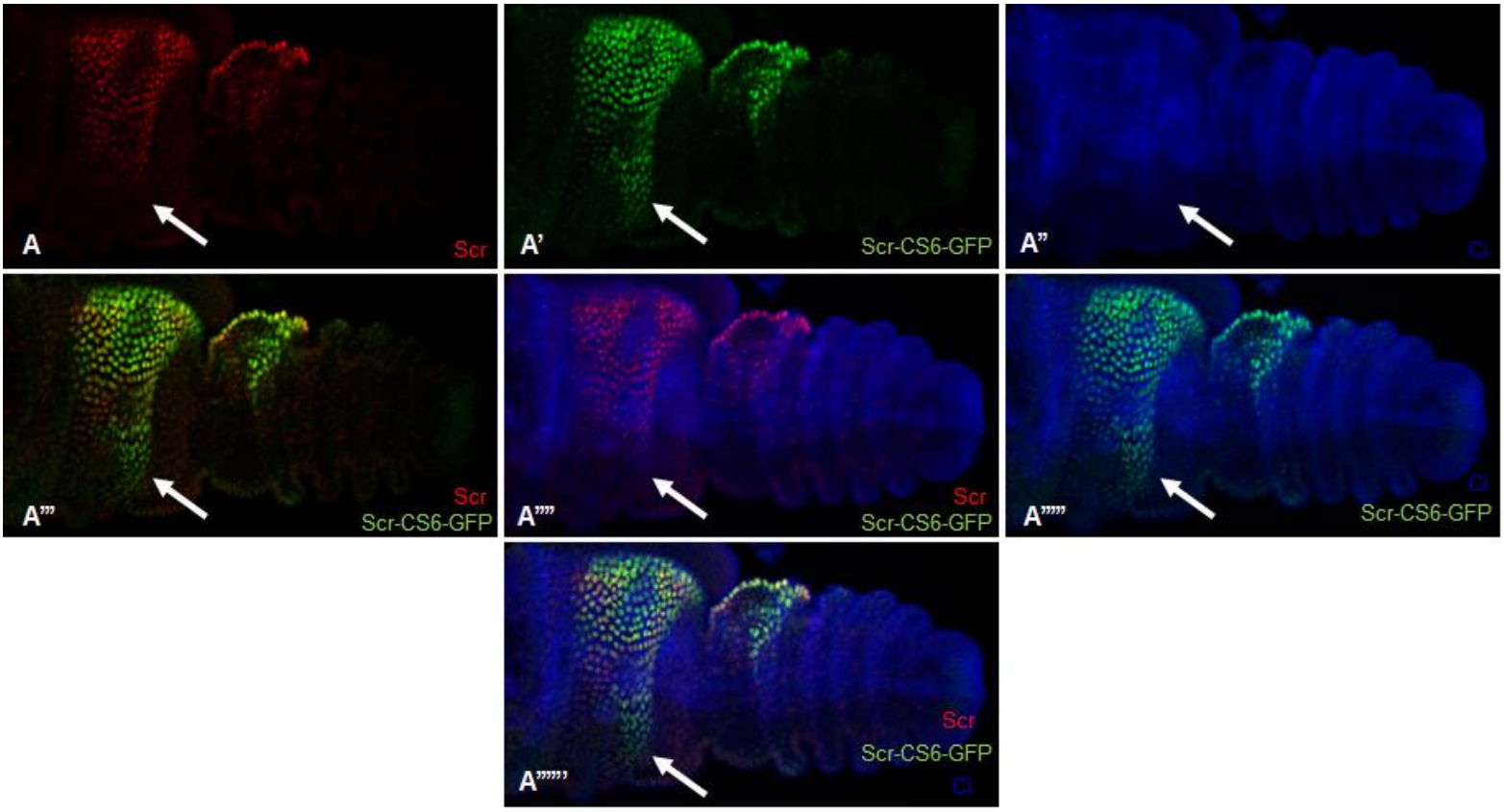


Figure 31 The CS6 deletion directs reporter expression that expands into the posterior compartment of the tibia

Prepupal leg (4-6 hrs. APF) is oriented such that proximal is to the left and distal is to the, and a ventral aspect is shown. All CREs were tested in in S3aG-*Scr-p-GFP* and the leg was labelled with anti-Scr and anti-Ci. The CS6 deletion fragment (A-A'''''') directs expression that recapitulates the endogenous *Scr* expression well, except that reporter expression extends into the posterior compartment of the tibia.

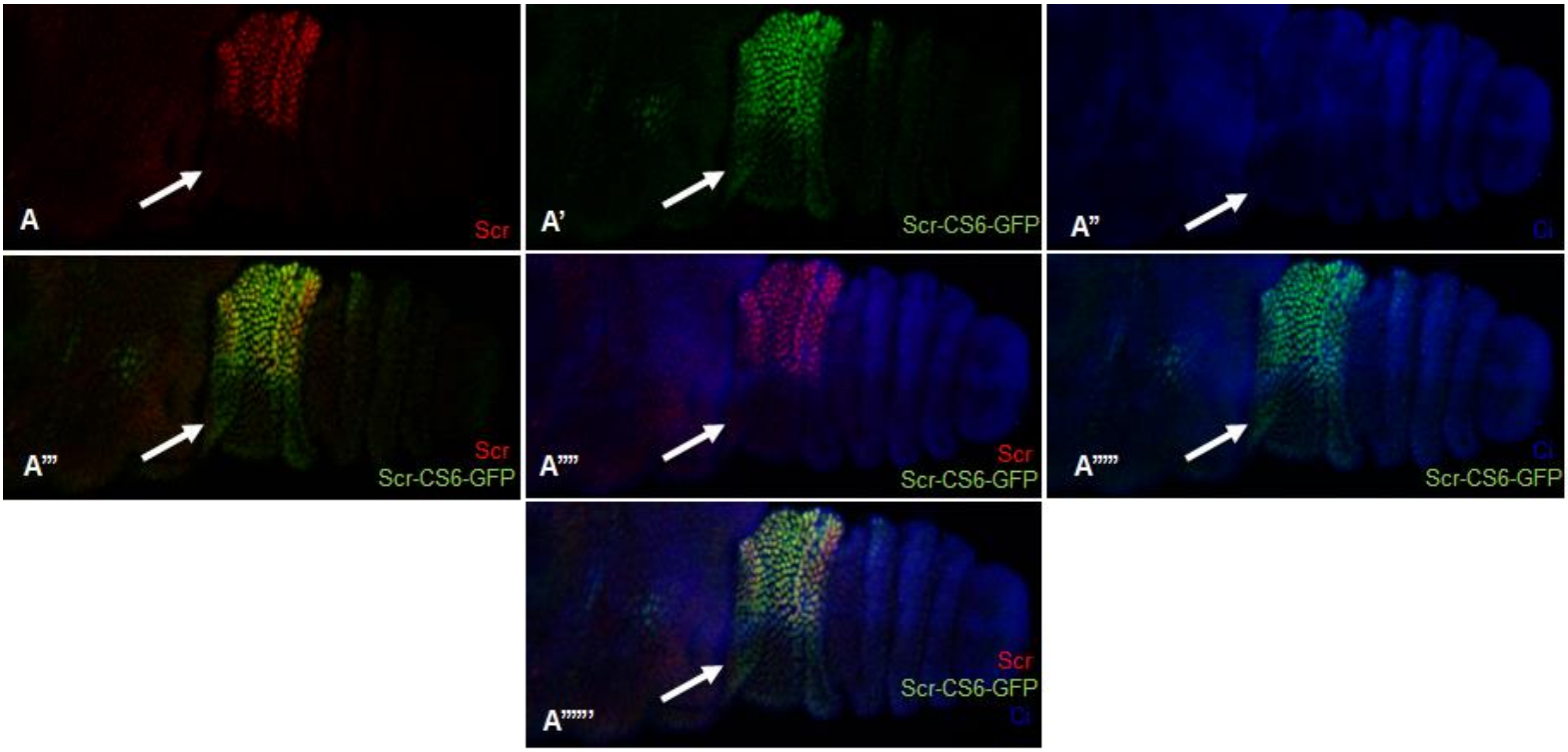


Figure 32 The CS6 deletion directs reporter expression that expands into posterior compartment of the basitarsus

Prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left and distal is to the right, and a ventral aspect is shown. All CREs were tested in in S3aG-*Scr-p-GFP* and the leg was labelled with anti-Scr and anti-Ci. The CS6 deletion fragment (A-A'''''') directs expression that recapitulates the endogenous *Scr* expression well, except that the reporter expression extends into the posterior compartment of the basitarsus.

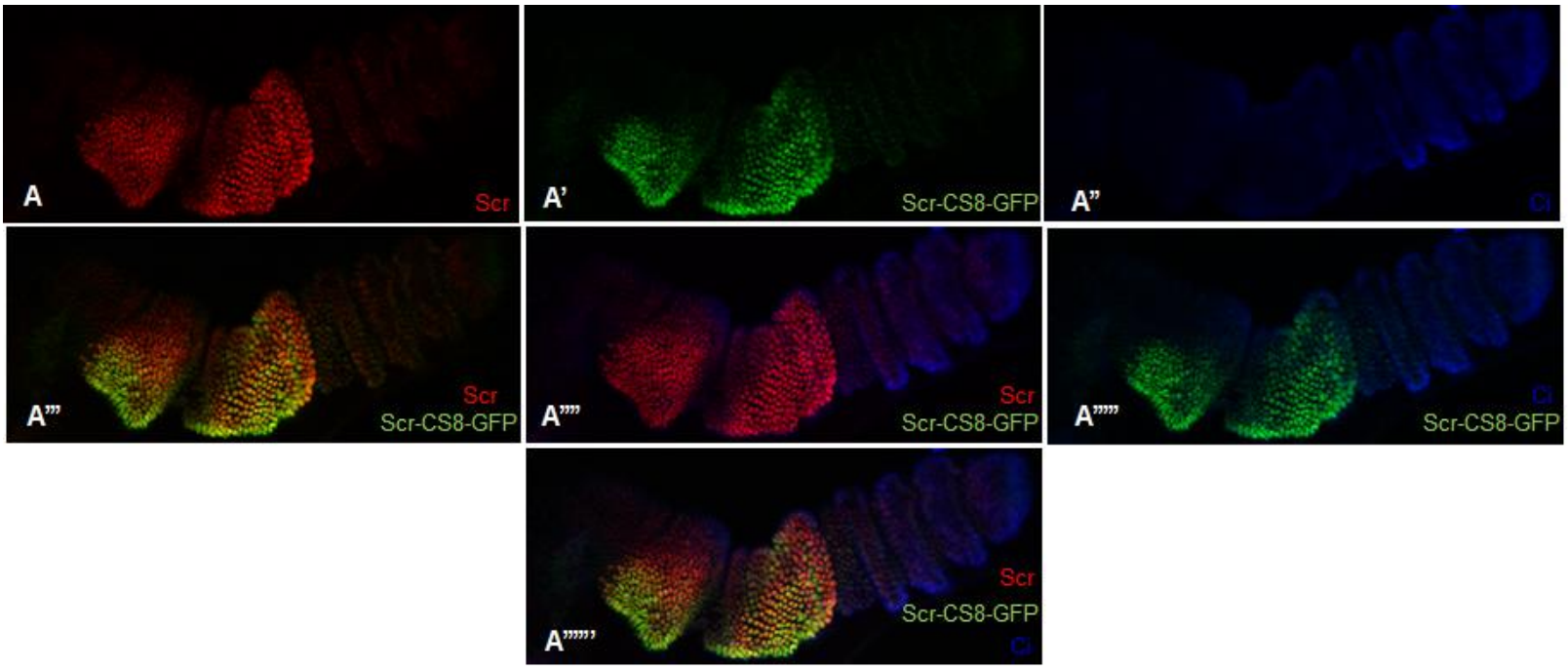


Figure 33 The CS8 deletion directs expression that partially recapitulates endogenous *Scr* expression

Prepupal leg (4-6 hrs. APF) is oriented such that proximal is to the left and distal is to the right, and a ventral aspect is shown. All CREs were tested in in S3aG-*Scr-p-GFP* and the leg was labelled with anti-Scr and anti-Ci. The CS8 deletion fragment (A-A'''''') directs expression that recapitulates the general pattern of endogenous *Scr* expression, but expression levels appear to be lower than that directed by the intact E fragment.

We established in chapter 3 that ectopic activation of the Dpp signaling pathway, using the constitutively active Thickveins (Tkv-act) receptor, represses endogenous *Scr* expression in the dorsal region of the T1 leg (Figure 18). To determine whether this signaling pathway acts through the *Scr* E fragment CRM, the effects of ectopic expression of Tkv-act on the CRM driven GFP expression pattern were examined. As shown in figure 50, the GFP reporter expression of the E fragment is repressed by this ectopic Dpp signaling (Figure 34). The E fragment response to Tkv-act mirrors the endogenous *Scr* reaction to the ectopic Dpp signaling in the T-row primordium.

Since both En and Bab encode transcription factors (TF), they have the potential to directly regulate the *Scr* expression pattern via *Scr* CRMs. Here we tested whether the E fragment *Scr* CRM is a target of each of these TFs. In the anterior compartment, ectopically expressed En represses the E fragment GFP reporter, mimicking the repressing of endogenous *Scr* expression by ectopic En (Figure 35). Similarly, when the E fragment expression pattern was examined in clones expressing ectopic Bab expression, Bab repressed the GFP reporter (Figure 36).

Together these data suggest that all three genetic regulators of *Scr* act via the Scr CRM within the E fragment. Furthermore, these data provide functional evidence to corroborate our expression data suggesting the upstream CRM is a bona fide regulator of *Scr* expression in the primordia of T1 leg-specific sensory organs.

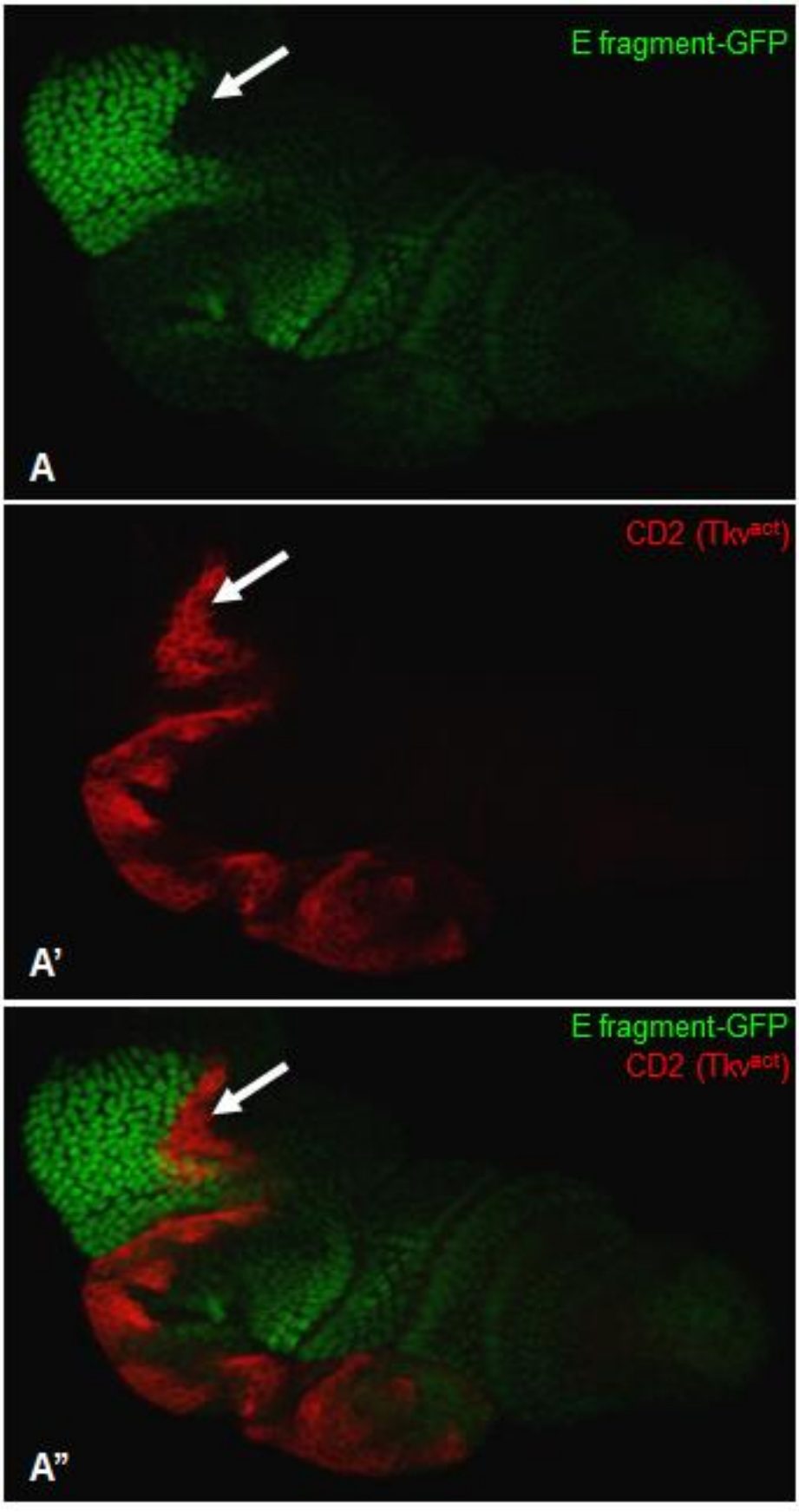

E fragment-GFP
A
CD2 (Tkv^{act})
A'
E fragment-GFP
CD2 (Tkv^{act})
A"

Figure 34 Reporter expression of the E fragment is repressed by ectopic Dpp signaling

Prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left and distal is to the right with ventral facing aspect of the leg labeled with anti-Scr and the construct driving GFP expression. A-A'' The E fragment expression of GFP is repressed by the constitutive active receptor Tkv which simulates Dpp signaling.

