Identification and Characterization of Genes Involved Sensory Organ Patterning in *Drosophila* Adult Legs

BY

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THESIS

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I would like to thank my family and friends.

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LIST OF ABBREVIATIONS

ac	*achaete*
Antp	*Antennapedia*
bHLH	basic Helix loop Helix
Ci	Cubitus interruptus
dac	*dachshund*
dm	*diminutive*
dE-Cad	*Drosophila E-Cadherins*
DI	Delta
Dll	*Distalless*
Dl/N	Delta/ Notch
dpp	*decapentaplegic*
dsx	*double sex*
en	*engrailed*
Egfr	*epidermal growth factor receptor*
GFP	Green Fluorescent protein
GMM	Gary's magic Mount
h	*hairy*
hh	*hedgehog*
L- row	Longitudinal row
mC	microchaete
MCs	macrochaetae
nej	*nejire*
P/D	proximal/distal
RNAi	RNA interference
rn	*rotund*
shg	*shotgun*
sc	*scute*
scr	*Sex combs reduced*
SOP	Sensory Organ Precursor
T1	First thoracic segment
T2	Second thoracic segment
T3	third thoracic segment
T-row	Transverse row
Tj	*traffic jam*
Ubx	*ultrabithorax*
Wg	*Wingless*

SUMMARY

The generation of morphological diversity among homologous animal structures is known to be controlled by the homeotic (Hox) genes. However, the molecular mechanisms underlying Hox gene function are not fully understood. This thesis addresses this issue by using the Drosophila adult legs as a model system. The Drosophila adult has three pairs of legs, one pair on each of its three thoracic segment, the T1, T2 and T3 segments. Each leg is identical but differs in size, shape and in the sense organ pattern. Our focus is on the differences in sensory organ patterns among the legs from different segments. The leg sensory organs include a group of small mechanosensory bristles (mCs), which on the T2 leg are precisely arranged in longitudinal rows, called the L-row bristles. The T1 and T3 legs also have L-rows, but in addition to the L-rows, T1 and T3 legs have mCs organized in transverse rows oriented orthogonal to the L-rows, which are called T-rows. The T-rows are found at specific positions along the circumference and proximal/distal axis of T1 and T3 legs. In addition, male T1 legs have a modified T-row called the sex comb, which consists of a group of peg like bristles and is used in mating. Our focus is on the mechanisms that generate the T1 leg specific T-row and sex comb.

Previous research in the lab has revealed the role of Drosophila Hox gene, *Sex combs reduced (Scr)*, in generating segment-specific sense organ patterns on the T1 leg by modulating the presumably default L-row patterning pathway. *Scr* function is necessary for T-row and sex comb development, but our studies suggest that *Scr* function, alone, is insufficient to form T-rows and sex combs and that additional genes are required for T-row/sex comb development. The goal of my research was to identify genes that function either downstream and/or in parallel to *Scr* to specify a T-row/sex comb fate. To do this we initiated an *in vivo* RNAi screen and identified several new genes that are required to form the T1 leg T-row and sex comb pattern.

1. INTRODUCTION

My studies have focused on the mechanisms underlying the generation of morphological diversity among homologous animal structures. The homeotic or Hox genes, which encode homeodomain transcription factors, have important functions in generating morphological diversity in multicellular organisms [11-13]. The goal of my project was to gain insight into the connection between Hox gene function and the formation of particular morphological features, a process that is not completely understood. Our focus is on the function of the Drosophila Hox gene, *Sex combs reduced (Scr)*, in generating segment-specific sense organ patterns on the adult legs of the first thoracic segment.

The Hox genes have conserved functions in patterning animal body plans, including vertebrates. In addition, altered Hox gene function is associated with genetic disorders and cancer in humans. Therefore, study of Hox gene function in Drosophila is likely to provide mechanistic insight into Hox function in human development and disease.

1.1 Morphology of adult legs

The Drosophila adult has three pairs of legs, one pair on each of the three thoracic segments, which are called the T1, T2 and T3 legs. Each leg is divided into nine segments along the proximal/distal segments (P/D) axis, which are: the coxa, trochanter, femur, tibia, basitarsus and four additional tarsal segments [20]. By convention, the basitarsus is considered the first tarsal segment (ta1) and the more distal tarsal segments are labeled, ta2-t5, from proximal to distal. The legs are homologous but differ in size, shape and in their sensory organ patterns. The sensory organs on the legs are a part of the peripheral nervous system (PNS) of the fly and are highly organized on each leg. The majority of sense organs on the legs are small bristles called the microchaetae (mCs).