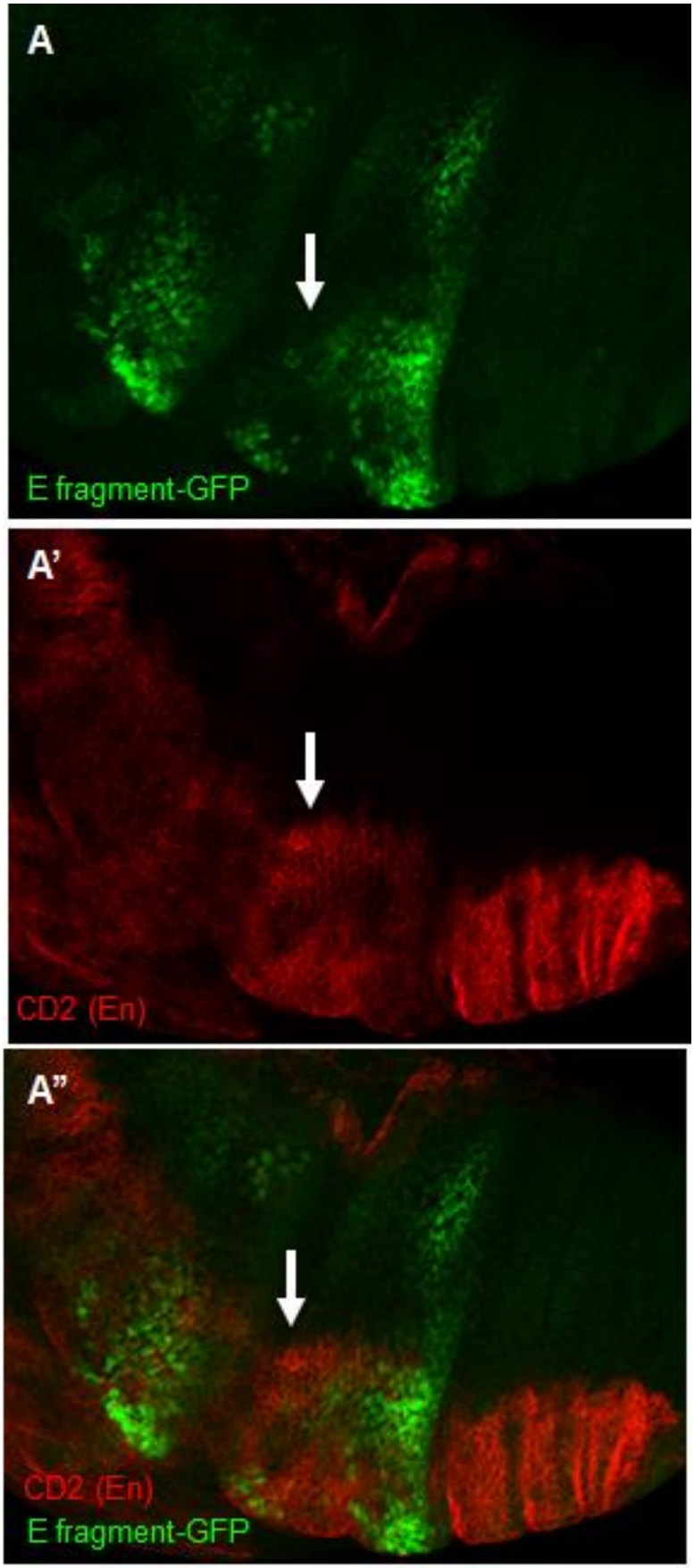

A
E fragment-GFP
A'
CD2 (En)
A"
CD2 (En)
E fragment-GFP

Figure 35 Reporter expression directed by the E fragment is repressed by *En*

Prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left and distal is to the and a ventral aspect is shown. All CREs were tested in in S3aG-*Scr-p-GFP* and the leg was labelled with anti-Scr and anti-Ci. A-A'' Ectopic expression of En in the anterior compartment represses E fragment reporter expression.

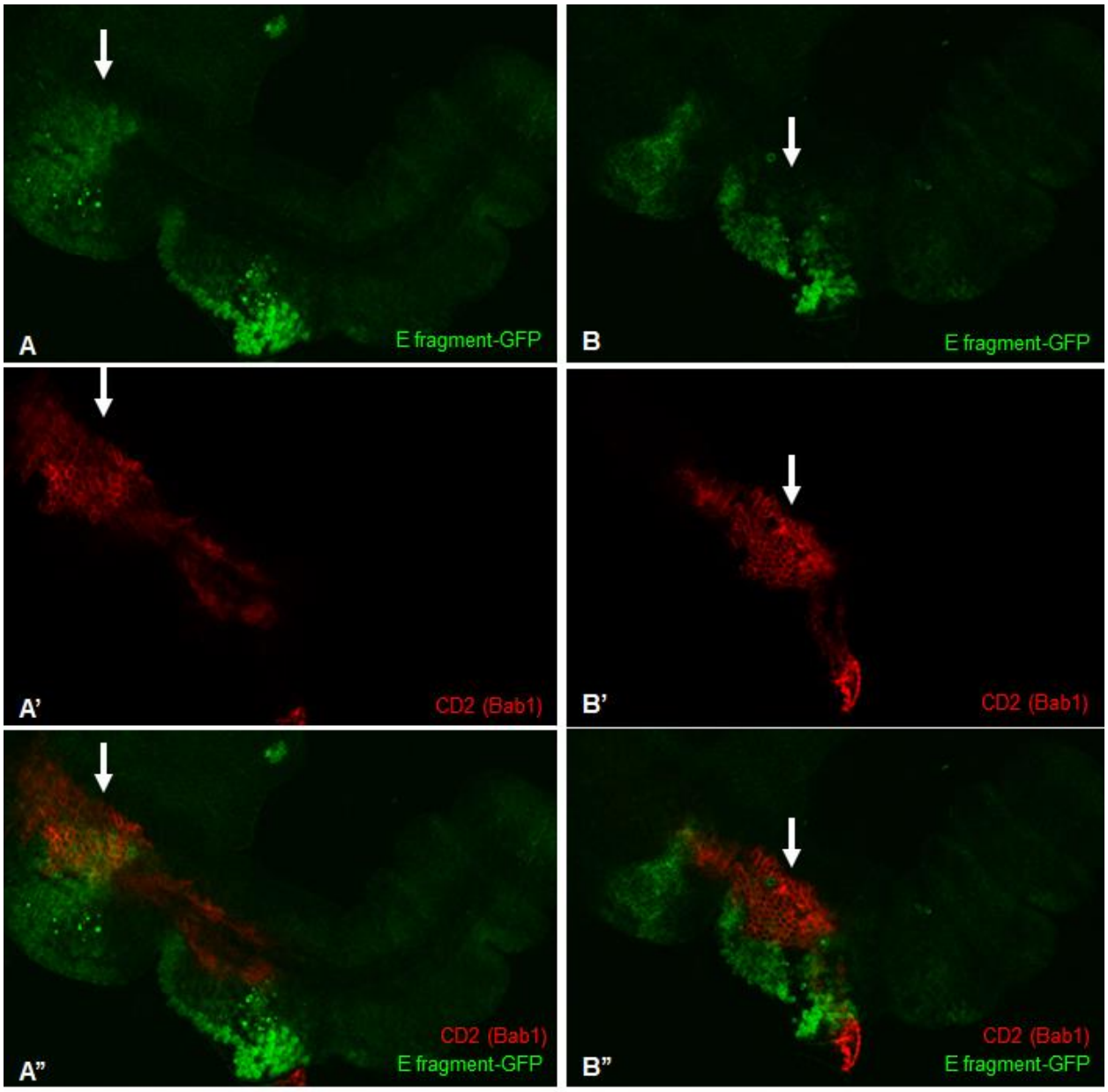


Figure 36 Reporter expression directed by the E fragment is repressed by *Bab*

Prepupal legs (4-6 hrs. APF) are oriented such that proximal is to the left and distal is to the right, and a ventral aspect is shown. All CREs were tested in in S3aG-*Scr-p-GFP* and the leg was labelled with anti-Scr and anti-Ci.' Ectopically expressed Bab does not affect E fragment reporter expression of GFP in the tibia (A-A'), but does inhibit E fragment reporter expression in the basitarsus (B-B'').

4.3 Discussion

4.3a Sex combs reduced activation is controlled by two functionally distant cis-regulatory modules

These studies identify two CRMS that direct expression in the T-row primordium and only one of which directs SC expression. Our observations raise multiple questions. Are the intronic and upstream enhancers functionally redundant with regard to expression in T-row primordia or do they function additively? Test for redundancy will require CRISPR deletion of each. Might expect redundancy with regard to expression in T-row primordia, but unless there is a 3rd CRM, we would expect sex comb development to be disrupted if the upstream enhancer is deleted. Our previous studies suggest that, while low levels of Scr are sufficient for development of most T1 leg specific features, a high level of Scr expression is necessary for T-row/sex comb development. Perhaps the two enhancers function additively to boost Scr expression in the T-row primordia to levels that are sufficient for T-row specification. The observation that the upstream enhancer better reproduces endogenous Scr expression implies that this might be the "primary" enhancer and the intronic enhancer might function to boost Scr expression.

Do the two enhancer respond to the same inputs? Although the two CRMs direct similar expression patterns in prepupal legs, it is plausible that they respond differentially to patterning inputs. For example, one enhancer could respond to patterning inputs along the three axes, as we have shown for endogenous Scr, while the other enhancer could respond to Scr itself, allowing for auto-regulatory stimulation of Scr expression. We have identified putative En (this study) and Dll binding sites (Eksi and Orenic). Furthermore, we have shown that the putative Dll binding sequences are necessary for activity of the

upstream enhancer, suggesting that patterning inputs are, at least part, integrated through the upstream enhancer. Nevertheless, it is important to determine whether the activity of either or both enhancers is dependent on Scr function and to perform mutational analyses of both to determine how each responds to the factors that we have shown are essential for directing upregulated Scr expression.

These observations of the two identified CRMs raise the question of whether or not one is a “shadow enhancer”. The three general characteristics of these CRMs are: both elements drives similar expression patterns, they bind the same TFs and lastly a shadow enhancer is located in an intron or near a neighboring gene (Hong et al., 2008; Barolo, 2012). This study shows that the two CRMs do indeed drive similar expression patterns with the upstream CRM driving expression in the sex comb primordia in addition to the T-rows like the intronic CRM. We have shown that putative Dll binding sequences are present in one of the CRMs which can easily be tested in the intronic CRM (Eksi and Orenic). As for the last feature of shadow enhancers, location, both fit the definition leaving the decision up to the results of which TFs bind and the two elements.

4.2b A conserved region in the upstream cis-regulatory module contain punitive binding sites for *Engrailed*

The Genetic studies in chapter two have identified multiple transcription factors that are regulators of Scr. This raises the question, do these factors act directly through the Scr enhancer? For En, we have identified a putative binding site within a short conserved sequence, which when deleted results in expression expanded to posterior compartment. Functional test of this site is needed. It would also be informative to test response of E-ΔCS6 to En, as done for wild type E. En is homedomin protein, all of which

share binding sites with a TAAT core. Dll, which genetic studies indicate is an activator is also a homedomain protein and studies in lab have identified multiple putative Dll binding sites (Eksi and Orenic), which appear to be required for activation.

In posterior compartment cells, En might repress Scr by competing with Dll for binding to same site. However, several observations argue against this: specific binding sequences have been identified for some homeodomain proteins, including Dll (Noyes et al., 2008). Two, ΔCS6 deletion suggests putative En response sites may be distinct from Dll binding sites. Lastly, none of putative Dll binding site mutations result in posterior compartment expansion.

4.3c Regulation of *Sex combs reduced* by Mothers against decapentaplegic, the Decapentaplegic transcriptional mediator, may be indirect

The EF2 fragment recapitulated endogenous Scr expression well except for the antero-dorsal expansion in the tibia. This suggests that there are sequences in the E fragment that mediates response to Dpp. This raises the question if the EF2 fragment is responsive to Dpp signaling still. We did not find any high scoring putative Mad binding sites in the region that is missing in EF2, suggesting Dpp might act indirectly by activing expression of putative repressor of Scr. Optomotor-blind (Omb), a Dpp target gene, is a candidate repressor, based on the expression pattern in the dorsal region of the leg primordium (Jackson and Hoffmann, 1994).

4.3d Bric-a- brac acts through the upstream cis-regulatory module to refine expression along the Proximal/Distal axis

In chapter two we show that Bab represses *Scr* expression in the T-row primordia in the basitarsus and that the E fragment reporter expression is a repressed by ectopicly expressed Bab. The ΔCS7, not shown here, causes reporter expansion into ta2 and ta3 in prepupal legs (Eksi and Orenic). This expansion of the reporter into the region were Bab is normal express suggests Bab acts through sites in the CS7 sequence that was deleted. The Bab1/2 TFs contain a BTB/POZ domain and both share the Bab conserved domain which binds TA rich sequences (Zollman et al., 1994, Lours et al., 2003). As Dll binds TAAT core sequence, Bab might repress by competing with Dll.

However, it is important to consider interactions between Dac and Bab. Bab and Dac are mutually repressive. (Chu et al., 2002; Greenberg and Hatini, 2009). Expression of each expands when the other is mutant. Therefore, it is plausible that Bab indirectly represses Scr effects on dac expression and vice versa. It will be important to test Scr expression in legs lacking bab1/2 and dac function to sort this out.

V. Discussion

5.1 *Sex combs reduced* integrates patterning information along all three axes

This study was conducted to elucidate the regulators of the Hox gene *Scr* expression at the genetic level. Previous studies in our lab illuminated the process of how L-row patterning takes place and how that pattern is modulated to produce a second patter, T-rows (Joshi et al., 2006; Shroff et al., 2007).

This investigation shows that upregulated Scr expression in the T1 leg receives patterning information on all three axes and uses these directional ques to produce two broad fields of upregulated expression that give rise to the T-rows bristle pattern. Activation of Scr takes place along the Pr/Di in the region of overlapping expression of the genes Dac and Dll. The Pr/Di gene Bab refines the distal edge of upregulated Scr expression. On the proximal edge the gene Lim1 defines the edge of expression. The circumferential axis in the Drosophila leg refines Scr expression to the antero-ventral region. The dorsal edge of Scr expression is refined by Dpp signaling with Wg preventing Dpp repression in the ventral region.

5.2 Regulation of *Sex combs reduced* is controlled by two discrete cis-regulatory modules

The CRM studies were undertaken to identify enhancers that direct Scr expression in the T1 leg and to test any identified for response to know regulators. A

previous study by the Kaufman lab dissected the *Scr* locus and identified CRMs that directed expression in the embryo (Gindhart et al., 1995).

Our investigation has identified two CRM that direct *Scr* expression in the prothoracic leg. The intronic CRM recapitulates *Scr* expression in the T-row primordium at 6 hAPF but it lacks the ability to reproduce the sex comb expression at 24 hAPF. A second identified CRM is upstream of *Scr* and recapitulates the T-row expression like the intronic CRM plus the sex combs expression that the intronic CRM lacks. After dissection of the upstream CRM we discovered that the 1.6kb E fragment is the smallest fragment that recapitulates endogenous *Scr* expression. This fragment responds to Dpp signaling, Bab and En just as endogenous *Scr* does. These observations give great insight into the genetic and molecular mechanisms involved in development and pattern formation.

APPENDICES

Appendix A Primers used for cloning *Scr* cis-regulatory modules

Scr promoter

Forward Primer

TTCTCGCTAGCTCCAAACTCCCAAAATGAGTCCC

NheI

Reverse Primer

ACTCGGATCCAACTCGTTTACTCACAATTTTAGTTAGCC

BamHI

5.4kb Intronic-CRM

Forward Primer

ACTCGGTACCCTCAGGCCGCAGTCGGCAAAATCCG

KpnI

Reverse Primer

CTCGGTACCCGACAGGCAACTCACGAAGTTGTTTTGGGG

KpnI

5'-CRM-A

Forward Primer

ACTCGGTACCCGCATCTGGCCCGTCAAAGTCAAAAGTC

KpnI

Reverse Primer

TTCTCGCTAGCTCGCCAACGTGTGACACATT

NheI

5'-CRM-A1

Forward Primer

TTGCCATACCATTTAGCCGATCAATTGTGC

KpnI site from 5’-CRM-A is downstream

Reverse Primer

CATACGCTAGCTAGTCCGAATGGACGTCAAT

NheI

5’-CRM-A2

Forward Primer

GGGACCAAATCATAAACATTCGAACTCCG

NheI site from 5’-CRM-A is downstream

Reverse Primer

ACTCACGGTACCACAGGCTAAATCGTTTTCTCTCC

KpnI

5’-CRM-D

Forward Primer

TTGCCATACCATTTAGCCGATCAATTGTGC

KpnI site from 5’-CRM-A1 is downstream

Reverse Primer

GCTAGCGCATTCTGAGCAGCTCATAA

NheI

5’-CRM-F

Forward Primer

GGTACCTCGCCATTATCTCGCTTA

KpnI

Reverse Primer

CATACGCTAGCTAGTCCGAATGGACGTCAAT

NheI

5’-CRM-F1

Forward Primer

GGTACCTCGCCATACTTCCGTTACAC

KpnI

Reverse Primer

CATACGCTAGCTAGTCCGAATGGACGTCAAT

NheI

5’-CRM-EF

Forward Primer

GGTACCTCGCCATTATCTCGCTTA

KpnI

Reverse Primer

GCTAGCGCATTCTGAGCAGCTCATAA

NheI

5’-CRM-EF1

Forward Primer

GGTACCTCGCCATTATCTCGCTTA

KpnI

Reverse Primer

GCTAGCGTTGACACAGCCATTACCCA