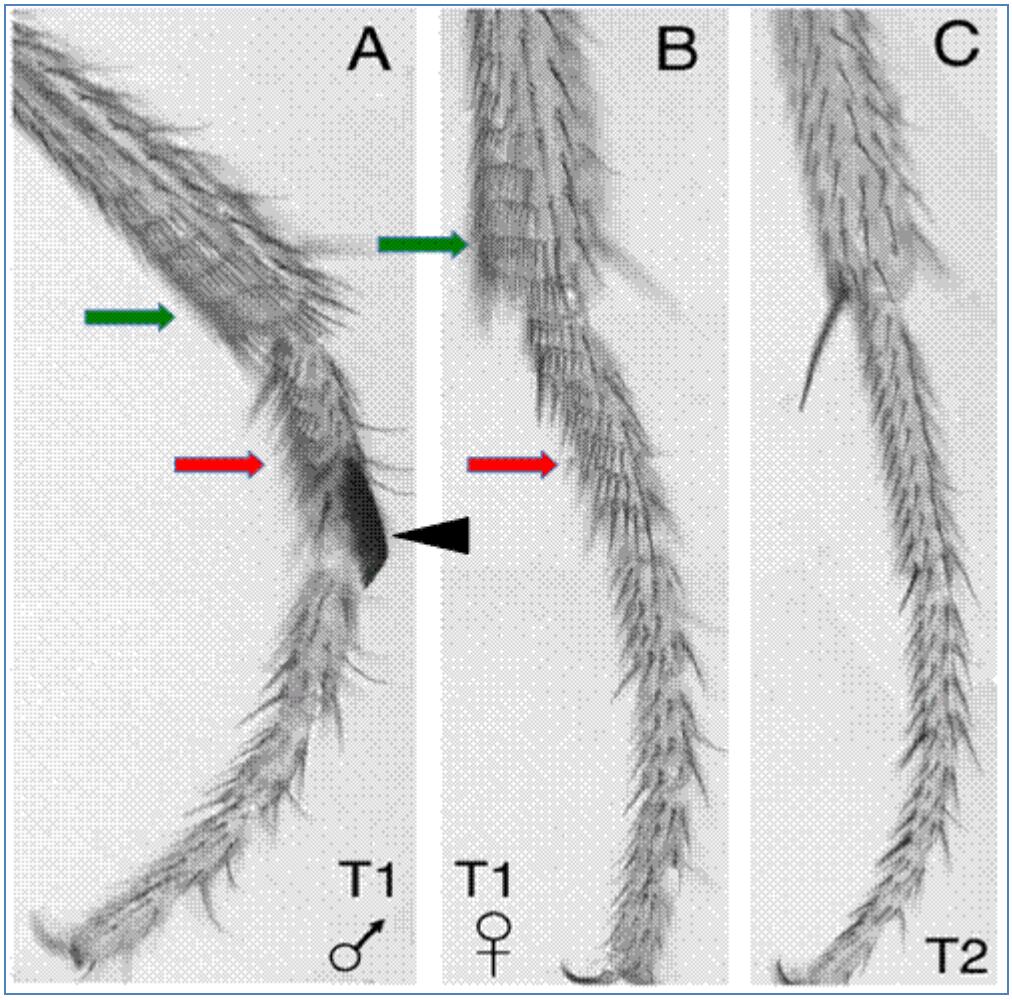

Figure 1: Bristle pattern of wild type legs the Drosophila melanogaster legs

Bristle pattern of wild type legs the Drosophila melanogaster legs from different thoracic segments exhibit distinct mC patterns. A) T1 male leg. In addition to L-rows, T1 male legs have T-rows in the tibia (green arrow) and basitarsus (red arrow) and a sex comb in the distal basitarsus (arrowhead). B) T1 female legs exhibit a similar T-row pattern to that observed on the male leg, but they lack a sex comb. C) T2 legs have the simplest mC pattern, consisting of only of L-rows

On the tibia and basitarsus of the T2 legs, which are thought to be primitive, the mCs are organized in a series of longitudinal rows (L-rows) located at defined positions along the leg circumference [1-3] (Figure 1C). The T1 and T3 legs have, in addition to the L-rows, a group of lightly pigmented and tightly packed set of bristles organized into transverse rows or T-rows. These T-rows are found at specific positions along the proximal/distal (P/D) axis and circumference of the leg and are oriented orthogonal to the L-rows. On the T1 legs, T-rows are seen on the tibia and basitarsus in an antero-ventral domain (Figure 1A,B). The mCs are lined up next to each other within the rows, but the rows are spaced at defined intervals along the P/D axis [1-3]. The T-row bristles are used by the fly as brushes for grooming [15]. In addition, on the T1 legs in males, the distal-most T-row rotates to form the sex comb, which consists of thick darkly pigmented bristles [16] (Figure 1A).

1.2 Drosophila leg development

The Drosophila adult limb primordia are the imaginal discs, which are sacs made of a folded epithelial monolayer. The leg imaginal discs arise from group of about 30 embryonic cells that have invaginated the from the first instar larval epidermis. During the three stages of larval development, 1st through 3rd instar, the leg discs undergo extensive growth, and by the 3rd larval instar consist of about 20,000 cells. The time at which a larva pupates, forming a white prepupa, is termed as 0 hrs after puparium formation (APF). At this time the leg disc begins to unfold and elongate, eventually acquiring the cylindrical shape of the adult leg [17]. The period between 0-12 hrs. APF is defined as the prepupal period, which is followed by pupal leg development, begining at 12 hrs APF.

Leg imaginal discs are divided into anterior and posterior compartments (A/P

compartments) from the time of their formation [18-21] (Figure 2).

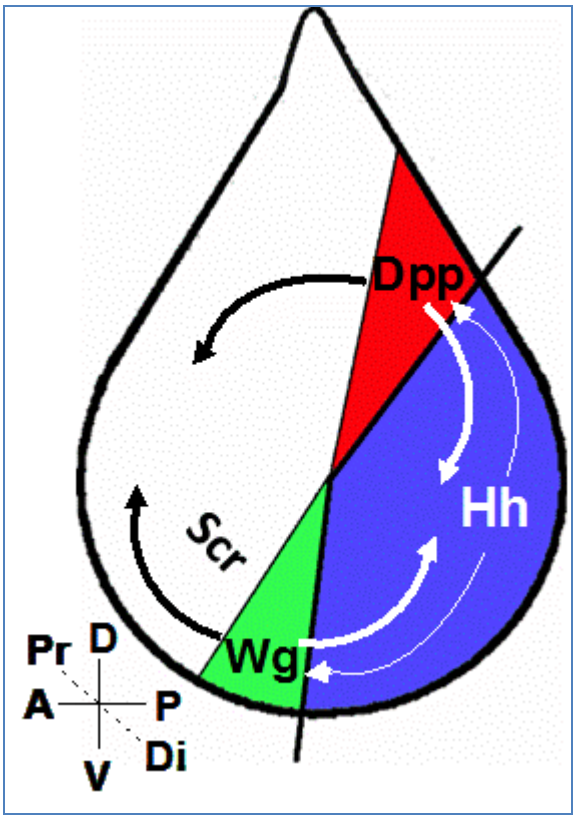

Figure 2: Patterning along the leg circumference

Regulatory interactions in a 3rd instar leg disc are depicted. In response to Hh secreted by posterior-compartment cells (blue), Dpp and Wg are expressed in dorsal (red) and ventral stripes (green), respectively. Dpp and Wg are morphogens that pattern the legs along the circumference. Scr (yellow) is expressed in the T-row/sex comb primordia, in a domain just anterior and slightly overlapping Wg expression.

Cells from the two compartments are separated by a boundary, which cells on either side never cross. Anterior compartment cells are lineally distinct from posterior compartment cells and vice versa. Posterior compartment cells secrete the Hedgehog (Hh) protein, which signals to a stripe of cells anterior and adjacent to the A/P compartment boundary. Hh controls the expression of genes in this stripe of anterior compartment cells through its transcriptional mediator, Cubitus interruptus (Ci), a zinc- finger transcription factor [28-31]. Ci then activates expression of other genes important for limb patterning in both vertebrates and invertebrates [33-3]. In leg discs, Ci activates the expression of the *wingless (wg)* gene in an antero/ventral stripe of cells and *decapentaplegic (dpp)* gene in an antero/dorsal stripe of cells. *wg* and *dpp,* which specify the ventral and dorsal fates, respectively, in the leg encode signaling molecules. Together Hh, Dpp and Wg act as morphogens that pattern the legs along the circumference in a concentration-dependent manner [3, 32] (Figure 2). The function of the conserved Hh, Dpp and Wg proteins is important for patterning vertebrate and invertebrate limbs. It has also been shown that Wg is important for the formation of sex combs and T-rows[3, 7] .