NheI

5’-CRM-EF2

Forward Primer

GGTACCTGGGTAATGGCTGTGTCAAC

KpnI

Reverse Primer

GCTAGCGCATTCTGAGCAGCTCATAA

NheI

5’-CRM-E-CS6Δ

Forward Primer

TCCTCTCTCCGGCAGTCTTGGAGCAGT

Reverse Primer

AGACTGCCGGAGAGAGGAGGCTCGCC

5’-CRM-E-CS8Δ

Forward Primer

TGCCTTCCCGCAGAGCCAGAAAAAGG

Reverse Primer

GGCTCTGCGGGAAGGCAGCACATCCAG

Appendix B Constructs generated in this study

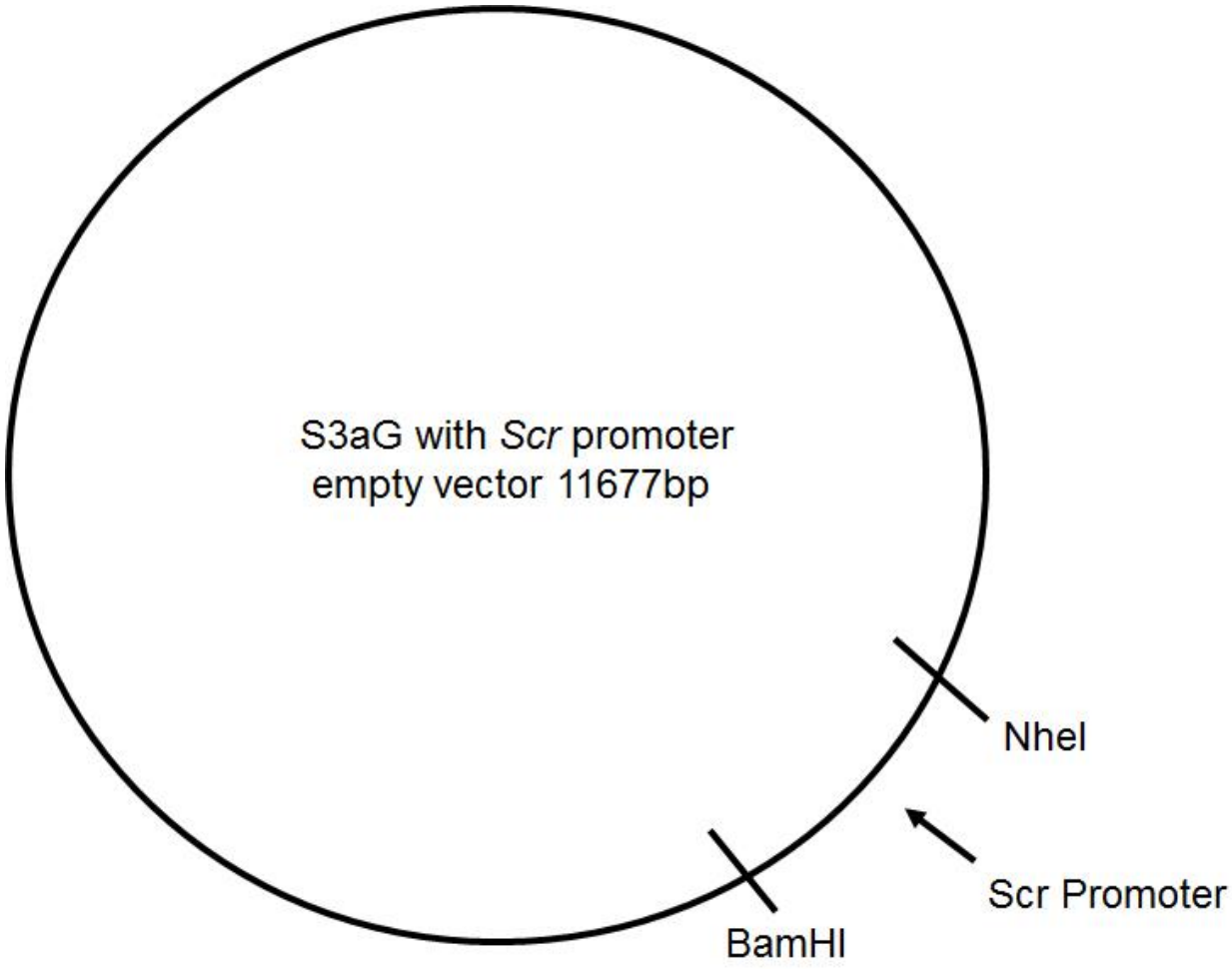


Figure 37 S3aG with *Scr* promoter

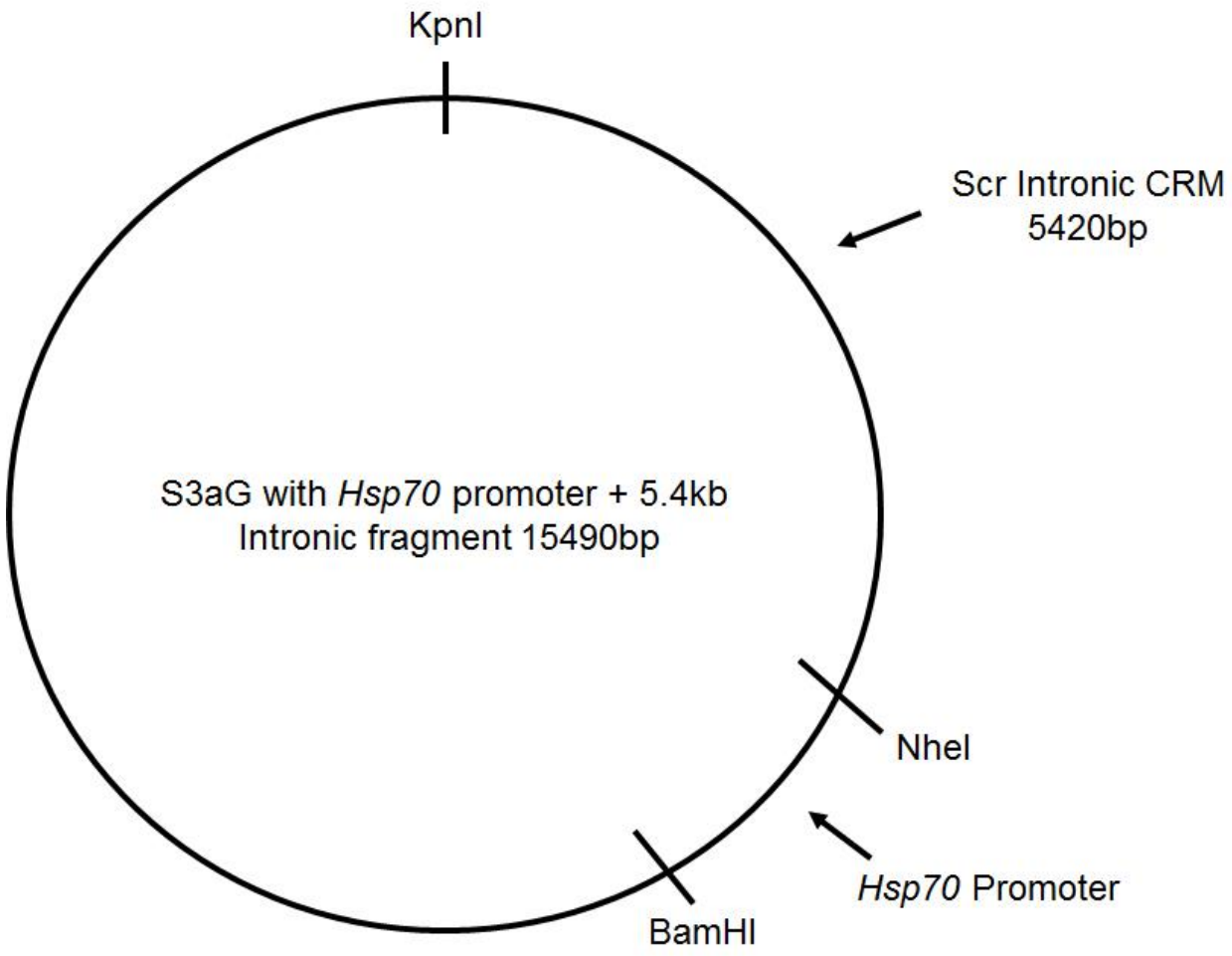


Figure 38 S3aG with *Hsp70* promoter + 5.4kb Intronic fragment

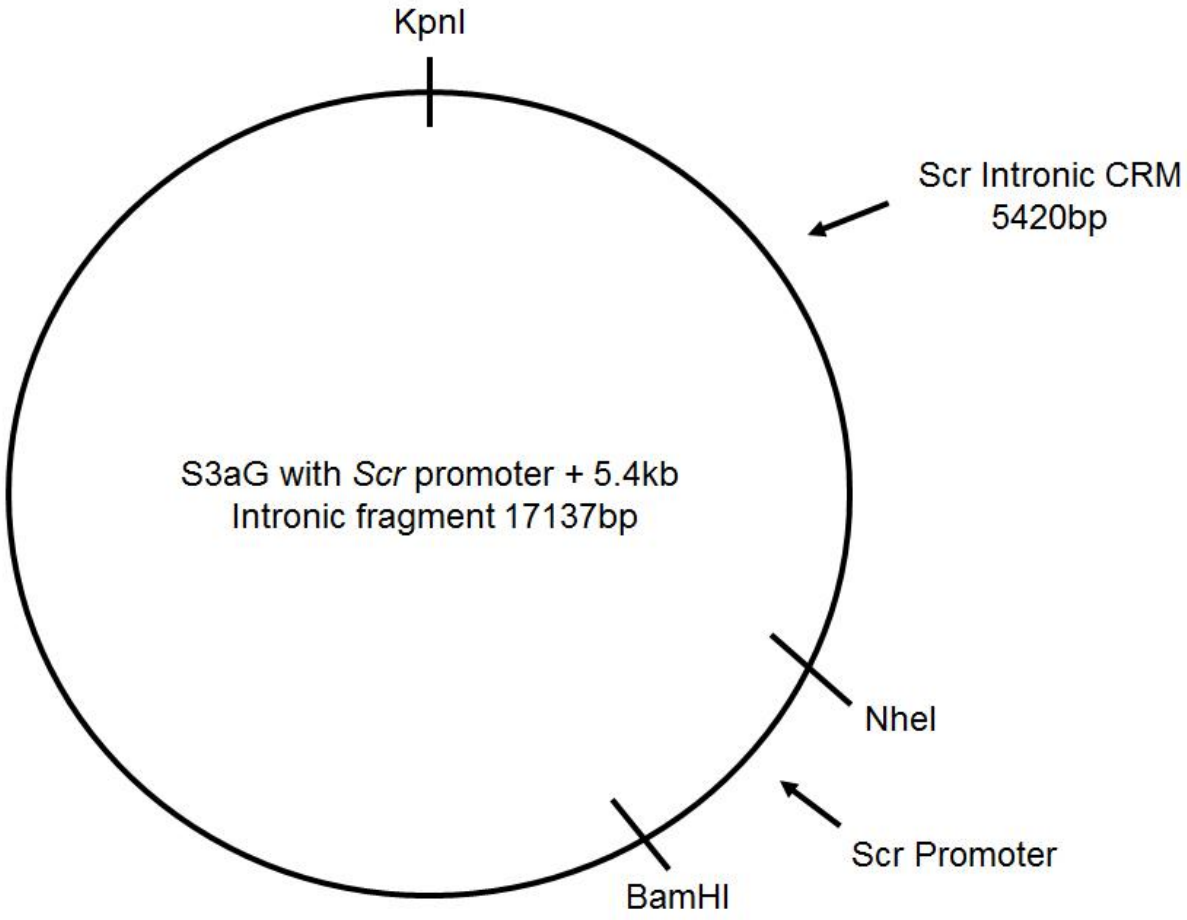


Figure 39 S3aG with *Hsp70* promoter + 5.4kb Intronic fragment

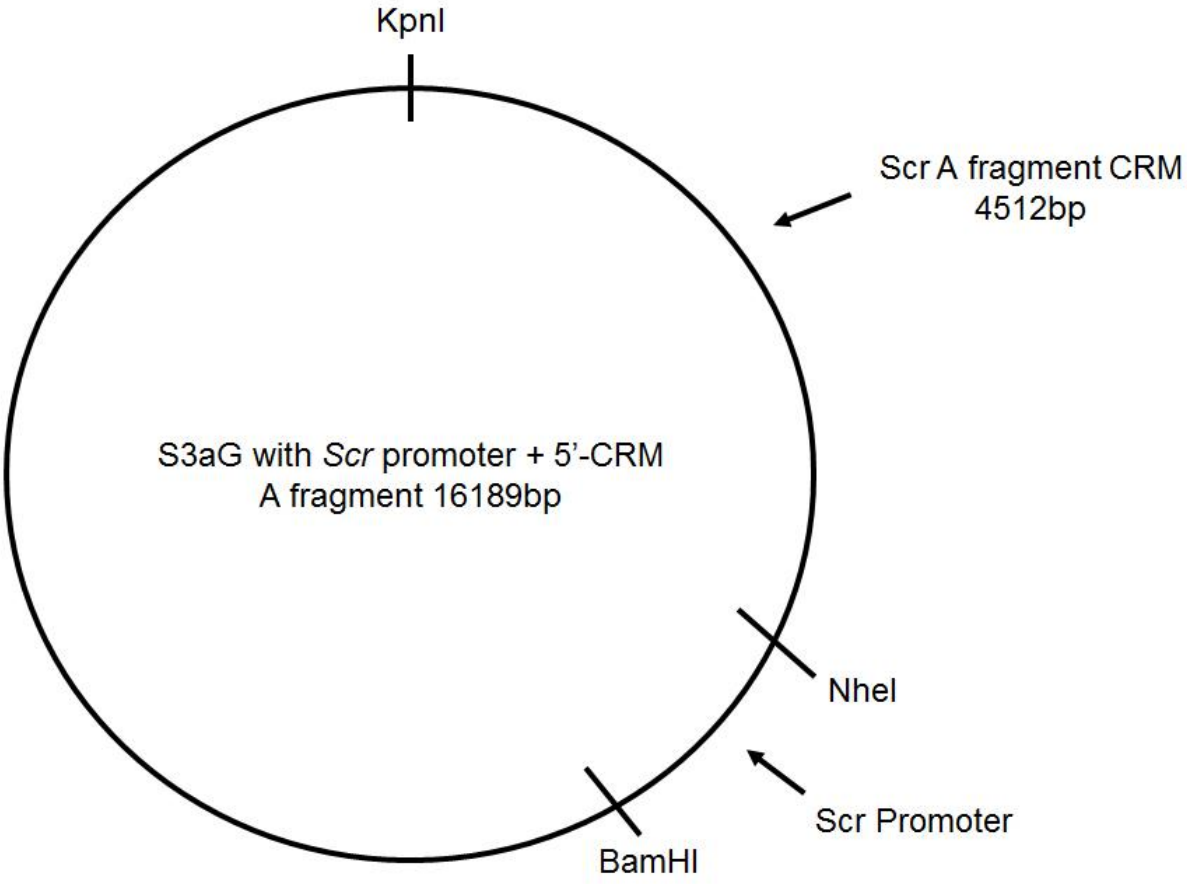


Figure 40 S3aG with *Scr* promoter + 5'-CRM A fragment

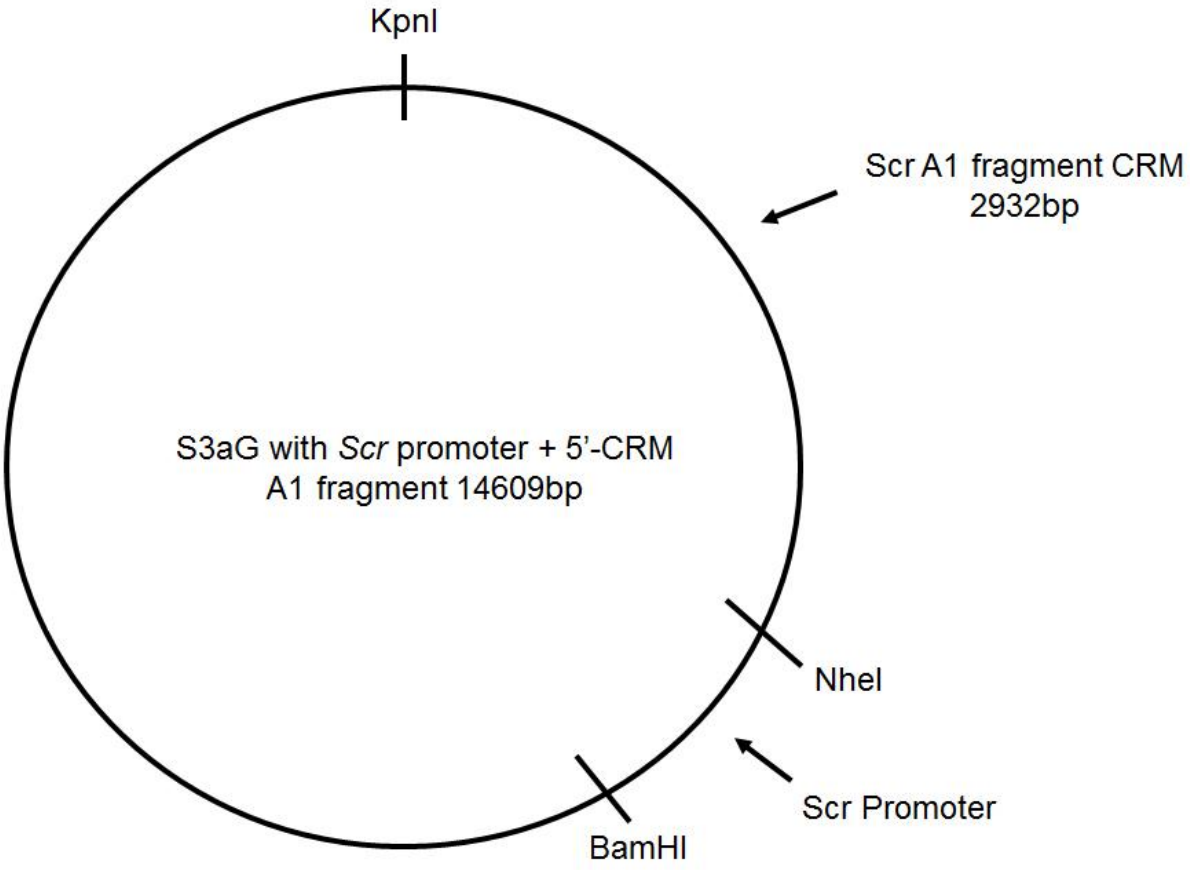


Figure 41 S3aG with *Scr* promoter + 5'-CRM A1 fragment

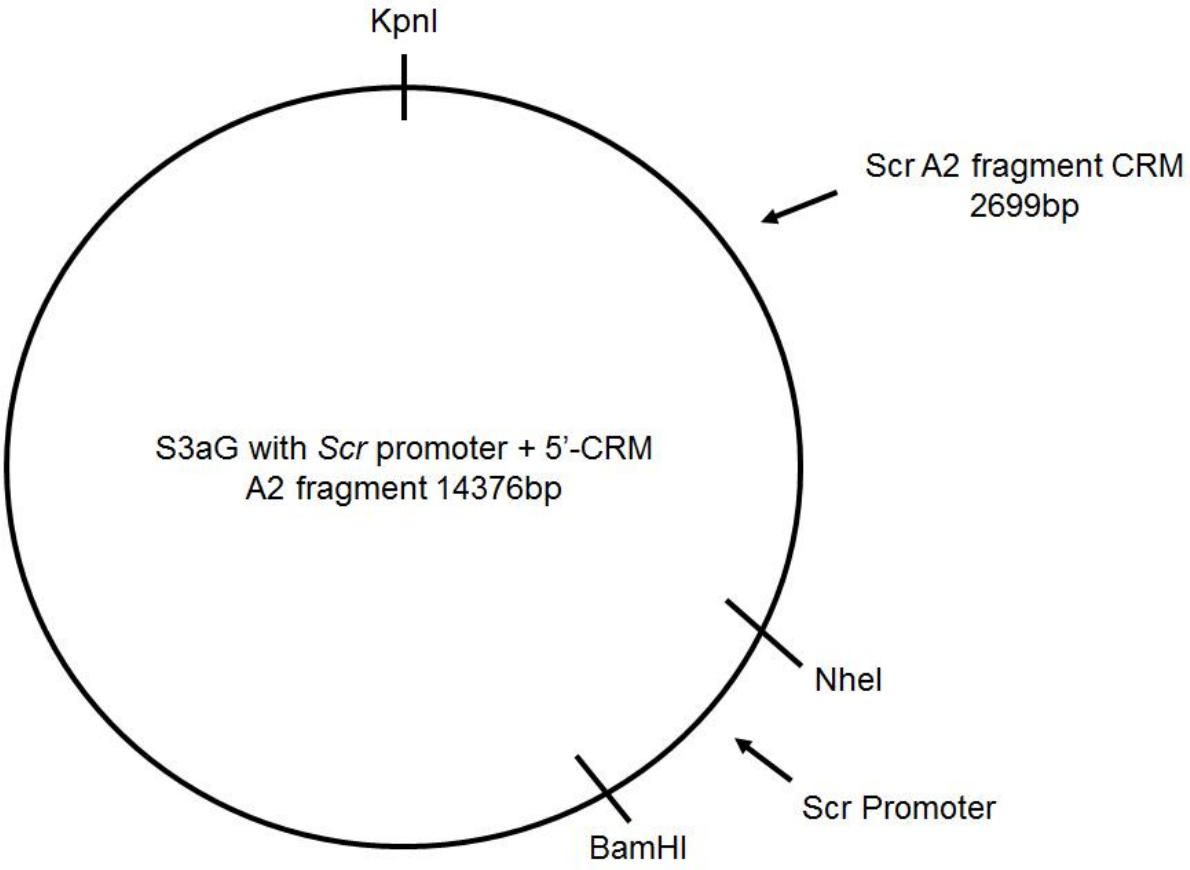


Figure 42 S3aG with *Scr* promoter + 5'-CRM A2 fragment

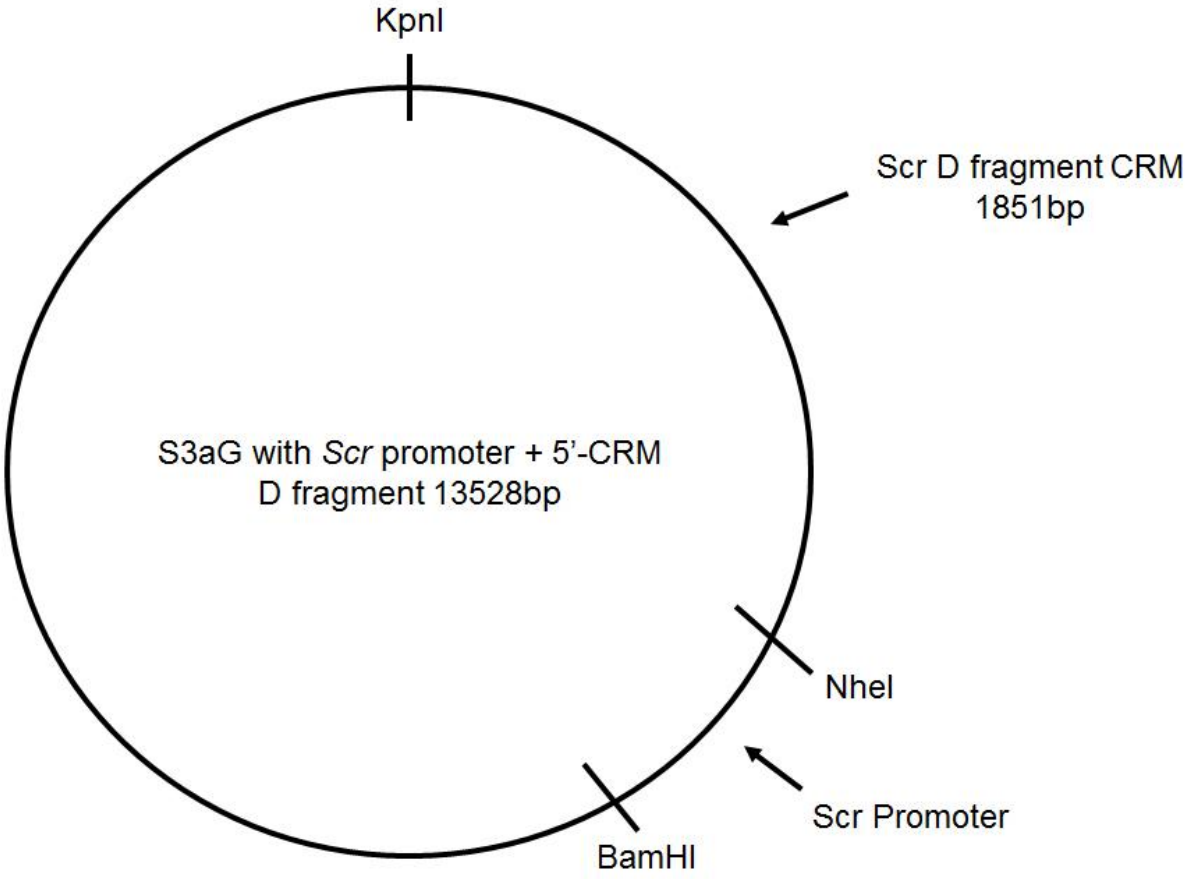


Figure 43 S3aG with *Scr* promoter + 5'-CRM D fragment

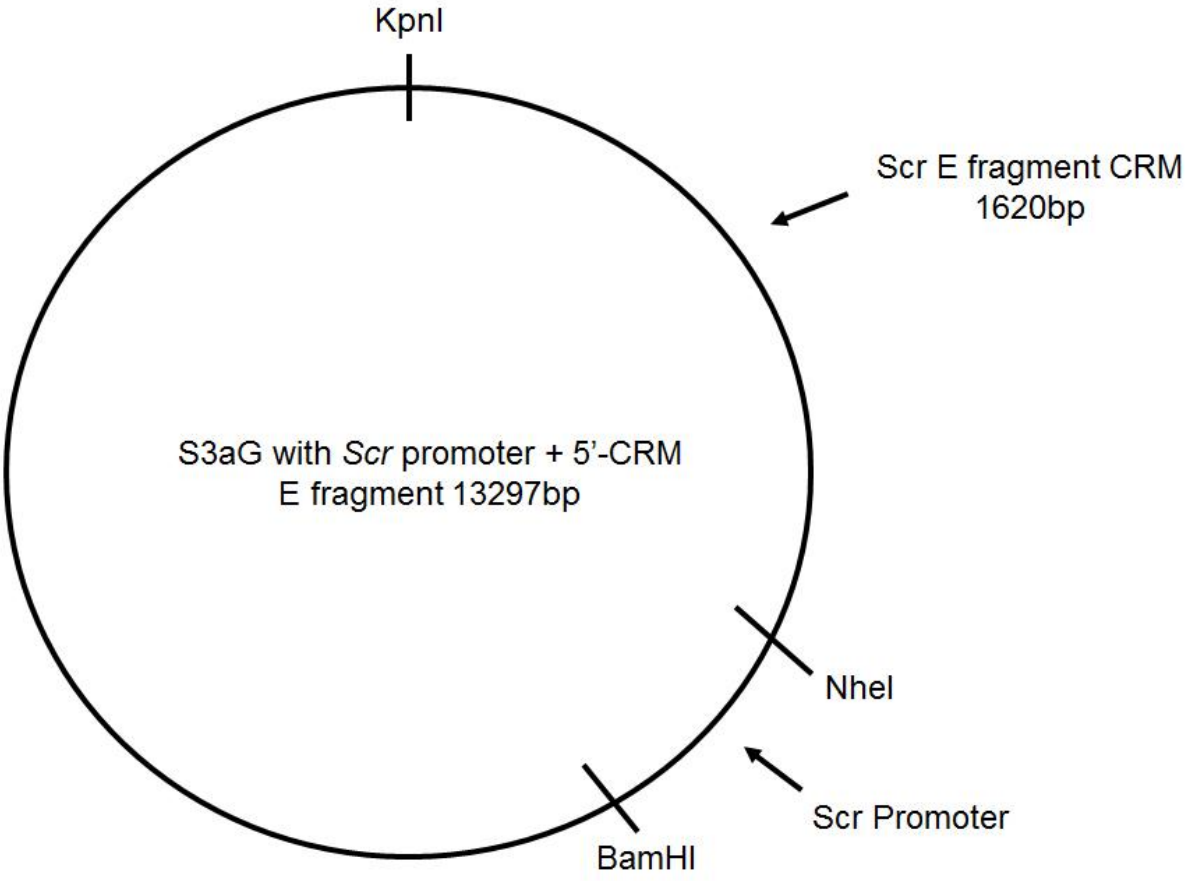


Figure 44 S3aG with *Scr* promoter + 5'-CRM E fragment

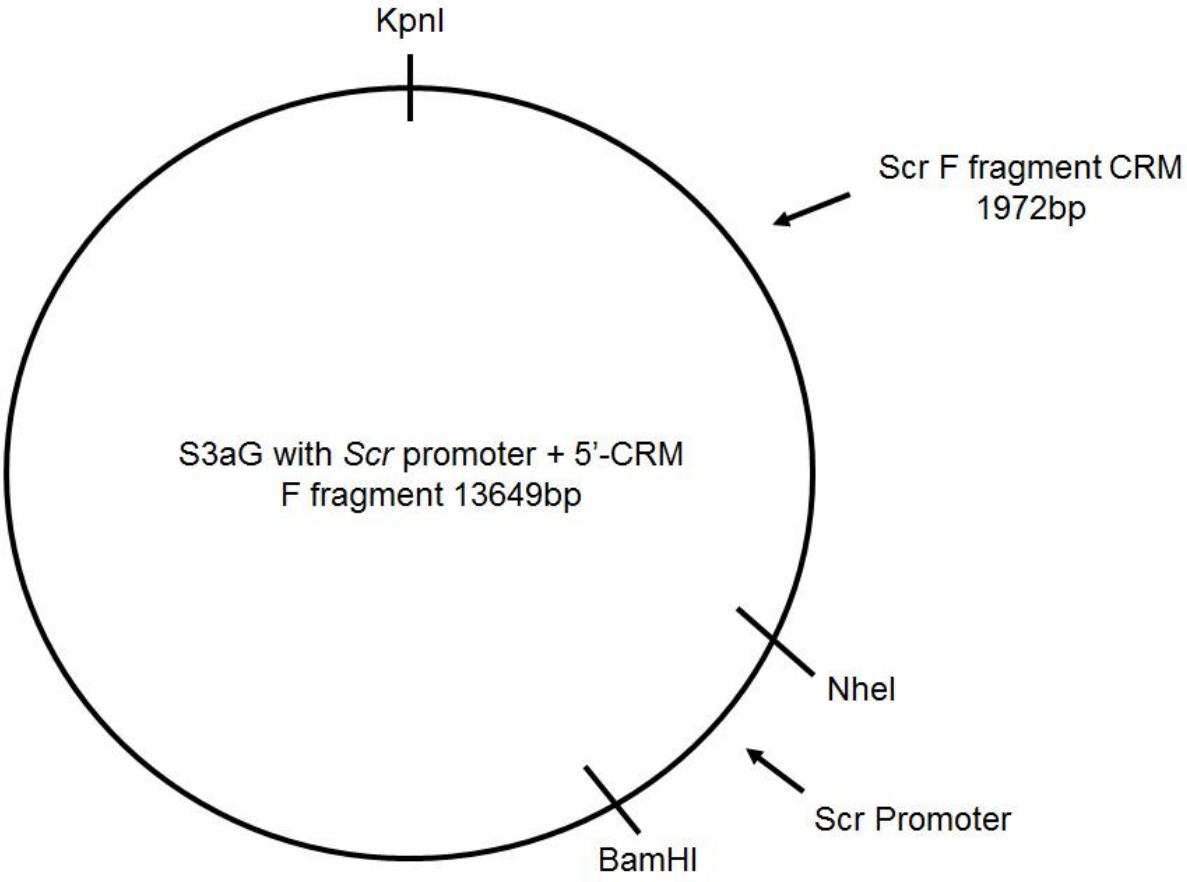


Figure 45 S3aG with *Scr* promoter + 5'-CRM F fragment

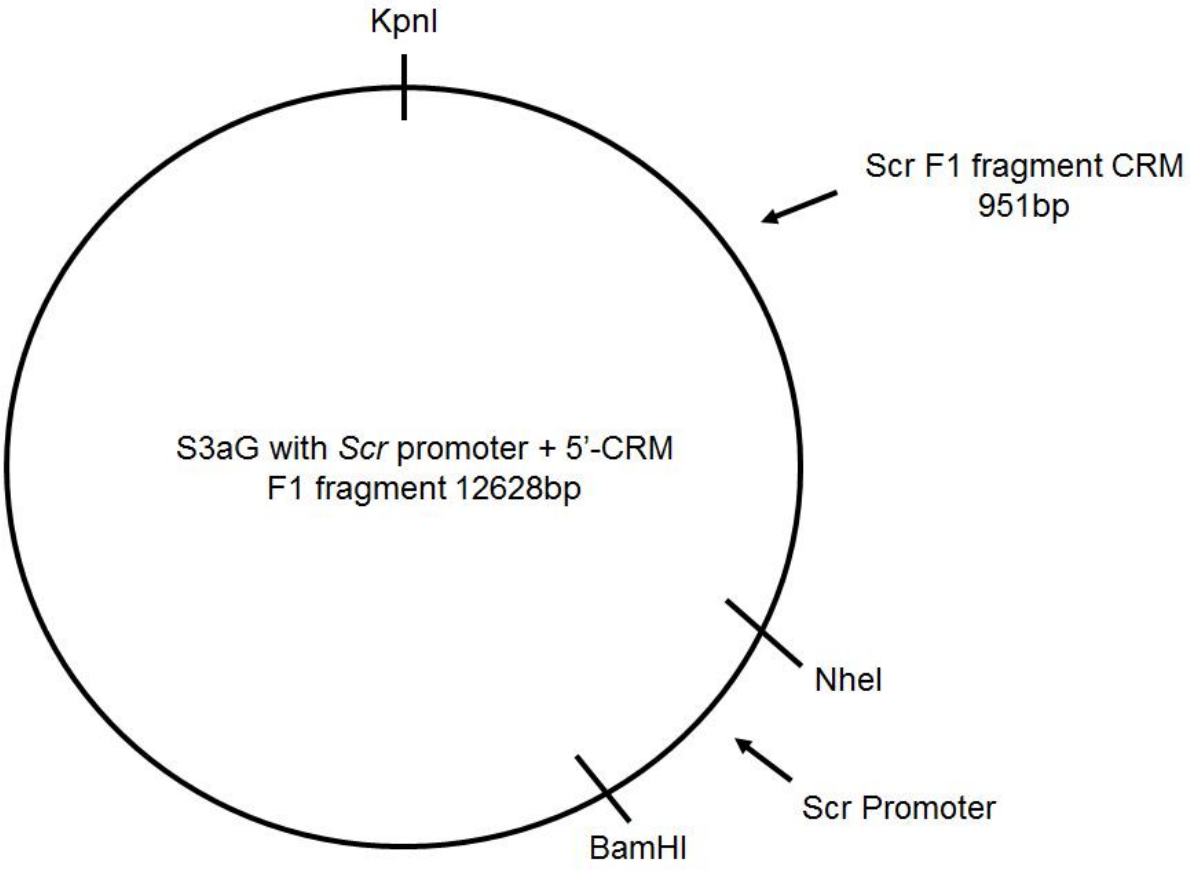


Figure 46 S3aG with *Scr* promoter + 5'-CRM F1 fragment

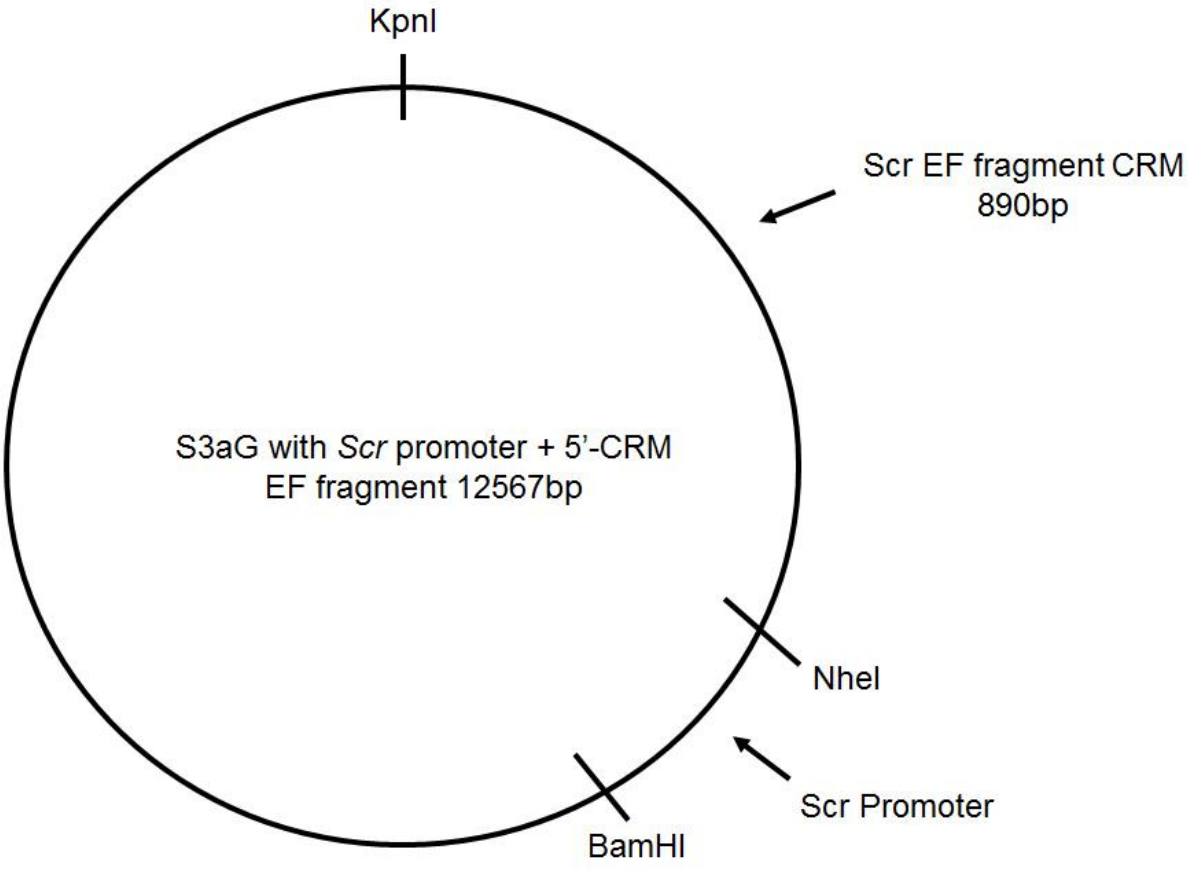


Figure 47 S3aG with *Scr* promoter + 5'-CRM EF fragment

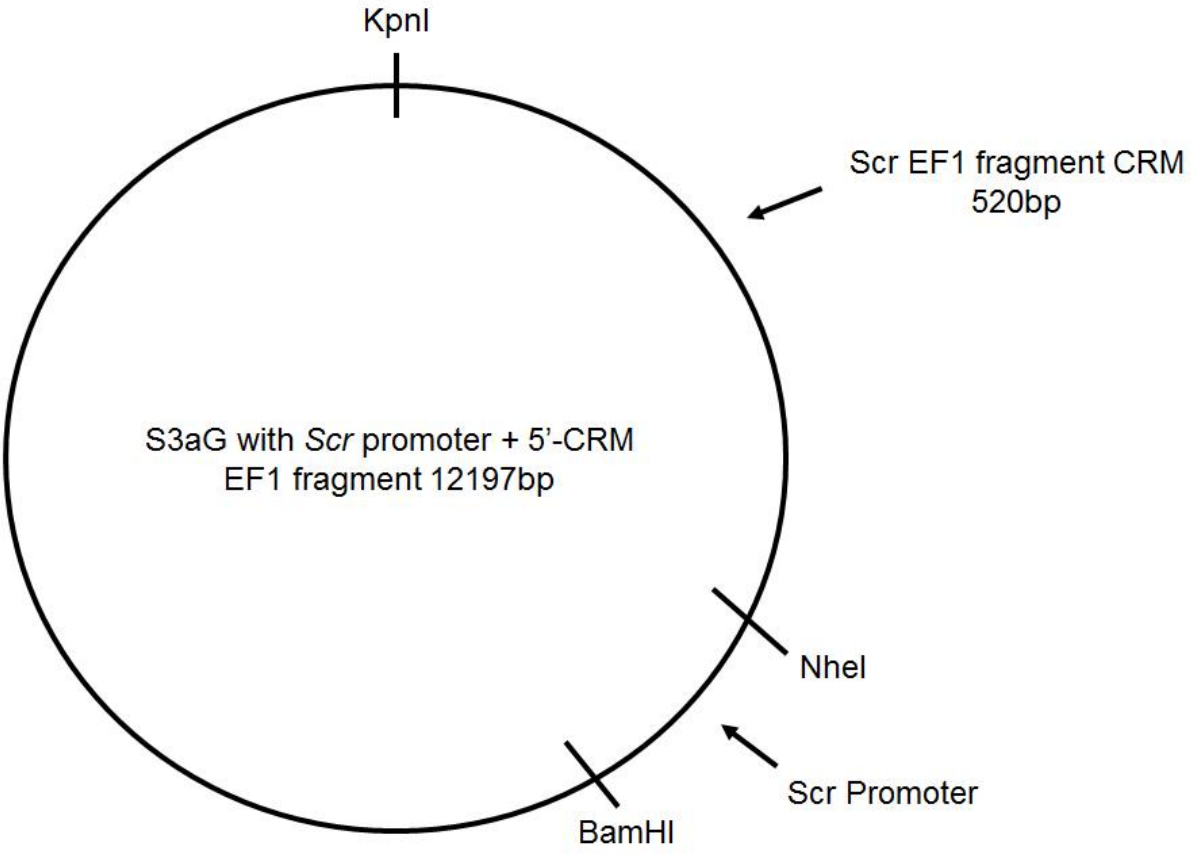


Figure 48 S3aG with *Scr* promoter + 5'-CRM EF1 fragment

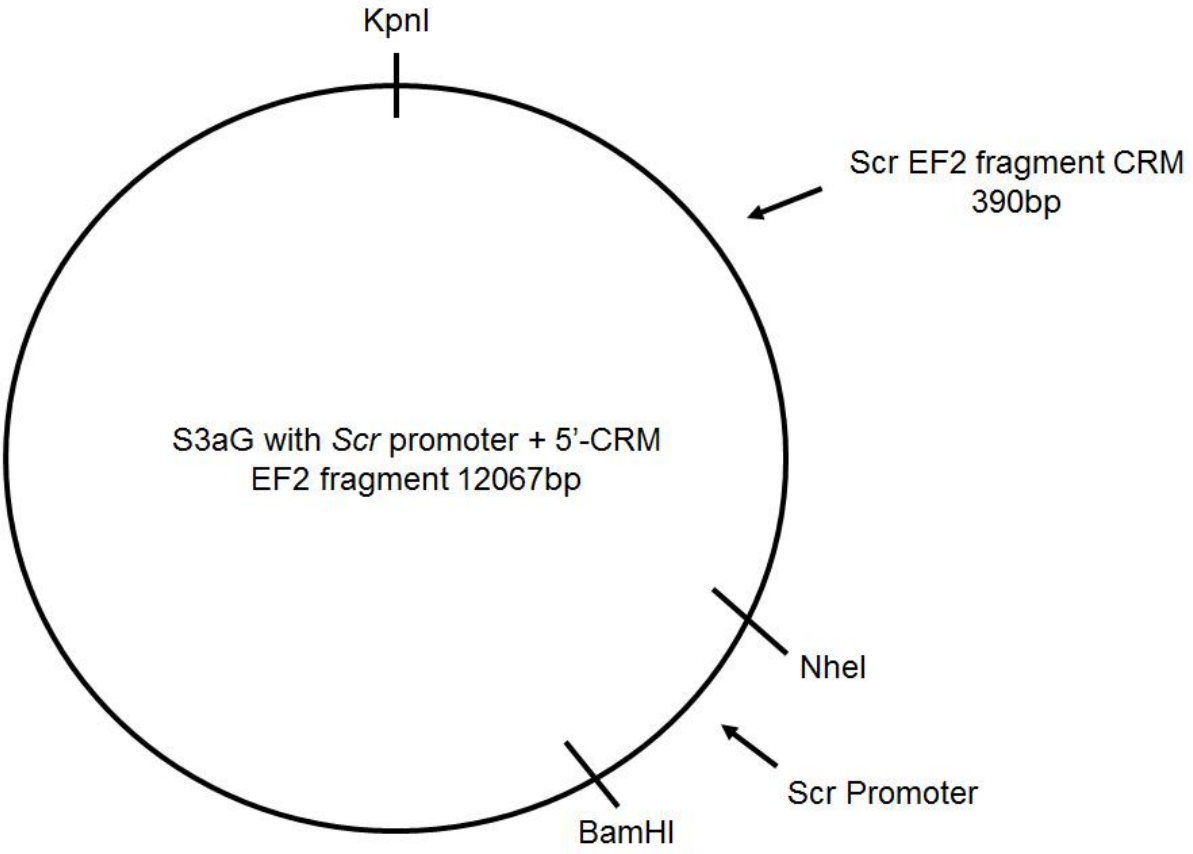


Figure 49 S3aG with *Scr* promoter + 5'-CRM EF2 fragment

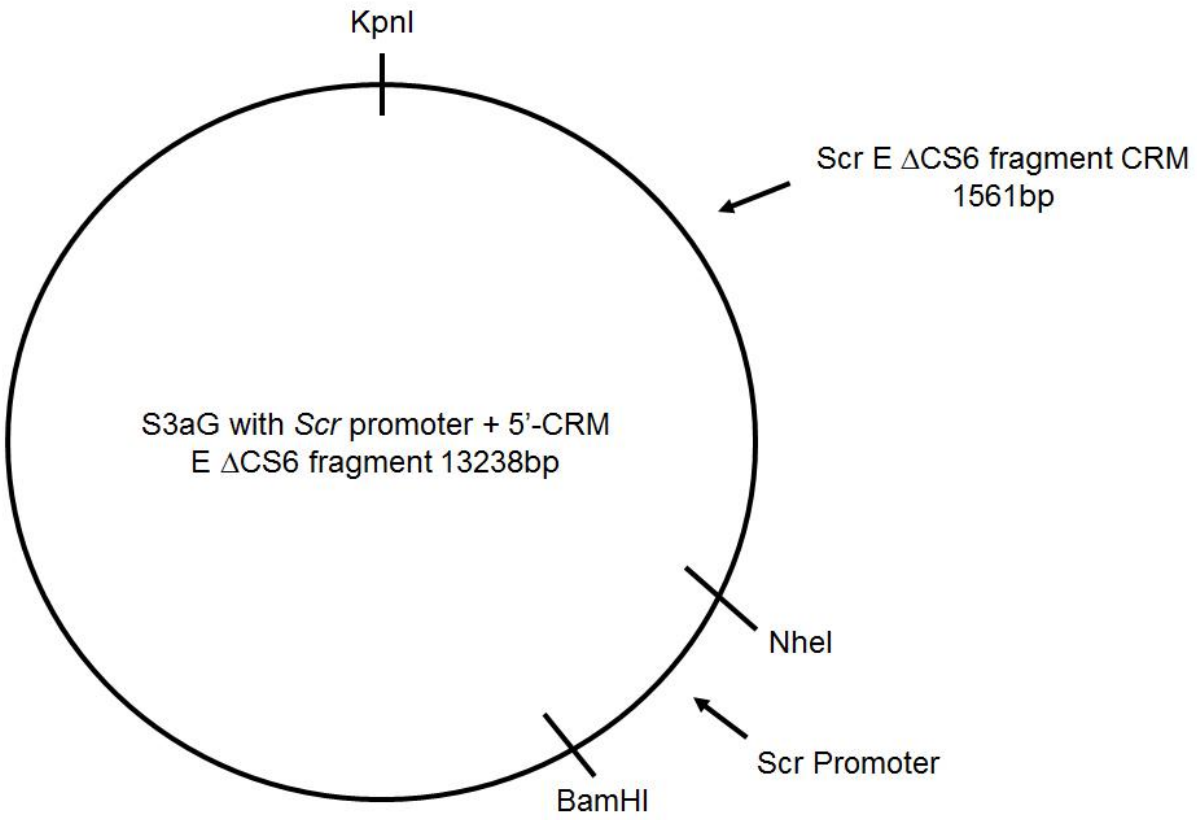


Figure 50 S3aG with *Scr* promoter + 5'-CRM E ΔCS6 fragment

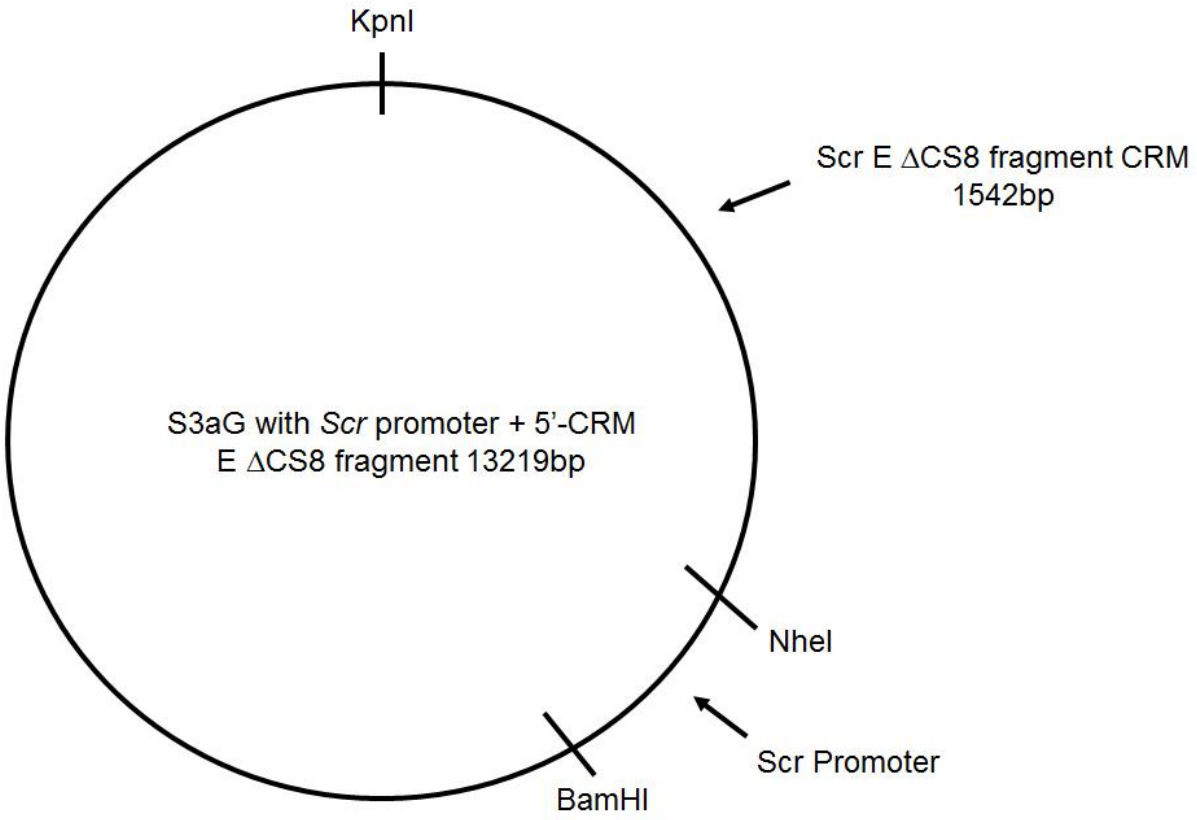


Figure 51 S3aG with *Scr* promoter + 5'-CRM E ΔCS8 fragment

Appendix C Joint pathway experiments

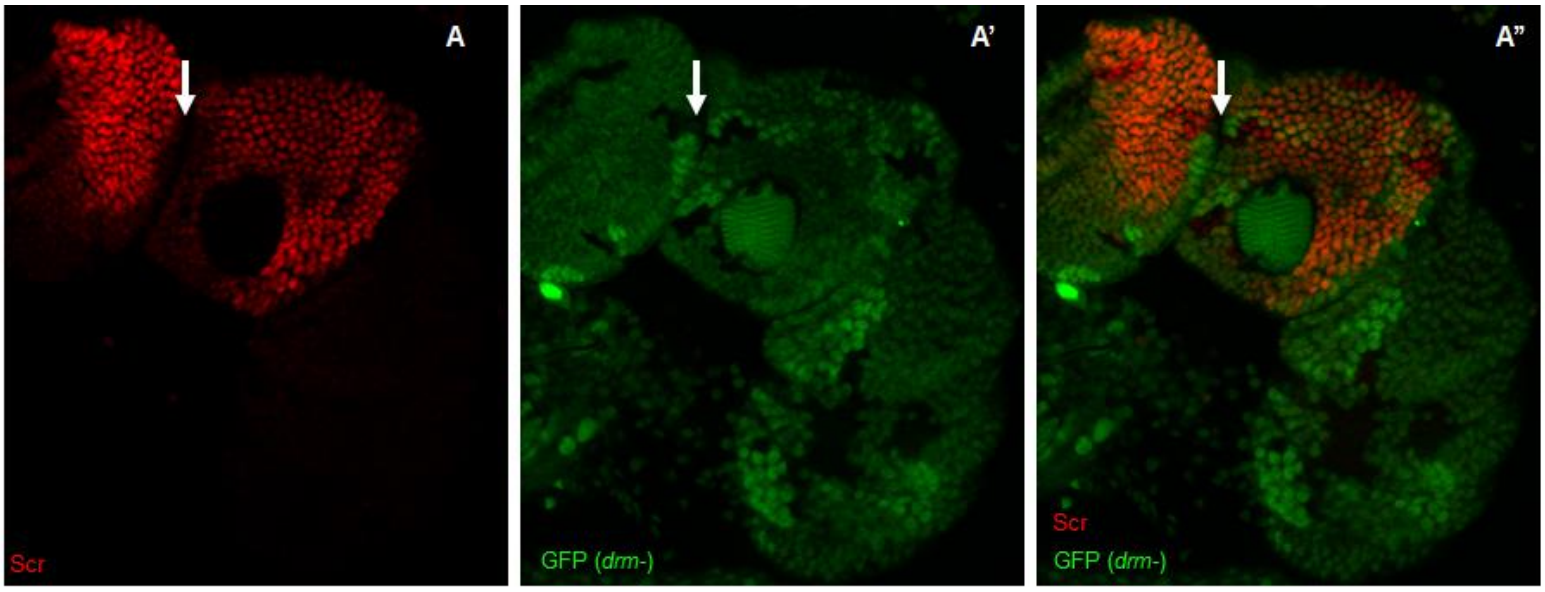


Figure 52 Loss of drm in the joint does not produce ectopic Scr

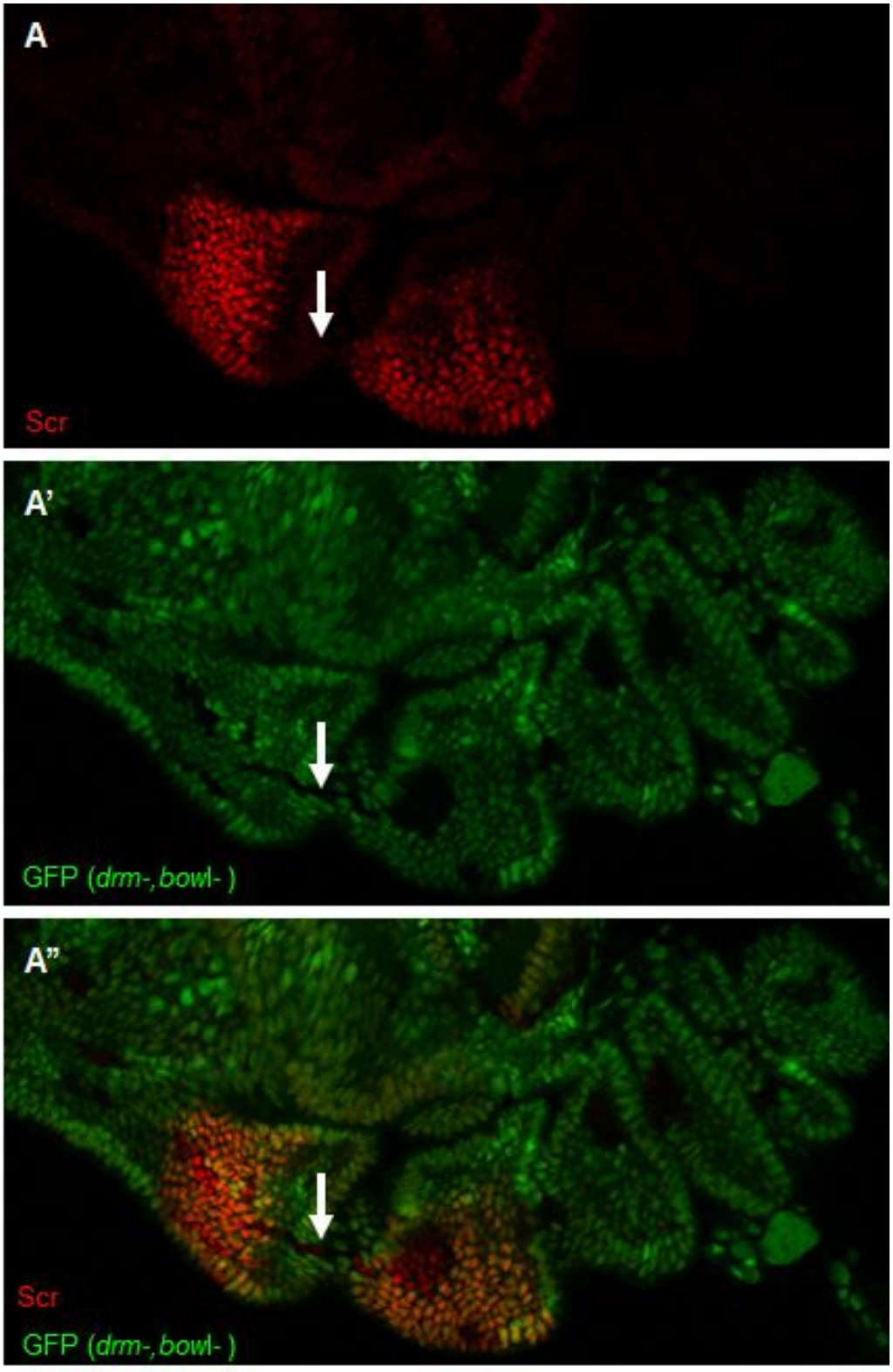


Figure 53 Loss of drm and bowl in the joint does not produce ectopic Scr

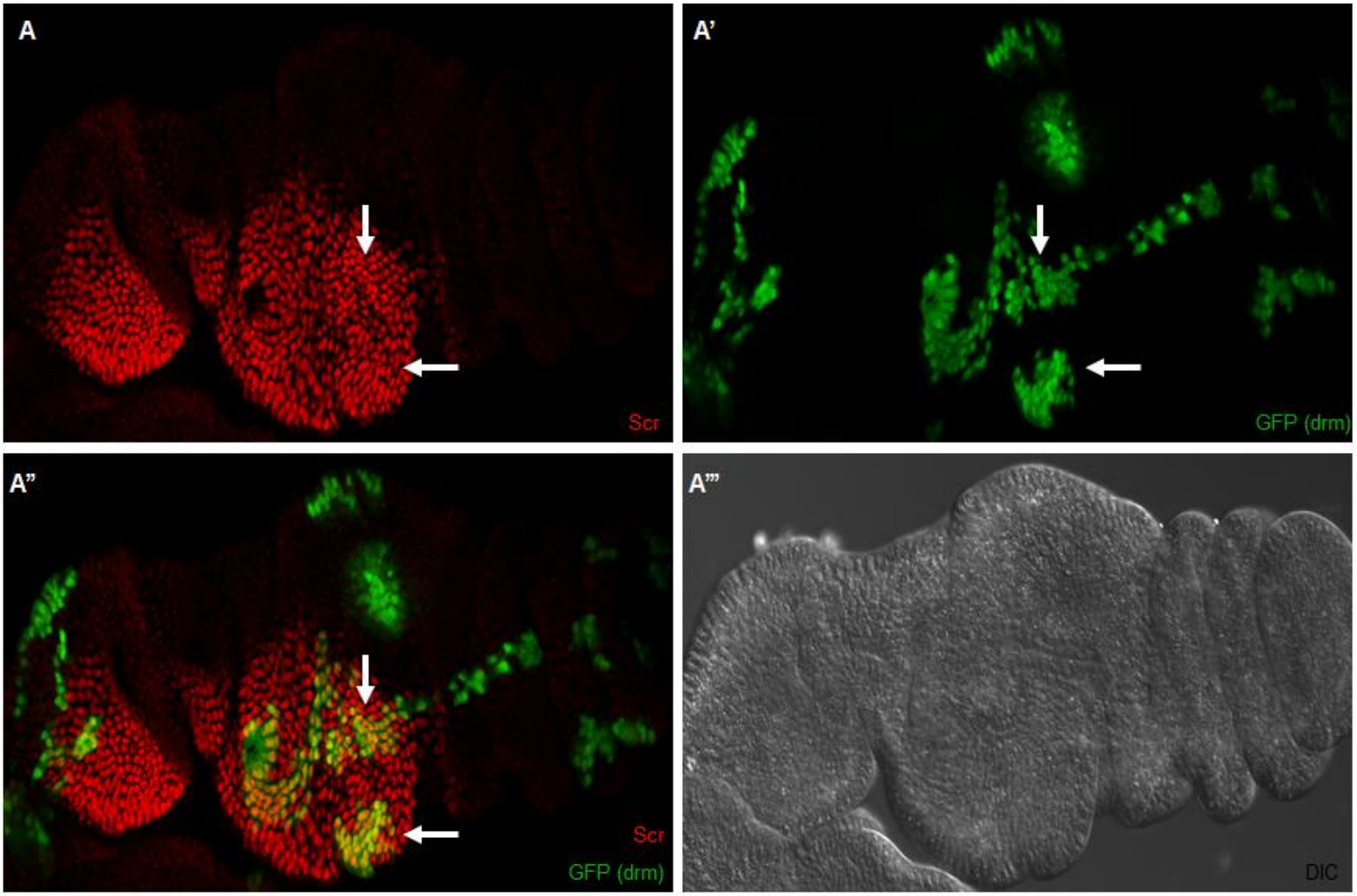


Figure 54 Upregulated *Scr* expression is unaffected by ectopic drm

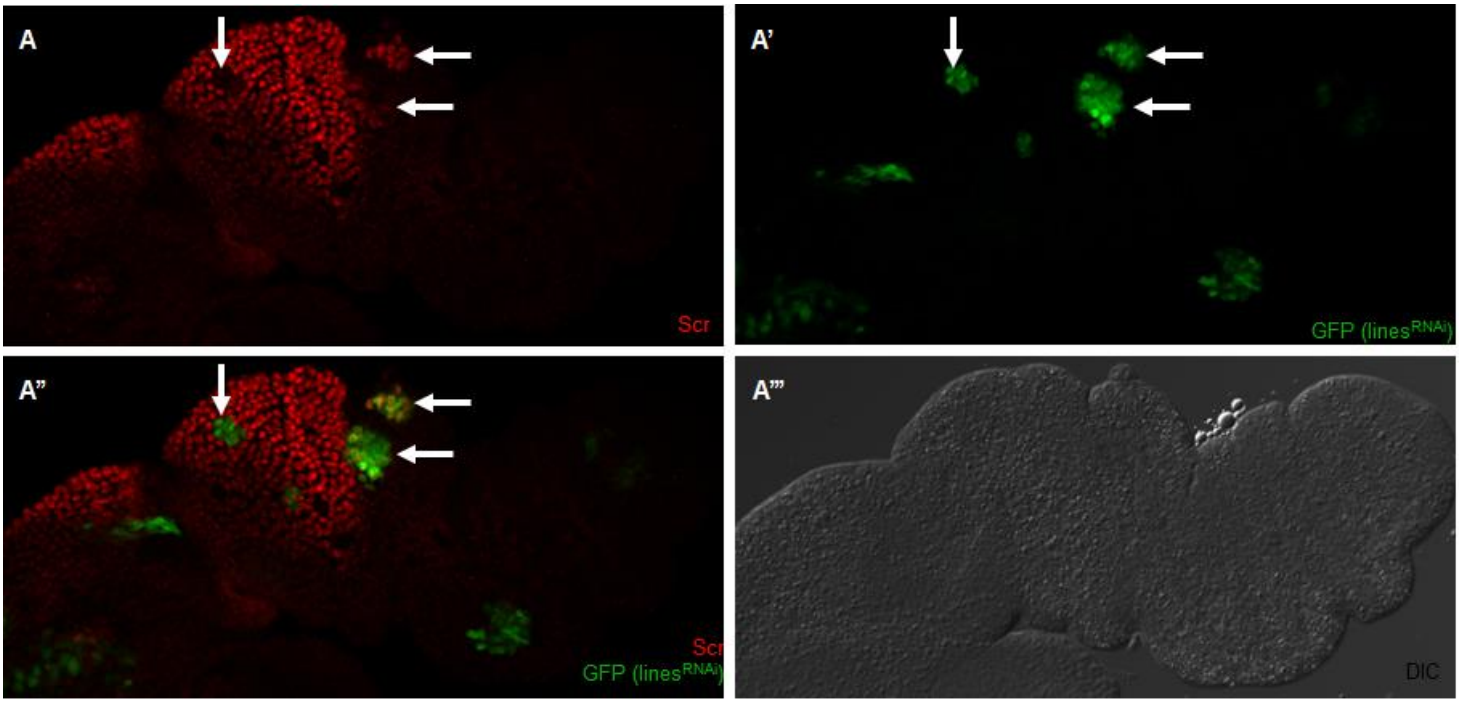


Figure 55 Effect of lines loss depends on which tarsal segment loss takes place in

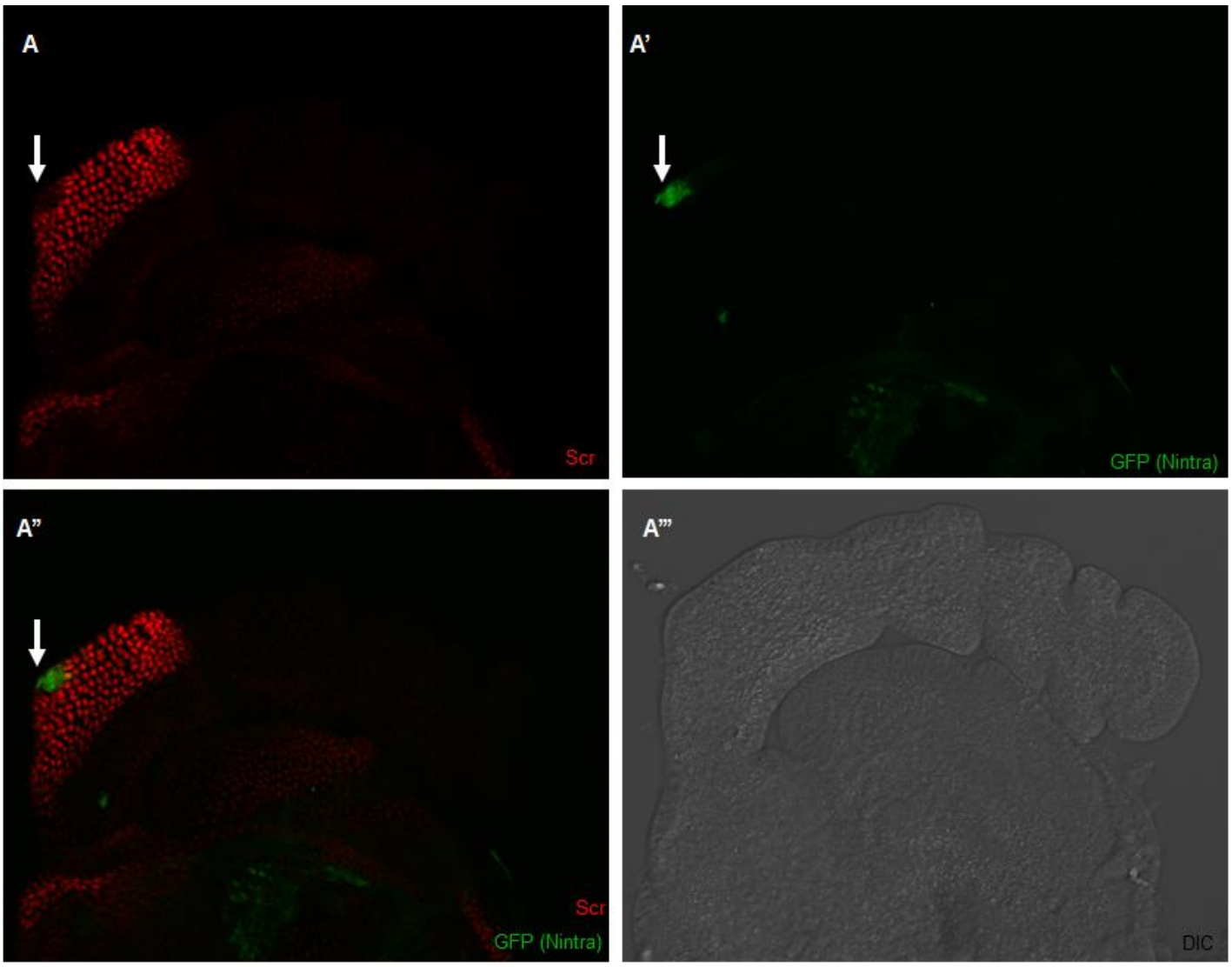


Figure 56 Notch signaling represses *Scr* expression in the basitarsus

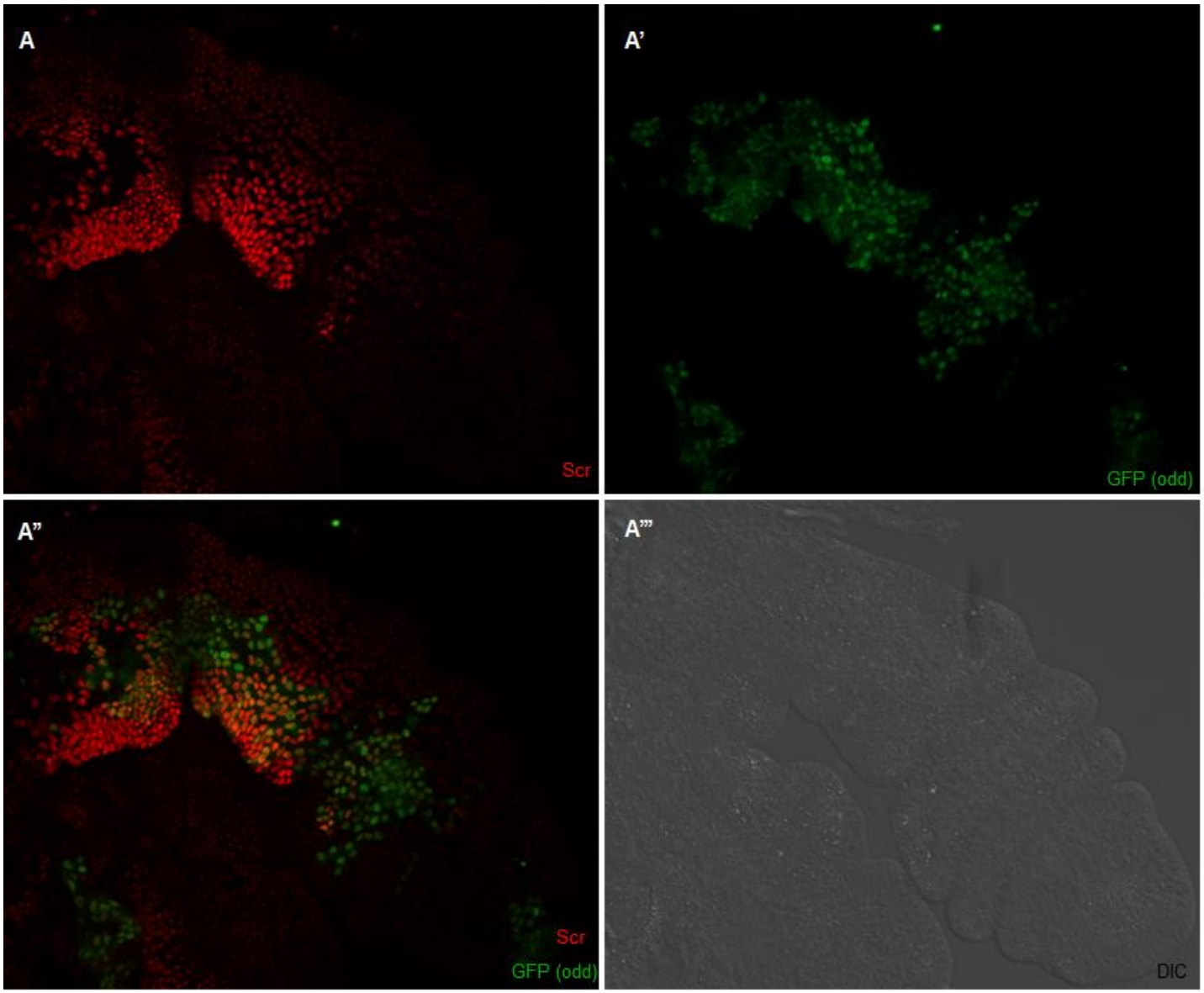


Figure 57 Ectopic odd expression has no effect on upregulated *Scr* expression

Cited Literature

Abu-Shaar, M., & Mann, R. (1998). Generation of multiple antagonistic domains along the proximodistal axis during Drosophila leg development. *Development*, *125*, 3821–3830.