Dpp and Wg also function in formation of the P/D axis of the legs. *dpp* and *wg* expression overlaps in the center of the leg disc, which defines the distal-most region of the leg. Dpp and Wg together regulate the expression of genes such as *Distal-less* (*Dll*) and *dachshund (dac),* which pattern the leg along the P/D axis. [25, 35]. *dac* encodes a nuclear protein [37] and is expressed in and required for formation of the femur, tibia and proximal tarsal segments [38, 39]. *Dll* is expressed in the distal tibia and the tarsus [40, 41] and encodes a homeodomain transcription factor [36] (Figure 3). Legs lacking *Dll* function fail to form distal leg structures. Together *Dll* and *dac* establish three domains of differential gene expression along the P/D axis of the leg: a proximal domain of *dac* expression, a medial domain in which *dac* and *Dll* expression

overlap and a distal domain in which only *Dll* is expressed (Figure 3).

1.3 Development of the Drosophila adult peripheral nervous system

Holometabolous insects, such as Drosophila, have two stages of PNS development; one during embryogenesis, which gives rise to the larval PNS and another during late larval, prepupal and pupal development, which gives rise to the adult sensory organs [42-47]. Selection of sensory organ precursors in the adult PNS is multi-step process. First, expression of two proneural genes, *achaete* (*ac*) and *scute* (*sc*), is activated in small groups of cells, called proneural clusters (PNC), at specific positions within the adult body wall and limb primordia. *ac* and *sc* function to specify a neural fate. All cells within the PNCs express *ac* and *sc* and are initially competent to acquire a sensory organ fate. However, expression of *ac/sc* within the PNCs is later refined to one or a few cells of the cluster, which will give rise to a sensory organ precursor(s) (SOPs), while the other cells acquire an epidermal fate [45-47]. The process through which *ac/sc* expression is refined to the SOP is called lateral inhibition and involves signaling from the SOP to neighboring cells. Lateral inhibition is mediated by the transmembrane ligand, Delta (Dl), which activates signaling by binding its receptor Notch (N) in adjacent cells [48, 49].

ac and *sc* are members of the *achaete/scute* complex (AS-C) complex, which has two additional members, *asense and lethal of scute*. Only *ac* and *sc* function in the development of the adult PNS. All the proneural genes encode transcription factors of the basic Helix loop Helix (bHLH) family [42-47]. They function by forming heterodimers with the protein product of another proneural gene, *daughterless*. These heterodimers then bind via the basic domain to specific sites on the DNA, called E boxes, to activate transcription of target genes [44].

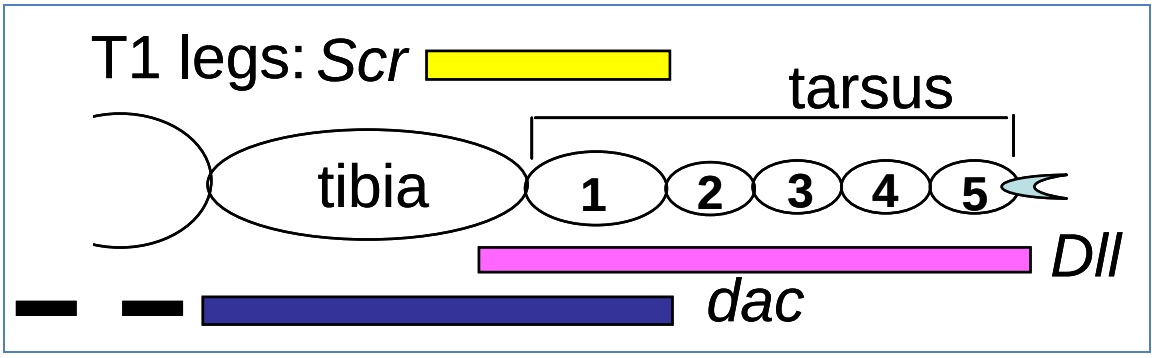

Figure 3: Patterning along the leg proximal/distal axis

Three regions of differential gene expression, defined by dac and Dll, subdivide the legs into proximal, medial and distal domains along the P/D axis (an everted leg is depicted). dac expression is expressed in the proximal domain. dac and Dll expression overlap in the medial domain, while Dll expression defines the distal domain. Scr is expressed in the region of dac and Dll overlap. The first tarsal segment is the basitarsus.

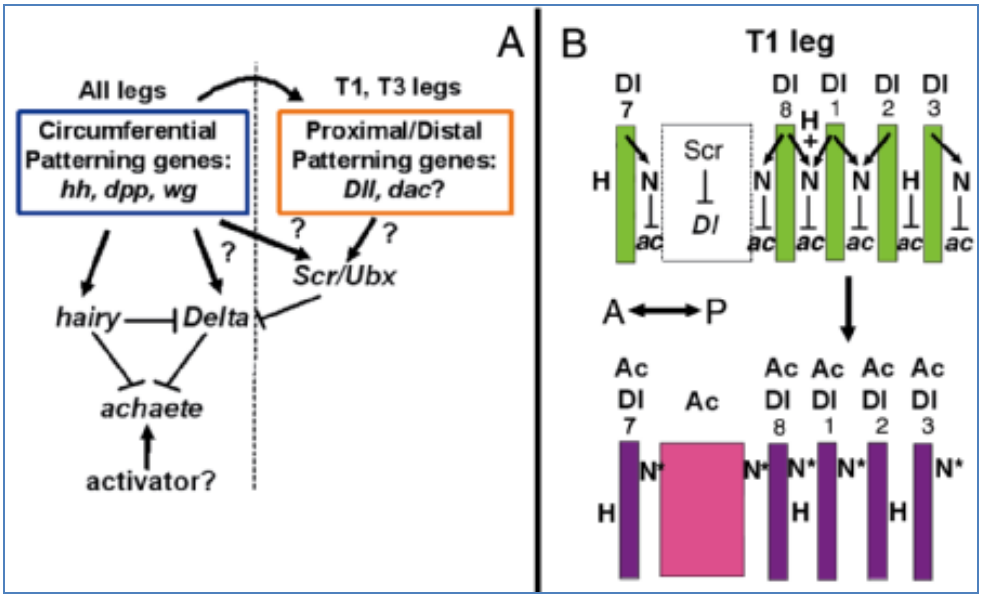

Figure 4: Hox genes modulate the mC sense organ patterning pathway

A) Pathways for mC patterning. In T1-T3 legs, Ac expression in the L-row primordia is established in response to the prepattern regulators Hairy and Delta. hairy expression is regulated by the circumferential patterning genes. Periodic Dl expression is regulated by Hairy and also likely by the circumferential patterning genes. In T1 and T3 egs, Scr and Ubx respectively modulate the pathway via repression of Dl expression. High-level Scr or Ubx expression in the T-row primordia is likely established in response to the leg circumferential and P/D patterning genes. B) Down-regulation of Dl expression is a prerequisite for formation of the T-row proneural fields. The narrow green or purple bars represent Dl expressing (green) or Dl + Ac expressing (purple) L-row primordia in the T1 basitarsus. N* designates N activation and H is Hairy. Dl and Ac expression overlap in the L-row primordia (purple bars) and Dl signals to adjacent cells to activate N signaling (*) and repress Ac expression. In T1 prepupal legs, Scr down-regulates Dl expression in a broad domain (white box) that corresponds to the T-row proneural fields between L-rows 7 and 8, resulting in Ac expression in a wide stripe of cells (pink rectangle) that presumably are out of range of Dl/N signaling.