Adam Oberstein, Adam Pare, L. K. and S. S. (2005). Site-specific transgenesis by Cre-mediated recombination in Drosophila. *Nature Methods*, *2*, 583–585.

Akam, M. (1987). The molecular basis for metameric pattern in the Drosophila embryo. *Development*, *101*(1), 1–22.

Alharbi, R., Pettengell, R., Pandha, H., & Morgan, R. (2012). The role of HOX genes in normal hematopoiesis and acute leukemia. *Leukemia*, *27*(5), 1000–1008.

Andrew P. Weng, Adolfo A. Ferrando, Woojoong Lee, John P. Morris, Lewis B. Silverman, Cheryll Sanchez-Irizarry, Stephen C. Blacklow, A. Thomas Look, J. C. A. (2004). Activating Mutations of NOTCH1 in Human T Cell Acute Lymphoblastic Leukemia. *Science*, *306*(5694), 269–271.

Ashburner, M., & Thompson, J. (1976). *The laboratory culture of Drosophila. In The Genetics and Biology of Drosophila (2nd ed.). Academic Press*.

Aza-Blanc, P., Ramírez-Weber, F. A., Laget, M. P. P., Schwartz, C., & Kornberg, T. B. B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell*, *89*(7), 1043–53.

Baanannou, A., Mojica-Vazquez, L. H., Darras, G., Couderc, J. L., Cribbs, D. L., Boube, M., & Bourbon, H. M. (2013). Drosophila Distal-less and Rotund Bind a Single Enhancer Ensuring Reliable and Robust bric-a-brac2 Expression in Distinct Limb Morphogenetic Fields. *PLoS Genetics*, *9*(6).