Previous research in our lab has elucidated the L-row patterning pathway. Briefly, *ac* and *sc* expression is activated in prepupal legs at 6 hrs. APF in eight longitudinal stripes that define the L-row mC proneural fields [50]. Expression of *ac* and *sc* in these stripes involves broad activation and repression by the bHLH repressor, Hairy, and Dl/N signaling [50, 51]. Hairy [52, 53], is expressed in and represses *ac/sc* in four of eight *ac*-interstripe regions, called the *"hairy-ON"* interstripes [50]. The other 4 interstripes, called the "*hairy-OFF*" interstripes, are established through repression by Dl/N signaling [51]. Dl, is up-regulated in the L-row proneural fields, overlapping *ac*-expression, but it does not-signal to *ac*-expressing cells. Rather, it signals to adjacent cells in the four *hairy-OFF* interstripes. Therefore, the periodic expression of *ac* in the L- row stripes is established by repression by Hairy and Dl. Expression of *hairy* and *Delta* is established prior to *ac* activation at 6 hrs. APF. Hence, they are called the pre-pattern genes. It should be noted that, as described above, Dl functions to mediate lateral inhibition. This function for Dl is observed in all adult sense organs, including the larger macrochaetae and the chemosensory bristles. However, during development of leg and notal mCs, Dl has an additional, earlier, function in establishing, together with Hairy, proneural expression of *ac* and *sc* expression.

Work in our lab has shown that development of T-rows on T1 legs involves the function of the Hox gene, *Scr*, which modifies the L-row patterning pathway [7] as discussed below.

1.4 *Scr* and T-row/sex comb development

The Hox or homeotic genes encode DNA binding helix-turn-helix homeodomain transcription factors that activate or repress downstream genes [11-13]. They are highly conserved

through the animal kingdom for their important role in generating morphological diversity. [11-13]. Hox genes were first discovered in Drosophila for their function in specifying segmental identity along the A/P axis. [13]. They are highly conserved in organization and in function. In Drosophila, there are eight Hox genes in two clusters, while mammals have 39 Hox genes organized in four complexes. Hox genes are known for their spatial co-linearity, which means that they are expressed in the order in which they are organized in their complexes [11-13]. In the fly, Hox genes confer distinct morphologies to homologous larval and adult structures by functioning embryonically, as well as post-embryonically as well [11, 12]. Perhaps one of the best studied Hox genes is *Antennapedia* (*Antp*), which acts in all legs to suppress antennal formation by repressing expression of genes that promote antennal development [54].

The Hox genes function in development of the distinct morphologies of the three homologous pairs of adult legs. For example, loss of *Scr* function causes the transformation of T1 legs toward a T2 leg morphology [55, 56], and *Ultrabithorax* (*Ubx*) function in the T3 segment prevents the T3 legs from acquiring a T2 leg identity [56]. In T1 larval and prepupal leg discs, from both males and females, *Scr* is expressed in all cells, but its levels are up-regulated in the basitarsus and tibia of, in ventro-lateral domains of the anterior compartment domain. These upregulated domains of *Scr* expression define the T-row/sex combs primordia [57, 58], and elevated *Scr* expression in these regions is required for formation of T-row bristles and sex combs [7].

Within the T-row/sex comb primordia, one important function of Scr is to repress expression of *Dl*. In the absence of repressive cues from Hairy and Dl, *ac/sc* expression is globally activated in prepupal legs. Since, there is no *hairy* expression in the T-row primordia, reduced expression of *Dl*, results in a region where there is no repression of *ac/sc*. This allows

expression of *ac/sc* in broad domains in the tibia and basitarsus, as opposed to the narrow L-row proneural fields, which correspond to the T-row proneural fields [7]. It is important to note that repression of *Dl* expression is not sufficient to specify a T-row fate, since we have observed that ectopic expression of Scr represses Dl in all cells, but only induces T-row/sex comb development in a subset of cells (discussed further below). This suggests that Scr function is required in a pathway independent of Dl to induce a T-row/sex comb fate (Figure 6).

Gain- and loss-of-function studies indicate that *Scr* function is necessary for T-row and sex comb development [7, unpublished observation, Shroff and Orenic]. Work done in the lab also suggests that *Scr* expression is insufficient for specification of a T-row/sex comb fate and that there are additional factors that are responsible for development of these mCs. For instance, when ectopically expressed all along the circumference of the T2 leg basitarsus, *Scr* induces formation of T-rows and sex combs but only in the ventral lateral domain at which they would have formed in T1 legs (Figure 5, Shroff and Orenic, unpublished observations). But when *Scr* is co-expressed with a constitutively active form of a transcriptional mediator of Wg signaling, Armadillo (Arm^{act}) [59], ectopic T-rows are formed all along the leg circumference (Figure 5, Shroff and Orenic, unpublished observations). This implies that Scr functions in parallel with Wg to specify a T-row fate (Figure 6)

Here I describe a screen to identify additional components of the pathway depicted in Figure 6, particularly targets of Scr and/or Wg, which function in T-row/sex comb development. Although a number of studies have provided insight into the molecular mechanisms of Hox gene function [54, 64], the mechanisms through which Hox genes direct formation of specific morphologies are only partially understood. In addition, only a few Hox gene target genes have

been identified. I initiated an *in vivo* RNAi screen and identified several genes that potentially function in T-row/sex comb development.

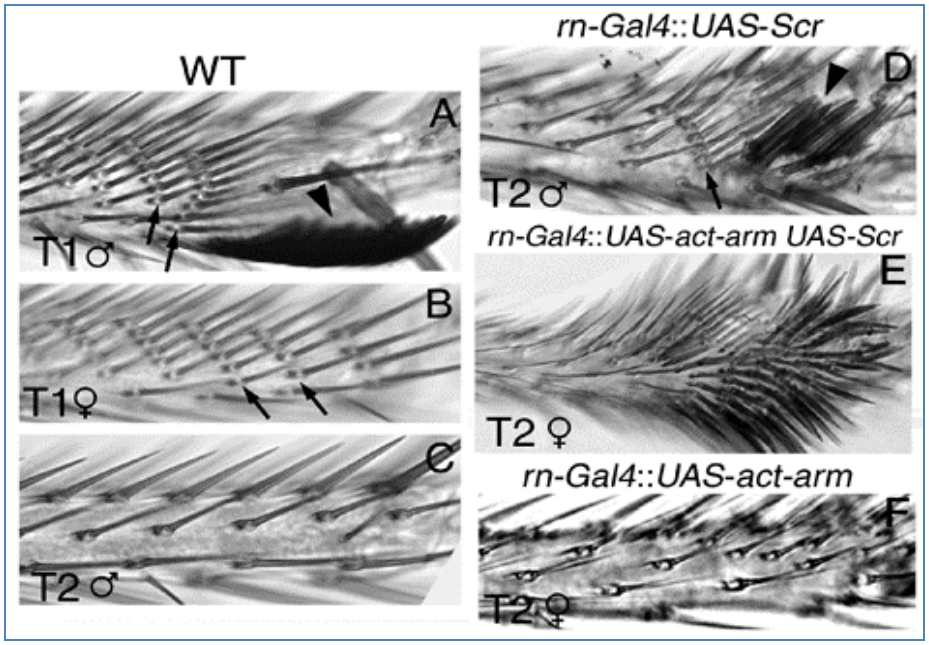

Figure 5: Scr function is not sufficient to specify a T-row and sex comb fate in T1 legs. Basitarsus of T1 and T2 legs is shown; ventral is up in all panels. Arrows indicate T-rows; arrowheads, the sex comb. A) Wild-type (WT) T1 male leg. B) WT T1 female leg. C) WT T2 male leg. Note that mCs are arranged in L-rows. D) Ectopic expression of Scr in T2 male legs results in formation of T-rows and a sex comb, but only in positions in which they are normally formed on T1 legs. On the other hand, combined expression of Scr and armS10 (E) results in a dramatic expansion of T-rows along the leg circumference. (F) Expression of armS10 causes disorganization of L-rows but does not transform them to T-rows.

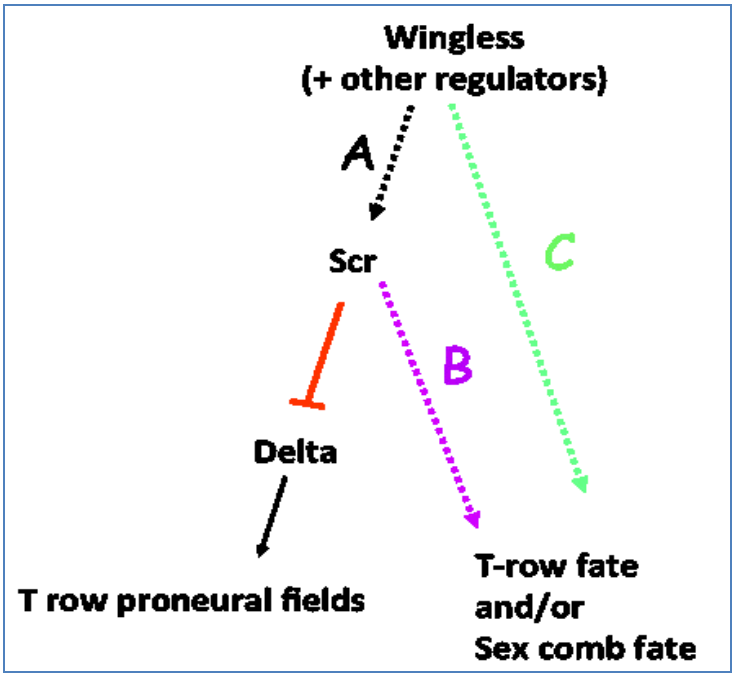

Figure 6: Model for T-row fate specification

Our preliminary studies suggest that Scr expression in the T-row primordia is regulated by Wg and that Wg and/or downstream targets of Wg function together with Scr to specify a T-row/sex comb fate. Scr function is also likely required in a pathway independent of Dl to specify a T-row/sex comb fate. This thesis describes an RNAi screen designed to identify genes that function in branches A, B, and C of the pathway shown.

2. Materials and Methods

2.1 Fly strains and genetics

The following fly strains were used in this study: *rotund*$^{Gal4-5}$/*TM6B, Tb*^{1}, *Antp*^{Hu}; *Dll*^{em212} (Dll-Gal4) *UAS-GFP*/*CyO; Scr*^{2} *p*^{p} *cu*^{1}/*TM6B Tb*^{1}, *Antp*^{Hu}; *ry*^{506} *P{PZ}Dl*^{05151}/*TM3, ry*^{RK} *Sb*^{1} *Ser*^{1}; various *UAS-RNAi*-Gene X lines from the Transgenic RNAi Project (TRiP) 73) and Vienna Drosophila RNAi Center (VDRC) (74). Standard genetic methods were used to generate a *Dll*^{em212} (Dll-Gal4) *UAS-GFP*/*CyO; ; Scr*^{2} *p*^{p} *cu*^{1}/*TM6B, Tb*^{1}, *Antp*^{Hu}, strain.

2.2 Immunofluorescence and imaging

Antibody staining of prepupal legs was done as follows: white prepupae were selected and allow to age until 5-6 h after puparium formation (APF). Dissection of prepupal legs and antibody staining were done as previously described (Carroll and Whyte, 1989). Prepupal legs were stained with anti-Scr [139], diluted 1:50, obtained from the Developmental Studies Hybridoma Bank.

All images were collected on a Zeiss Axiovert 200M equipped with ApoTome and a digital camera. Fluorescent images were collected as Z-stacks and subjected to 3-D deconvolution or directly collected as apotomized Z-stacks.

2.3 RNAi screen and UAS/Gal4 system

RNAi was used to knock down expression of various mutant genes. An RNAi is a double-stranded hairpin RNA that when expressed in a cell, will bind to mRNA of the appropriate sequence and target it for degradation (reviewed in 140). RNAi expression was driven by the use

of the binary UAS/Gal4 system (Brand et al., 1994). The first component of this system is the Gal4 driver. In our lab we use *rotund-Gal4* (*rn-Gal4*) [141] , which expresses Gal4 in the distal half of the basitarsus and extends distally to the 5th tarsal segment (Figure 7). Gal4 is a transcriptional activator in yeast but is not normally expressed in *Drosophila* and has no deleterious effects in flies [142] . It binds to an upstream activating sequence (UAS) and drives expression of an RNAi that is under UAS control (Figure 7).

Virgin *rn-Gal4*/*TM6B, Tb*$^{1\ 1}$, *Antp*^{Hu} females were crossed to males homozygous for a different *UAS-RNAi* insertion lines, which were obtained from Transgenic RNAi Project (TRiP) [143] or the Vienna Drosophila Resource Center (VDRC) [73-74] (Figure 8). For each cross, five males and ten virgin females were placed in a yeasted vial. The vials were incubated until larvae were visible. Once larvae were visible, the adults were transferred to a fresh vial.