Bainbridge, S. P., & Bownes, M. (1981). Staging the metamorphosis of Drosophila melanogaster. *Journal of Embryology and Experimental Morphology*, *66*, 57–80.

Baker, N. (1988). Transcription of the segment-polarity gene wingless in the imaginal discs of Drosophila, and the phenotype of a pupal-lethal wg mutation. *Development*, *102*, 489–497.

Bardot, O., Godt, D., Laski, F. a., & Couderc, J.-L. (2002). Expressing UAS-bab1 and UAS-bab2: A comparative study of gain-of-function effects and the potential to rescue the bric a brac mutant phenotype. *Genesis*, *34*(1-2), 66–70.

Barolo, S. (2012). Shadow enhancers: Frequently asked questions about distributed cis-regulatory information and enhancer redundancy. *BioEssays*, *34*(2), 135–141.

Basler, K. and G. S. (1994). Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. *Nature*, *368*(6468), 208–14.

Bateman, J. R., Lee, A. M., & Wu, C. T. (2006). Site-specific transformation of Drosophila via PhiC31 integrase-mediated cassette exchange. *Genetics*, *173*(2), 769–777.

Bhatlekar, S., Fields, J. Z., & Boman, B. M. (2014). HOX genes and their role in the development of human cancers. *Journal of Molecular Medicine*, *92*(8), 811–823.

Bischof, J., Maeda, R. K., Hediger, M., Karch, F., & Basler, K. (2007). An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(9), 3312–7.

Blair, S. S., & Ralston, a. (1997). Smoothened-mediated Hedgehog signalling is required for the maintenance of the anterior-posterior lineage restriction in the developing wing of Drosophila. *Development (Cambridge, England)*, *124*, 4053–4063.

Blair, S. S. (2003). Genetic mosaic techniques for studying Drosophila development. *Development*, *130*(21), 5065–72.

Blair, S. (1995). Compartments and appendage development in Drosophila. *BioEssays*, *17*, 299–309.

Bodenstein, D. (1950). *The postembryonic development of Drosophila. In Biology of Drosophila*. New York, NY: John Wiley and Sons.

Brand, A., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, *118*, 401–415.

Brook, W., & Cohen, S. (1996). Antagonistic interactions between wingless and decapentaplegic responsible for dorsal-ventral pattern in the Drosophila leg. *Science*, *273*(5280), 1373–1377.

Brower, D. L. (1987). Ultrabithorax gene expression in Drosophila imaginal discs and larval nervous system. *Development (Cambridge, England)*, *101*(1), 83–92.

Brower, D. L. (1986). engrailed gene expression in Drosophila imaginal discs. *The EMBO Journal*, *5*(10), 2649–2656.

Brummel, T. J., Twombly, V., Marqu??s, G., Wrana, J. L., Newfeld, S. J., Attisano, L., … Gelbart, W. M. (1994). Characterization and relationship of dpp receptors encoded by the saxophone and thick veins genes in Drosophila. *Cell*, *78*(2), 251–261.

Butler, J. E. F., & Kadonaga, J. T. (2001). Enhancer-promoter specificity mediated by DPE or TATA core promoter motifs. *Genes & Development*, *15*(19), 2515–2519.

Cabrera, C. (1990). Lateral inhibition and cell fate during neurogenesis in Drosophila: the interactions between scute, Notch and Delta. *Development*, *109*, 733–742.

Caestecker, M. P. De, Piek, E., & Roberts, A. B. (2000). Role of Transforming Growth Factor- ␣ Signaling in Cancer AS, *92*(17).

Calleja, M., Renaud, O., Usui, K., Pistillo, D., Morata, G., & Simpson, P. (2002). How to pattern an epithelium: lessons from achaete-scute regulation on the notum of Drosophila. *Gene*, *292*(1-2), 1–12.

Campbell, G., & Tomlinson, A. (1995). Initiation of the proximodistal axis in insect legs. *Development*, *121*(3), 619–628.

Campbell, G., & Tomlinson, A. (1999). Transducing the Dpp Morphogen Gradient in the Wing of Drosophila: Regulation of Dpp Targets by brinker. *Cell*, *96*, 553–562.

Casanueva, M. O., & Ferguson, E. L. (2004). Germline stem cell number in the Drosophila ovary is regulated by redundant mechanisms that control Dpp signaling. *Development*, *131*(9), 1881–90.

Caubit, X., Thangarajah, R., & Theil, T. (1999). Mouse Dac, a novel nuclear factor with homology to Drosophila dachshund shows a dynamic expression in the neural crest, the eye, the neocortex, and the. *Developmental*, *214*, 66–80.

Chou, T., & Perrimon, N. (1996). The autosomal FLP-DFS technique for generating germline mosaics in Drosophila melanogaster. *Genetics*, *144*(December), 1673–1679.

Chu, J., Dong, P. D. S., & Panganiban, G. (2002). Limb type-specific regulation of bric a brac contributes to morphological diversity. *Development (Cambridge, England)*, *129*(3), 695–704.

Clarkson, M., & Saint, R. (1999). A His2AvDGFP fusion gene complements a lethal His2AvD mutant allele and provides an in vivo marker for Drosophila chromosome behavior. *DNA and Cell Biology*, *18*(6), 457–62.

Cohen, B., McGuffin, M. E., Pfeifle, C., Segal, D., & Cohen, S. M. (1992). apterous, a gene required for imaginal disc development in Drosophila encodes a member of

the LIM family of developmental regulatory proteins. *Genes & Development*, *6*(5), 715–729.

Cohen, B., Simcox, A., & Cohen, S. (1993). Allocation of the thoracic imaginal primordia in the Drosophila embryo. *Development*, *117*, 597–608.

Cohen, S., Brönner, G., Küttner, F., Jürgens, G., & Jäckle, H. (1989). Distal-less encodes a homoeodomain protein required for limb development in Drosophila. *Nature*, *338*, 432–434.

Couderc, J., Godt, D., Zollman, S., & Chen, J. (2002). The bric a brac locus consists of two paralogous genes encoding BTB/POZ domain proteins and acts as a homeotic and morphogenetic regulator of imaginal. *Development*, *129*, 2419–2433.

Couso, J. P., Bishop, S. a, & Martinez Arias, a. (1994). The wingless signalling pathway and the patterning of the wing margin in Drosophila. *Development (Cambridge, England)*, *120*(3), 621–636.

Crick, F., & Lawrence, P. (1975). Compartments and polyclones in insect development. *Science*, *189*, 340–347.

Cubas, P., De Celis, J. F., Campuzano, S., & Modolell, J. (1991). Proneural clusters of achaete-scute expression and the generation of sensory organs in the Drosophila imaginal wing disc. *Genes and Development*, *5*(6), 996–1008.

Diaz-Benjumea, F.J., Cohen, B., Cohen, S. M. (1994). Cell interaction between compartments establishes the proximal-distal axis of Drosophila legs. *Nature*, *372*(6502), 175–179.

Dong, P., Chu, J., & Panganiban, G. (2000). Coexpression of the homeobox genes Distal-less and homothorax determines Drosophila antennal identity. *Development*, *127*, 209–216.

Dong, P., Chu, J., & Panganiban, G. (2001). Proximodistal domain specification and interactions in developing Drosophila appendages. *Development*, *128*, 2365–2372.

Duboule, D., & Morata, G. (1994). Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends in Genetics*, *10*(10), 358–364.

Duncan, D. M., Burgess, E. a., & Duncan, I. (1998). Control of distal antennal identity and tarsal development in Drosophila by spineless-aristapedia, a homolog of the mammalian dioxin receptor. *Genes & Development*, *12*(9), 1290–1303.

Emerald, B. S., & Cohen, S. M. (2004). Spatial and temporal regulation of the homeotic selector gene Antennapedia is required for the establishment of leg identity in Drosophila. *Developmental Biology*, *267 462-47*.

Estella, C., Voutev, R., & Mann, R. (2012). A dynamic network of morphogens and transcription factors patterns the fly leg. *Current Topics in Developmental*, *98*, 173–198.

Fichelson, P., Audibert, A., Simon, F., & Gho, M. (2005). Cell cycle and cell-fate determination in Drosophila neural cell lineages. *Trends in Genetics : TIG*, *21*(7), 413–20.

Fisher, A., & Caudy, M. (1998). The function of hairy-related bHLH repressor proteins in cell fate decisions. *Bioessays*, *20*, 298–306.

Fristrom, D., and Fristrom, J. (1993). *The Metamorphic Development of the adult epidermis*. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Fristrom, D., & Fristrom, J. W. (1975). The Mechanism of Evagination of Imaginal Discs of Drosophila melanogaster. *Developmental Biology*, *43*, 1–23.

Galindo, M. I., Bishop, S. a, Greig, S., & Couso, J. P. (2002). Leg patterning driven by proximal-distal interactions and EGFR signaling. *Science*, *297*(5579), 256–9.

Garcia-Bellido A, R. P. (1978). The number of genes in Drosophila melanogaster. *Nature*, *273*(5661), 399–400.

Garcia-Bellido, A. (1979). Genetic analysis of the achaete-scute system of Drosophila melanogaster. *Genetics*, *91*, 491–520.

Garcia-Bellido, A., Ripoll, P., & Morata, G. (1976). Developmental compartmentalization of the wing disk of Drosophila. *Developmental Biology*, *48*, 132–147.

Geer, B., Lischwe, T. D., & Murphy, K. G. (1983). Male fertility in Drosophila melanogaster: genetics of the vermilion region. *Journal of Experimental …*, *118*, 107–118.

Gellon, G., & McGinnis, W. (1998). Shaping animal body plans in development and evolution by modulation of Hox expression patterns. *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology*, *20*(2), 116–25.

Gindhart-Jr, J., King, A., & Kaufman, T. (1995). Characterization of the cis-regulatory region of the Drosophila homeotic gene Sex combs reduced. *Genetics*, *139*, 781–795.

Giorgianni, M. W., & Mann, R. S. (2011). Establishment of medial fates along the proximodistal axis of the Drosophila leg through direct activation of dachshund by Distalless. *Developmental Cell*, *20*(4), 455–68.

Glicksman, M., & Brower, D. (1988). Expression of the Sex combs reduced protein in Drosophila larvae. *Developmental Biology*, *127*, 113–118.

Godt, D., Couderc, J., Cramton, S., & Laski, F. (1993). Pattern formation in the limbs of Drosophila: bric a brac is expressed in both a gradient and a wave-like pattern and is required for specification and proper. *Development*, *119*, 799–812.

Golic, K. G., & Lindquist, S. (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the drosophila genome. *Cell*, *59*(3), 499–509.

Golic, K. (1991). Site-specific recombination between homologous chromosomes in Drosophila. *Science*, *252*, 958–961.

González, F., Swales, L., & Bejsovec, A. (1991). Secretion and movement of wingless protein in the epidermis of the Drosophila embryo. *Mechanisms of …*, *35*, 43–54.

González-Crespo, S., & Abu-Shaar, M. (1998). Antagonism between extradenticle function and Hedgehog signalling in the developing limb. *Nature*, *394*(July), 196–200.

Gorfinkiel, N., Morata, G., & Guerrero, I. (1997). The homeobox gene Distal-less induces ventral appendage development in Drosophila. *Genes & Development*, *11*(17), 2259–2271.

Goto, S., & Hayashi, S. (1997). Cell migration within the embryonic limb primordium of Drosophila as revealed by a novel fluorescence method to visualize mRNA and protein. *Development Genes and Evolution*, *207*, 194–198.

Goto, S., & Hayashi, S. (1999). Proximal to distal cell communication in the Drosophila leg provides a basis for an intercalary mechanism of limb patterning. *Development*, *126*, 3407–3413.

Goto, S., & Hayashi, S. (1997). Specification of the embryonic limb primordium by graded activity of Decapentaplegic. *Development*, *124*, 125–132.

Green, R. B., Hatini, V., Johansen, K. A., Liu, X.-J., & Lengyel, J. A. (2002). Drumstick is a zinc finger protein that antagonizes Lines to control patterning and morphogenesis of the Drosophila hindgut. *Development*, *129*(15), 3645–3656.

Greenberg, L., & Hatini, V. (2009). Essential roles for lines in mediating leg and antennal proximodistal patterning and generating a stable Notch signaling interface at segment borders. *Developmental Biology*, *330*(1), 93–104.

Groth, A. C., Fish, M., Nusse, R., & Calos, M. P. (2004). Construction of Transgenic Drosophila by Using the Site-Specific Integrase from Phage PhiC31. *Genetics*, *166*(4), 1775–1782.

Hafen, E., Kuroiwa, A., & Gehring, W. (1984). Spatial distribution of transcripts from the segmentation gene fushi tarazu during Drosophila embryonic development. *Cell*, *37*(July), 833–841.

Handler, A. (1982). Ecdysteroid titers during pupal and adult development in Drosophila melanogaster. *Developmental Biology*, *93*, 73–82.

Hannah-Alava, A. (1958). Developmental genetics of the posterior legs in Drosophila melanogaster. *Genetics*, *43*, 878–905.

Hanson, I. M. (2001). Mammalian homologues of the Drosophila eye specification genes. *Seminars in Cell & Developmental Biology*, *12*(6), 475–84.

Hao, I., Green, R. B., Dunaevsky, O., Lengyel, J. A., & Rauskolb, C. (2003). The odd-skipped family of zinc finger genes promotes Drosophila leg segmentation. *Developmental Biology*, *263*(2), 282–295.

Harding K., McGinnis W., W. C. and L. (1985). Spatially Regulated Expression of Homeotic Genes in Drosophila. *Science*, *229*(4719), 1236–1242.

Hartenstein, V., & Campos-Ortega, J. (1986). The peripehal nervous system of mutants of early neurogenesis in Drosophila melanogaster. *Roux's Archives of Developmental Biology*, *195*, 210–221.

Hartenstein, V., & Campos-Ortega, J. (1984). Early neurogenesis in wildtype Drosophila melanogaster. *Roux's Archives of Developmental Biology*, *193*, 308–325.

Hartenstein, V. (1993). *Atlas of Drosophila Development*. Long Island, New York: Cold Spring Harbor Laboratory Press.

Hartenstein, V., & Posakony, J. W. (1990). Sensillum development in the absence of cell division: The sensillum phenotype of the Drosophila mutant string. *Developmental Biology*, *138*(1), 147–158.

Hartenstein, V., & Posakony, J. (1989). Development of adult sensilla on the wing and notum of Drosophila melanogaster. *Development*, *107*, 389–405.

Hays, R., Buchanan, K., Neff, C., & Orenic, T. (1999). Patterning of Drosophila leg sensory organs through combinatorial signaling by hedgehog, decapentaplegic and wingless. *Development*, *126*, 2891–2899.

Held, L. I., & Heup, M. a. (1996). Genetic mosaic analysis of decapentaplegic and wingless gene function in the Drosophila leg. *Development Genes and Evolution*, *206*(3), 180–94.

Held, L. I. (1990). Arrangement of bristles as a function of bristle number on a leg segment in Drosophila melanogaster. *Roux's Archives of Developmental Biology*, *199*(1), 48–62.

Held, L. I. (2002). Bristles induce bracts via the EGFR pathway on Drosophila legs. *Mechanisms of Development*, *117*(1-2), 225–234.

Held, L. (1995). Axes, boundaries and coordinates: the ABCs of fly leg development. *BioEssays*, *17*(8), 721–732.

Higashijima, S., Kojima, T., Michiue, T., Ishimaru, S., Emori, Y., & Saigo, K. (1992). Dual Bar homeo box genes of Drosophila required in two photoreceptor cells, R1 and R6, and primary pigment cells for normal eye development. *Genes & Development*, *6*, 50–60.

Hong, J.-W., Hendrix, D. A., & Levine, M. S. (2008). Shadow Enhancers as a Source of Evolutionary Novelty. *Science*, *321*(5894), 1314.

Horn, C., & Handler, A. M. (2005). Site-specific genomic targeting in Drosophila. *Proc Natl Acad Sci U S A*, *102*(35), 12483–12488.

Hughes, C., & Kaufman, T. (2002). Hox genes and the evolution of the arthropod body plan. *Evolution & Development*, *499*, 459–499.

Irvine, K., & Rauskolb, C. (2001). Boundaries in development: formation and function. *Annual Review of Cell and …*, *17*, 189–214.

Jackson, P. D., & Hoffmann, F. M. (1994). Embryonic expression patterns of the Drosophila decapentaplegic gene: separate regulatory elements control blastoderm expression and lateral ectodermal expression. *Developmental Dynamics : An Official Publication of the American Association of Anatomists*, *199*(1), 28–44.

Jiang, J., & Struhl, G. (1996). Complementary and Mutually Exclusive Activities of Decapentaplegic and Wingless Organize Axial Patterning during Drosophila Leg Development. *Cell*, *86*(3), 401–409.

Joshi, M., Buchanan, K. T., Shroff, S., & Orenic, T. V. (2006). Delta and Hairy establish a periodic prepattern that positions sensory bristles in Drosophila legs. *Developmental Biology*, *293*(1), 64–76.

Joutel A, Corpechot C, Ducros A, Vahedi K, Chabriat H, Mouton P, Alamowitch S, Domenga V, Cécillion M, Marechal E, Maciazek J, Vayssiere C, Cruaud C, Cabanis EA, Ruchoux MM, Weissenbach J, Bach JF, Bousser MG, Tournier-Lasserve E.Joutel A1, Corpechot C, D, T.-L. E. (1996). Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature*, *383*(6602), 707–710.

Kassis, J. A., Nollt, E., Vansickle, E. P., Odenwald, W. F., & Perrimon, N. (1992). Altering the insertional specificity of a Drosophila transposable element. *Proceedings of the …*, *89*(March), 1919–1923.

Kent, W. J. (2002). BLAT- The BLAST -Like Alignment Tool. *Genome Research*, *12*, 656–664.

Kim, Jaeseob, Johnson, Kirby, Chen, Hui Ju, Carroll, Sean and Laughon, A. (1997). Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. *Nature*, *388*, 304–308.

Kojima, T., Ishimaru, S., Higashijima, S., Takayama, E., Akimaru, H., Sone, M., … Saigo, K. (1991). Identification of a different-type homeobox gene, BarH1, possibly causing Bar (B) and Om (1D) mutations in Drosophila. *Proceedings of the …*, *88*(May), 4343–4347.

Kojima, T. (2004). The mechanism of Drosophila leg development along the proximodistal axis. *Development, Growth & Differentiation*, *46*(February), 115–129.

Kubota, K., Goto, S., & Hayashi, S. (2003). The role of Wg signaling in the patterning of embryonic leg primordium in Drosophila. *Developmental Biology*, *257*(1), 117–126.