The *rn-Gal4* driver is a homozygous lethal insertion that is maintained over a *TM6* balancer carrying the *Tb*^{1} and *Antp*^{Hu} alleles. When adults emerged from the crosses described above, those carrying the *rn-Gal4* driver were identified by selecting against the dominant *Antp*^{Hu} phenotype. The distal half of the basitarsal segment through the more distal tarsal segments of progeny from the cross were examined for phenotypes. Progeny were examined under a dissecting microscope, and if a phenotype was visible, they were preserved in 70% ethanol, prior to dissection and mounting of the legs (see below).

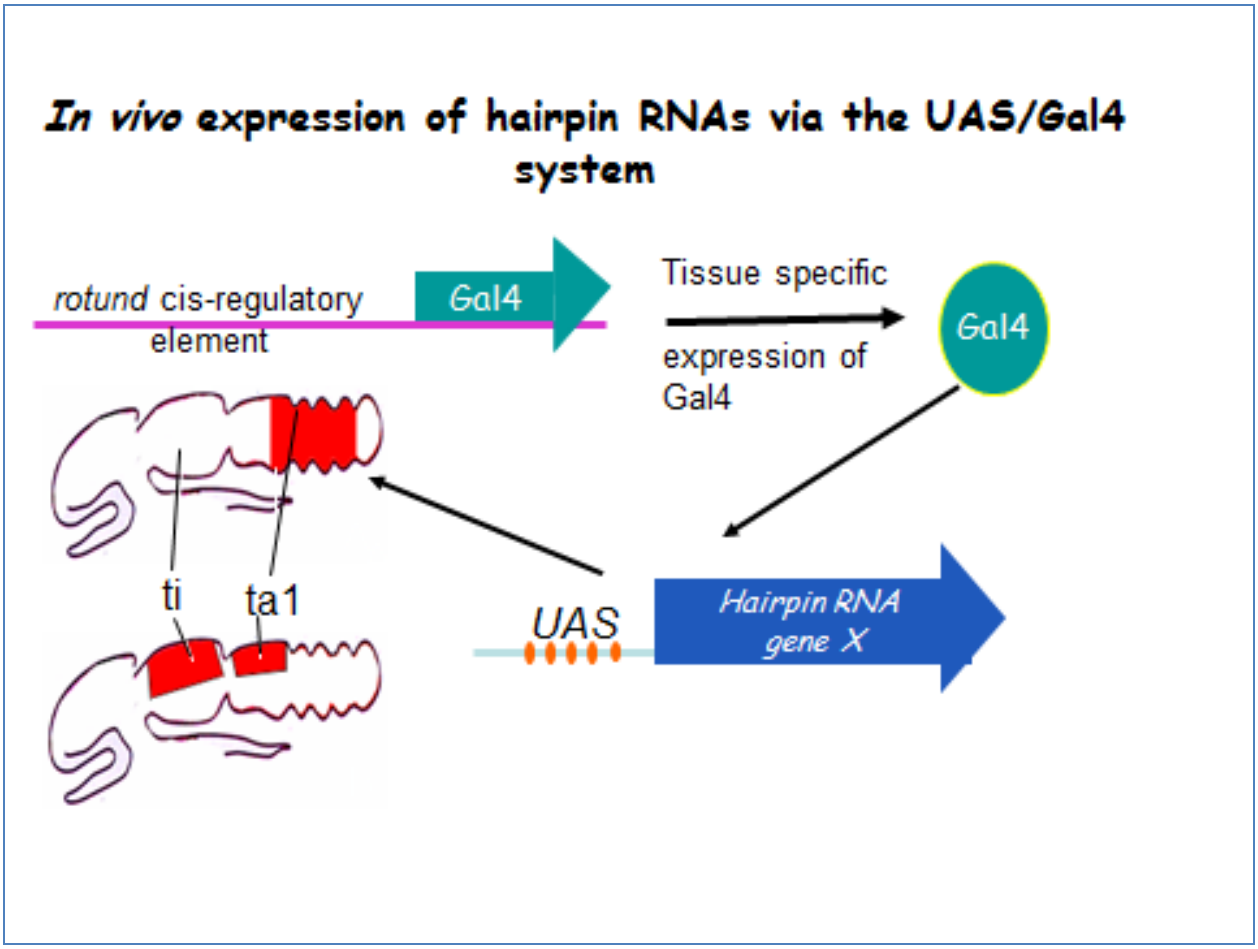

Figure 7: In vivo expression of hairpin RNAs via the UAS/Gal4 system

Hairpin RNAs (RNAi) corresponding to genes of interest were expressed in the distal half of the basitarsus via the UAS/Gal4 system. In animals carrying UAS-RNAi-Gene-X and rn-Gal4 transgenes, the Gal4 activator binds its target sequence, UAS and activates expression of a hairpin RNA corresponding to Gene X in the domain of rn expression. This should result in knock-down of Gene X function in the distal half of the basitarsus, while the proximal half of the basitarsus and the tibia are wild type for Gene X function, serving as an internal control.

2.4 Method of analyzing leg phenotypes

The adult legs are collected and preserved in 70% ethanol. Before mounting, the legs were gradually dehydrated by incubation in solutions with increasing concentrations of ethanol, 80%, 90%, 95%, for 5 min. each and finally transferred to 100% ethanol. Using forceps, the T1 legs were carefully removed so as not to disturb the bristles. The legs were placed on a slide and were oriented such that the T rows and the sex combs were easily visible. A coverslip with a drop of in Gary's Magic Mount (GMM) [144] was placed on the legs, and the slides were placed in a 65°C to bake overnight. T2 and T3 legs were also mounted to observe potential transformations to a T1 fate, and T3 legs were examined for potential T-row phenotypes. Mounted legs were examined by light microscopy on a Zeiss Axiovert 200mot; images were collected on a color digital camera.

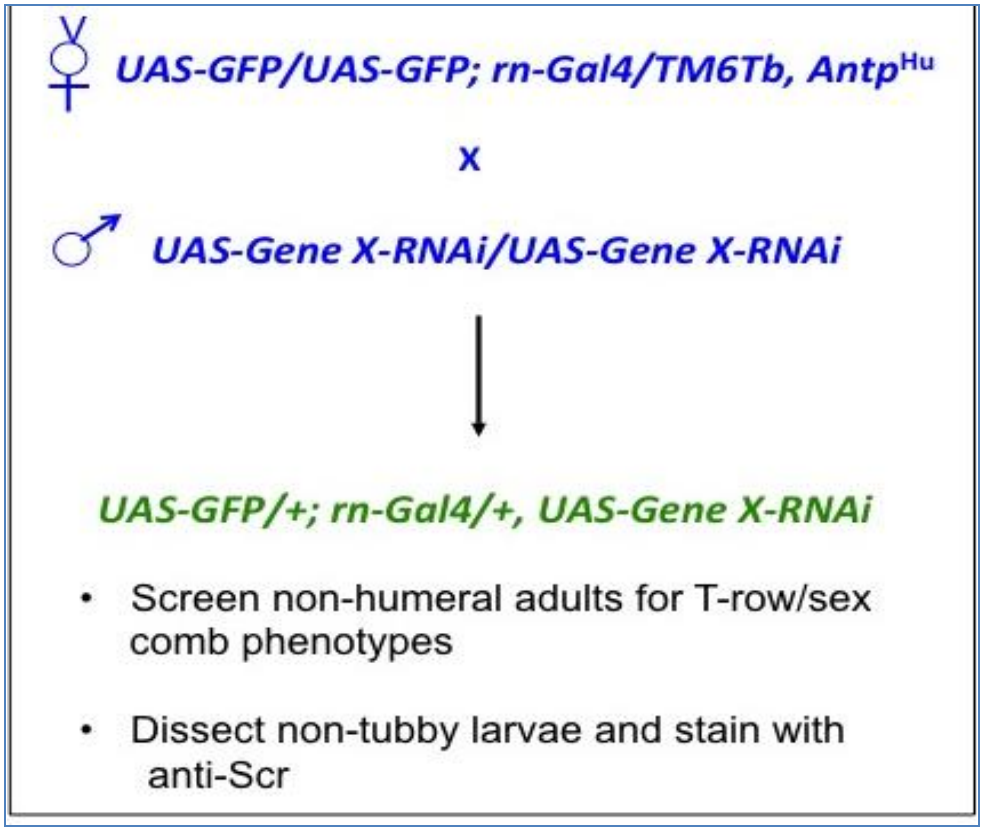

Figure 8: Scheme for the RNAi screen.

Separate vials with 10 virgin females carrying a UAS-GFP transgene and the rn-Gal4 driver are crossed to males carrying a UAS-RNAi-GeneX transgene. To assay effects on Scr expression, prepupal legs are dissected from non-tubby prepupae at 5-6 hrs. APF and stained for anti-Scr. Cells expressing the RNAi transgene are marked by GFP expression. To screen for T-row/sex comb phenotypes, legs are dissected from non-humeral adults, mounted and visualized on a compound microscope (see text for details on materials and methods).

3. RESULTS

3.1 Rationale for genes used in the screen

Transcriptional regulatory networks have been shown to regulate specification of cell fates during development. Hox genes function by activating downstream genes that act as "realizators" to regulate morphogenesis [90]. Many known Hox downstream target genes encode transcription factors [91], and sometimes Hox downstream targets are components of a network of transcription factors and signaling molecules that in turn act on realizators genes [90]. *Scr* is a Hox gene. Therefore, our screen included RNAi insertions that target transcription factors, starting with transcription factors known to be expressed in leg imaginal discs. We are interested in identifying genes responsible for generating the very unique morphological features of T-rows/Sex comb. These features include the tandem arrangement of the mCs within rows, the precise alignment of bristles and the regular spacing of the T-rows, the pigmentation and sex comb rotation. The tandem arrangement of the mCs could be mediated by homophilic adhesion between adjacent bristle precursor cells. We, hence, chose to screen genes encoding adhesion molecules, such as the Cadherins. It has been observed in a previous microarray analysis in which the homeotic gene, *labial*, was ubiquitously expressed many of the genes that were up-regulated belonged to the class of cell adhesion molecules (CAM's) [90].

Phenotypes expected were 1) Replacement of T-rows with L-row type bristles resulting from loss of *Scr* function or expression or compromised function of factors that function downstream or in parallel to *Scr* to specify a T-row vs. L-row fate 2) Transformation of T-rows to sex combs or vice versa by knock-down of genes that function in specifying a T-row vs. sex comb fate 3) Ectopic formation of T-row bristles along the leg circumference or P/D axis and/or in T2 legs due to knockdown of genes that spatially repress expression of *Scr* or *wg* 4) We might also

observe ectopic sex combs in females due to reduced function of genes involved in sexual dimorphism 5) Perturbation of the tandem arrangement of T-row mCs resulting in misaligned T-rows due to knockdown of genes regulating adhesion between bristle precursors 6) Ectopic formation of T-row bristles between the rows by compromised function of genes that have a role in generating the spacing of T-rows along the P/D axis of the leg.

3.2 Description of the screen

Originally discovered by Craig Mello and Andrew Fire, RNA interference (RNAi) is a mechanism of blocking cellular gene function by mRNA cleavage and degradation, through a sequence specific double stranded RNA (dsRNA) [87-88]. RNAi is a powerful tool for gene knock-down experiments, in functional genomics to identify genes in a specific pathway, in gene therapeutics with small interfering (siRNA) based drugs for treatment of disorders (neurological conditions like Alzheimer's) and in biotechnology for producing disease resistant crops [87-88]. Studying genes involved in human diseases, identifying genes that are involved in host-pathogen interactions, signal transduction, transcription and translation processes, and metabolism regulation are a few instances of successful applications of *in vivo* Drosophila RNAi experiments [89]. With the aim of identifying genes that are required for transverse rows (T-row)/sex comb fate specification an *in vivo* RNAi screen in Drosophila was initiated. We were interested in target genes that function downstream or in parallel with *Scr* in T-row specification pathway and possible regulators of *Scr* expression.

Our screen involves use of the bipartite UAS/Gal4 system [73] to direct expression of hairpin RNAis against specific genes functioning in the T-row/sex comb primordia. Transgenic fly lines carrying *UAS-RNAi* constructs against the majority of Drosophila genes are available

from multiple sources, including TRiP and VDRC [74, 75]. For this screen, males carrying a *UAS-RNAi* against a gene of interest will be crossed to virgin females carrying the *rotund (rn)-Gal4* driver [76]. This will direct the expression of the yeast transcriptional activator, Gal4, from the distal half of the basitarsus to the tarsal segment. This enables the RNAi to be expressed in this region of the leg, resulting in spatially restricted gene knockdown. As shown in Figure 7, the domain of *rn-Gal4* expression overlaps the distal half of the T-row/sex comb primordium in the basitarsus. Legs from adults that arise from this cross will be compared to negative control legs from adults carrying the driver but not the *UAS-RNAi* transgene. Leg phenotypes were analyzed as described in the materials and methods. The stock lines used in the assay are given in table 1.