Kumarasinghe, D. D., Hopwood, B., Kuliwaba, J. S., Atkins, G. J., & Fazzalari, N. L. (2011). An update on primary hip osteoarthritis including altered wnt and TGF-?? associated gene expression from the bony component of the disease. *Rheumatology*, *50*(12), 2166–2175.

Kwon, C., Hays, R., Fetting, J., & Orenic, T. V. (2004). Opposing inputs by Hedgehog and Brinker define a stripe of hairy expression in the Drosophila leg imaginal disc. *Development (Cambridge, England)*, *131*(11), 2681–92.

Lai, E. C., & Orgogozo, V. (2004). A hidden program in Drosophila peripheral neurogenesis revealed: fundamental principles underlying sensory organ diversity. *Developmental Biology*, *269*(1), 1–17.

LaJeunesse, D., & Shearn, A. (1995). Trans-regulation of thoracic homeotic selector genes of the Antennapedia and bithorax complexes by the trithorax group genes: absent, small, and homeotic discs 1 and 2. *Mechanisms of Development*, *53*(1), 123–139.

Lawrence, P. A. (1992). The making of a fly: The genetics of animal design. *Oxford: Blackwell Scientific Publications*.

Lawrence, P., Casal, J., & Struhl, G. (2002). Towards a model of the organisation of planar polarity and pattern in the Drosophila abdomen. *Development*, *129*, 2749–2760.

Lawrence, P., & Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from drosophila? *Cell*, *85*, 951–961.

Lecuit, T., Brook, W., Ng, M., Calleja, M., Sun, H., & Cohen, S. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature*, *381*, 387–393.

Lecuit, T., & Cohen, S. (1997). Proximal–distal axis formation in the Drosophila leg. *Nature*, *388*(July), 139–145.

Lee, T., & Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron*, *22*, 451–461.

Lewis, E. (1978). A gene complex controlling segmentation in Drosophila. *Nature*, *276*(5688), 565–570.

Lewis, R., Wakimoto, B., Denell, R., & Kaufman, T. (1980). Genetic analysis of the Antennapedia gene complex (ANT-C) and adjacent chromosomal regions of Drosophila melanogaster. II. Polytene chromosome segments. *Genetics*, *95*, 383–397.

Lours, C., Bardot, O., Godt, D., Laski, F. A., & Couderc, J. L. (2003). The Drosophila melanogaster BTB proteins bric à brac bind DNA through a composite DNA binding domain containing a pipsqueak and an AT-Hook motif. *Nucleic Acids Research*, *31*(18), 5389–5398.

Mann, R. S. (2014). Hth, Dac, & Dll expression throughout Drosophila leg development. Retrieved from http://themannlab.org/wp-content/plugins/slideshow-gallery/vendors/timthumb.php?src=wp-content/uploads/slideshow-gallery/12.jpg&w=600&h=450&q=100&a=t

Mardon, G., Solomon, N., & Rubin, G. (1994). dachshund encodes a nuclear protein required for normal eye and leg development in Drosophila. *Development*, *120*, 3473–3486.

Martinez-Arias, A., Ingham, P. W., Scott, M. P., & Akam, M. E. (1987). The spatial and temporal deployment of Dfd and Scr transcripts throughout development of Drosophila. *Development (Cambridge, England)*, *100*(4), 673–83.

McDaniell, R., Warthen, D. M., Sanchez-Lara, P. A., Pai, A., Krantz, I. D., Piccoli, D. A., & Spinner, N. B. (2006). NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. *American Journal of Human Genetics*, *79*(1), 169–73.

McGuire Sean E., Phuong T. Le, Alexander J. Osborn, Kunihiro Matsumoto, R. L. D. (2003). Spatiotemporal Rescue of Memory Dysfunction in Drosophila. *Science*, *302*(5651), 1765–1768.

Merli, C., Bergstrom, D.E., Cygan, J.A., Blackman, R. K. (1996). Promoter specificity mediates the independent regulation of neighboring genes. *Genes & Development*, *10*(10), 1260–1270.

Morel, V., & Arias, A. M. (2004). Armadillo/beta-catenin-dependent Wnt signalling is required for the polarisation of epidermal cells during dorsal closure in Drosophila. *Development (Cambridge, England)*, *131*(14), 3273–83.

Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, K. K. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science*, *275*(5307), 1787–1790.

Motzny, C. K., & Holmgren, R. (1995). The Drosophila cubitus interruptus protein and its role in the wingless and hedgehog signal transduction pathways. *Mechanisms of Development*, *52*(1), 137–150.

Nellen, D., Affolter, M., & Basler, K. (1994). Receptor serine/threonine kinases implicated in the control of Drosophila body pattern by decapentaplegic. *Cell*, *78*(2), 225–237.

Nellen, D., Burke, R., Struhl, G., & Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell*, *85*, 357–368.

Neul, J. L., & Ferguson, E. L. (1998). Spatially Restricted Activation of the SAX Receptor by SCW Modulates DPP/TKV Signaling in Drosophila Dorsal–Ventral Patterning. *Cell*, *95*(4), 483–494.

Ni, J.-Q., Markstein, M., Binari, R., Pfeiffer, B., Liu, L.-P., Villalta, C., … Perrimon, N. (2008). Vector and parameters for targeted transgenic RNA interference in Drosophila melanogaster. *Nature Methods*, *5*(1), 49–51.

Ni, J.-Q., Zhou, R., Czech, B., Liu, L.-P., Holderbaum, L., Yang-Zhou, D., … Perrimon, N. (2011). A genome-scale shRNA resource for transgenic RNAi in Drosophila. *Nature Methods*, *8*(5), 405–407.

Noyes, M. B., Christensen, R. G., Wakabayashi, A., Stormo, G. D., Brodsky, M. H., & Wolfe, S. A. (2008). Analysis of Homeodomain Specificities Allows the Family-wide Prediction of Preferred Recognition Sites. *Cell*, *133*(7), 1277–1289.

Nusslein-Volhard, C. (1991). Determination of the embryonic axes of Drosophila. *Development*, *113*, (Suppl. 1), 1–10.

Ohsako, S., Hyer, J., Panganiban, G., Oliver, I., & Caudy, M. (1994). Hairy function as a DNA-binding helix-loop-helix repressor of Drosophila sensory organ formation. *Genes & Development*, *8*(22), 2743–2755.

Orenic, T., Held, L., Paddock, S., & Carroll, S. (1993). The spatial organization of epidermal structures: hairy establishes the geometrical pattern of Drosophila leg bristles by delimiting the domains of achaete expression. *Development*, *118*, 9–20.

Pirrotta, V., Chan, C., McCabe, D., & Qian, S. (1995). Distinct parasegmental and imaginal enhancers and the establishment of the expression pattern of the Ubx gene. *Genetics*, *141*, 1439–1450.

Prestwich Tyler C, M. O. A. (2007). Wnt/β-catenin signaling in adipogenesis and metabolism. *Current Opinion in Cell Biology*, *19*(6), 612–617.

Pueyo, J., Galindo, M., Bishop, S., & Couso, J. (2000). Proximal-distal leg development in Drosophila requires the apterous gene and the Lim1 homologue dlim1. *Development*, *127*, 5391–5402.

Qian, S., Capovilla, M., & Pirrotta, V. (1991). The bx region enhancer, a distant cis-control element of the Drosophila Ubx gene and its regulation by hunchback and other segmentation genes. *The EMBO Journal*, *10*(6), 1415–25.

Randsholt, N. B., & Santamaria, P. (2008). How Drosophila change their combs: the Hox gene Sex combs reduced and sex comb variation among Sophophora species. *Evolution & Development*, *10*(1), 121–33.

Reddy, G., & Rodrigues, V. (1999). Sibling cell fate in the Drosophila adult external sense organ lineage is specified by prospero function, which is regulated by Numb and Notch. *Development*, *126*, 2083–2092.

Romani, S., Campuzano, S., Macagno, E. R., & Modolell, J. (1989). Expression of achaete and scute genes in Drosophila imaginal discs and their function in sensory organ development. *Genes & Development*, *3*(7), 997–1007.

Ruberte, E., Marty, T., Nellen, D., Affolter, M., & Basler, K. (1995). An absolute requirement for both the type II and type I receptors, punt and thick veins, for Dpp signaling in vivo. *Cell*, *80*(6), 889–897.

Rushlow, C., Hogan, A., & Pinchin, S. (1989). The Drosophila hairy protein acts in both segmentation and bristle patterning and shows homology to N-myc. *The EMBO …*, *8*(10), 3095–3103.

Schneitz, K., Spielmann, P., & Noll, M. (1993). Molecular genetics of aristaless, a prd-type homeo box gene involved in the morphogenesis of proximal and distal pattern

elements in a subset of appendages in Drosophila. *Genes & Development*, *7*(1), 114–129.

Shroff, S., Joshi, M., & Orenic, T. V. (2007). Differential Delta expression underlies the diversity of sensory organ patterns among the legs of the Drosophila adult. *Mechanisms of Development*, *124*(1), 43–58.

Simcox, A., Hersperger, E., & Shearn, A. (1991). Establishment of imaginal discs and histoblast nests in Drosophila. *Mechanisms of …*, *34*, 11–20.

Simcox, A., & Roberts, I. (1989). Imaginal discs can be recovered from cultured embryos mutant for the segment-polarity genes engrailed, naked and patched but not from wingless. *Development*, *107*, 715–722.

Simpson, P. (1990). Notch and the choice of cell fate in Drosophila neuroepithelium. *Trends in Genetics*, *6*(11), 343–345.

Simpson, P., & Marcellini, S. (2006). The origin and evolution of stereotyped patterns of macrochaetes on the nota of cyclorraphous Diptera. *Heredity*, *97*(3), 148–56.

Skeath, J., & Carroll, S. (1994). The achaete-scute complex: generation of cellular pattern and fate within the Drosophila nervous system. *The FASEB Journal*, 714–721.

Skeath, J., & Carroll, S. (1992). Regulation of proneural gene expression and cell fate during neuroblast segregation in the Drosophila embryo. *Development*, *114*, 939–946.

Spradling, A., Stern, D., & Beaton, A. (1999). The Berkeley Drosophila Genome Project gene disruption project: single P-element insertions mutating 25% of vital Drosophila genes, *153*(September), 135–177.

St. Pierre, S. E., Galindo, M. I., Couso, J. P., & Thor, S. (2002). Control of Drosophila imaginal disc development by rotund and roughened eye : differentially expressed transcripts of the same gene encoding functionally distinct zinc finger proteins, *1281*, 1273–1281.

Stern, C. (1954). Two or three bristles. *American Scientist*, *42*, 213–247.

Struhl, G. (1982). Genes controlling segmental specification in the Drosophila thorax. *Proceedings of the National Academy of …*, *79*(December), 7380–7384.

Struhl, G. (1981). A homoeotic mutation transforming leg to antenna in Drosophila. *Nature*, *292*, 635–638.

Struhl, G., & Basler, K. (1993). Organizing activity of wingless protein in Drosophila. *Cell*, *72*(February 26), 527–540.

Szabad, J., Bellen, H. J., & Venken, K. J. T. (2012). An assay to detect in vivo Y chromosome loss in Drosophila wing disc cells. *G3 (Bethesda, Md.)*, *2*(9), 1095–102.

Szidonya, János and Reuter, G. (1988). Cytogenetic analysis of the echinoid (ed), dumpy (dp) and clot (cl) region in Drosophila melanogaster. *Genetic Research*, *51*(3), 197–208.

Talora, C., Sgroi, D. C., Crum, C. P., & Paolo Dotto, G. (2002). Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation. *Genes and Development*, *16*(17), 2252–2263.

Tavsanli, B. C., Ostrin, E. J., Burgess, H. K., Middlebrooks, B. W., Pham, T. a, & Mardon, G. (2004). Structure-function analysis of the Drosophila retinal determination protein Dachshund. *Developmental Biology*, *272*(1), 231–47.

Thorpe, H. M., Wilson, S. E., & Smith, M. C. M. (2000). Control of directionality in the site-specific recombination system of the streptomyces phage PhiC31. *Molecular Microbiology*, *38*(2), 232–241.

van Arensbergen, J., van Steensel, B., & Bussemaker, H. J. (2014). In search of the determinants of enhancer-promoter interaction specificity. *Trends in Cell Biology*, *24*(11), 695–702.

Van Doren, M., Bailey, A. M., Esnayra, J., Ede, K., & Posakony, J. W. (1994). Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of achaete. *Genes & Development*, *8*, 2729–2742.

Vandervorst, P., & Ghysen, A. (1980). Genetic control of sensory connections in Drosophila. *Nature*, *286*, 65–67.

Venken Koen J. T., Yuchun He, Roger A. Hoskins, H. J. B. (2006). P[acman]: A BAC Transgenic Platform for Targeted Insertion of Large DNA Fragments in D. melanogaster. *Science*, *314*(5806), 1747–1751.

Villares, R., & Cabrera, C. V. (1987). The achaete-scute gene complex of D. melanogaster: Conserved Domains in a subset of genes required for neurogenesis and their homology to myc. *Cell*, *50*(3), 415–424.

Wang, L., & Coulter, D. E. (1996). bowel, an odd-skipped homolog, functions in the terminal pathway during Drosophila embryogenesis. *The EMBO Journal*, *15*(12), 3182–96.

Weatherbee, S. D., Halder, G., Kim, J., Hudson, a., & Carroll, S. (1998). Ultrabithorax regulates genes at several levels of the wing-patterning hierarchy to shape the development of the Drosophila haltere. *Genes & Development*, *12*(10), 1474–1482.

Weigmann, K., Klapper, R., Strasser, T., Rickert, C., Technau, G., Jackle, H., Janning W, Klambt, C. (2003). FlyMove - A new way to look at development of Drosophila. *Trends in Genetics*, (9), 310–311.

Westhoff, B., Colaluca, I. N., D'Ario, G., Donzelli, M., Tosoni, D., Volorio, S., … Di Fiore, P. P. (2009). Alterations of the Notch pathway in lung cancer. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(52), 22293–22298.

White, R., & Akam, M. (1985). Contrabithorax mutations cause inappropriate expression of Ultrabithorax products in Drosophila. *Nature*, *318*, 567–569.

Wiersdorff, V., Lecuit, T., Cohen, S., & Mlodzik, M. (1996). Mad acts downstream of Dpp receptors, revealing a differential requirement for dpp signaling in initiation and propagation of morphogenesis in the Drosophila eye. *Development*, *122*, 2153–2162.

Wodarz, A., & Huttner, W. B. (2003). Asymmetric cell division during neurogenesis in Drosophila and vertebrates. *Mechanisms of Development*, *120*(11), 1297–1309.

Xu, T., & Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development (Cambridge, England)*, *117*(4), 1223–1237.

Yoffe, K. B., Manoukian, A. S., Wilder, E. L., Brand, A. H., & Perrimon, N. (1995). Evidence for engrailed-independent wingless autoregulation in Drosophila. *Developmental Biology*, *170*(2), 636–50.

Zákány, J., Fromental-Ramain, C., Warot, X., & Duboule, D. (1997). Regulation of number and size of digits by posterior Hox genes: a dose-dependent mechanism with potential evolutionary implications. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(December), 13695–13700.

Zákány, J., & Duboule, D. (1999). Hox genes in digit development and evolution. *Cell and Tissue Research*, *296*(1), 19–25.

Zollman, S., Godt, D., Privé, G. G., Couderc, J. L., & Laski, F. A. (1994). The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(22), 10717–21.

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To:	"McCallough, Christopher Lee" <cmccal2@uic.edu>,"Olga Barmina" <oyBarmina@ucdavis.edu>

Hi Christopher,

Of course, you are welcome to use these images as far as I am concerned. Olga, are you OK with this?

I sure hope the paper will be published in less than a year :-)

Artyom

On 8/7/16 18:19, McCallough, Christopher Lee wrote:

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>
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> this consideration.
>
> Sincerely,
> Christopher McCallough

--

Artyom Kopp
Professor, Department of Ecology and Evolution
Director, Center for Population Biology
University of California - Davis
One Shields Ave
Davis CA 95616

Subject: Re: thesis permissions
From: "Olga" <oybarmina@ucdavis.edu>
Date: Tue, August 9, 2016 3:07 pm
To: "Artyom Kopp" <akopp@ucdavis.edu>
Cc: "McCallough, Christopher Lee" <cmccal2@uic.edu>

Go ahead.
Olga

> On Aug 9, 2016, at 11:43 AM, Artyom Kopp <akopp@ucdavis.edu> wrote:
>
> Hi Christopher,
>
> Of course, you are welcome to use these images as far as I am concerned.
Olga,

are you OK with this?
>
> I sure hope the paper will be published in less than a year :-)
>
> Artyom
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>
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>> should be published before my actual thesis is published. Thank you for
>> this consideration.
>>
>> Sincerely,
>> Christopher McCallough
>
> --
> Artyom Kopp
> Professor, Department of Ecology and Evolution
> Director, Center for Population Biology
> University of California - Davis
> One Shields Ave
> Davis CA 95616
> office (530) 752-8657
> lab (530) 752-8328
> fax (530) 752-9014
> akopp@ucdavis.edu
> http://kopplab.ucdavis.edu/

From: "Richard Mann" <rsmann10@gmail.com>

Subject: Re: image use request

Date: Thu, July 28, 2016 1:39 pm

To: "McCallough, Christopher Lee" <cmccal2@uic.edu>

Dear Christopher,

Sure, that would be fine with me.

Best,

Richard

Richard S. Mann
Professor

Dept. Biochemistry and Molecular Biophysics and Systems Biology

Zuckerman Mind Brain Behavior Institute
Columbia University
701 W. 168th Street
HHSC 1104
New York, NY 10032

email: rsmann10@gmail.com
Office: 212-305-7731
Lab: 212-305-2111
Fax: 212-305-7924

http://themannlab.org/

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Sincerly,

Christopher Mccallough
Laboratory of Dr. Teresa Orenic
University of Illinois at Chicago

Dear Christopher,
no problem to use the images.
Good luck for your thesis
all the best christian

Am 26.07.2016 um 21:43 schrieb "McCallough, Christopher Lee"
<cmccal2@uic.edu>:

> Dear Dr. Klambt,
>
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1. Christopher Lee Mccallough, PhD student at University of Illinois at Chicago
2. my address that the internet doesn't need
3. christophermccallough@yahoo.com
4. PNAS vol. 104 no. 9 pages 3312-3317
5. An optimized transgenesis system for Drosophila using germ-line-specific PhiC31 integrases
6. Johannes Bischof, Robert K. Maeda, Monika Hediger, Francois Karch, and Konrad Basler
7. 3313

8. Figure 1 a,b, and c unaltered

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