BL#	TRiP #	CG #	Gene Name
25780	JF01365	CG1725	*discs large (dlg)*
25781	JF01368	CG10079	EGFR
25783	JF01761	CG10798	*diminutive*
25784	JF01762	CG10798	*diminutive*
25786	JF01792	CG2028	*Cklalpha*
25787	JF01793	CG8942	*Nimrod C1(NIMC1)*
25788	JF01794	CG18247	*shark*
25789	JF01795	CG34418	*still life (sif)*
25790	JF01796	CG8967	*Offtrack (otk)*
25791	JF01797	CG8049	*Btk family kinsae at 29A Btk29A*
25792	JF01798	CG30388	*Magi*
25793	JF01799	CG7892	*nemo (nmo)*
25987	JF02009	CG10034	*traffic jam (tj)*
26226	JF02124	CG10488	*eyegone (eyg)*
26229	JF02127	CG1046	*zerknullt (zen)*
26738	JF02300	CG1007	*extra macrochaete (emc)*
27060	JF02402	CG10571	*araucan (ara)*
27072	JF02417	CG10002	*fork head (fkh)*
27074	JF02419	CG10021	*brother of odd with entrails limited(bowl)*
25795	JF01805	CG6338	*Ets at 97D (Ets97D)*
25973	JF01995	CG5441	*delilah (dei)*

Table 1a: Stock lines used in the *In vivo* RNAi screen.

Stock lines used in the RNAi project. RNAi lines were made by the Transgenic RNAI Project (TRiP) and were obtained from the Bloomington Stock Center. BL stands for Bloomington stock number and CG# is the Computed Gene number assigned by FlyBase. Table continues on the next page.

25974	JF01996	CG6913	*48 related 3 Fer3*
25975	JF01997	CG8522	*Helix loop helix protein 106 (HLH106)*
25976	JF01998	CG3052	*Helix loop helix protein 4C (HLH4C)*
25977	JF01999	CG8346	*E(spl) region transcript m3 (HLHm3)*
25978	JF02000	CG8333	*E(spl) region transcript mγ (HLHmγ)*
25979	JF02001	CG5545	*Olig family (Oli)*
25980	JF02002	CG12952	*sage*
26743	JF02306	CG34403	*pangolin (pan)*
26759	JF02323	CG3166	*anterior open (aop)*
26202	JF02100	CG14548	E(spl) region transcript mβ (HLHmβ)
26203	JF02101	CG8328	E(spl) region transcript mδ (HLHmδ)
27037	JF02363	CG11561	*smoothened (smo)*
26752	JF02316	CG9015	*engrailed (en)*
25781	JF01368	CG10079	*Egfr*
25981	JF02003	CG2956	*twist (twi)*
25982	JF02004	CG17592	*upstream transcription factor (Usf)*
25983	JF02005	CG13624	CG13624
25984	JF02006	CG17894	*cap-n-collar (cnc)*
25985	JF02007	CG8669	*cryptocephal (crc)*
25986	JF02008	CG9954	*musculo aponeurotic fibrosarcoma-s (maf-S)*
25987	JF02009	CG10034	*traffic jam (tj)*
25988	JF02010	CG2848	*Transportin-Serine/Arginine rich (Trn-SR)*
25989	JF02011	CG14029	*vrille (vri)*
25990	JF02012	CG9415	*X-box binding protin 1 (Xbp1)*
25991	JF02013	CG3891	CG3891
25992	JF02014	CG7839	CG7839
25994	JF02016	CG5591	CG5591
25995	JF02017	CG5067	capicua (cic)
25996	JF02018	CG18024	*SoxNeuro (SoxN)*
27689	JF02769	CG3722	*shotgun (shg)*

Table 1b: Stock lines used in the *In vivo* RNAi screen.

3.3 Genes identified in the screen

In a preliminary RNAi screen, we have identified several genes that when knocked down result in T-row and/or sex comb phenotypes. One of these genes is *diminutive* (*dm*), which encodes the Drosophila dMyc transcription factor [65]. In our RNAi screen, *dm* knock-down causes failure of sex comb rotation in males. In addition, knock-down of *shotgun* (*shg*), which encodes Drosophila E-cadherin (DE-cad) [67], causes a T-row/sex comb phenotype. The Cadherin family of proteins function in cell adhesion molecules and maintain homophilic interaction between cells [68, 69]. We observed that knock-down of *shg* causes disorganization of T-rows and sex combs in the T1 basitarsus. Compromised function of a third gene identified in the screen, *traffic jam* (*tj*) causes conversion of T-rows to ectopic sex comb bristles. This effect is seen only in males, and in females, *tj* knock-down mild disorganization of T-rows. *nejire* (*nej*) encodes the drosophila homolog of CREB binding protein (dCBP) [124-125] , and its knockdown causes truncation of legs, resulting in loss of tarsal segments. The males lose the sex combs and there is general disorganization of the T-rows in females and males.

3.3.1 Traffic jam

The *tj* gene encodes the only known Drosophila large Musculoaponeurotic fibrosarcoma (Maf factor). Tj is a b-ZIP protein [106], which has a basic domain for specific DNA binding and a leucine zipper domain that allows protein dimerization. It also has an extended Maf specific homology domain for additional DNA binding. This domain is rich with acidic amino acids, like glycine and tyrosine, and functions in transactivation of transcription. Tj is 85% similar to its

mammalian counterparts c-Maf and MafB [107]. Another class of Mafs includes the small Mafs. The large Mafs, unlike the small Mafs, have an additional amino terminal transactivation domain. Drosophila has one small Maf, which is involved in head development [108,111]. In development, Maf proteins are involved in early specification and later in terminal differentiation [109]. They are also known to be associated with signal transduction pathways during development.

Tj is a nuclear protein and was first isolated in a screen to identify genes responsible for female sterility on the second chromosome [110]. Tj is expressed in the somatic cells of the gonads and is required for gonad development in both males and females. It was shown that *tj* mutants are viable but are sterile, as their gonads do not develop normally. Tj loss of function results in loss of adhesion between the somatic and germ line cells during larval development. Hence, TJ might regulate the expression of genes encoding homophilic and heterophilic adhesion molecules, such FasciclinIII and DE-cad [111].

TJ was chosen as a candidate gene for several reasons. It encodes a transcription factor expressed in the leg imaginal discs that, as mentioned above, is thought to regulate expression of adhesion molecules [111]. The tandem arrangement of the T-row bristles suggests that homophilic adhesion may be important in generating their organization. As a potential regulator of the expression of *DE-cad*, which is known to mediate hemophilic adhesion, we hypothesized that TJ might be involved in T-row/sex comb patterning.

We drove expression of *UAS-tj-RNAi* in the distal basitarsus and distal tarsal segments, using a *rn-Gal4* driver. Consistent with our hypothesis that Tj might regulate genes that mediate cell-adhesion, we observe a disorganization of T-rows in the distal basitarsus of female legs (Figure 9D,E). Surprisingly, however, in males we observed formation of supernumerary sex combs and fewer T-rows (Figure 9A-C), suggesting that one or more T-row have acquired a sex

comb fate. The effect observed due to *tj* knockout is limited to the basitarsus region, and the T-rows in the tibia remain intact in both males and females. This is indeed what is expected as the driver is only expressed from the basitarsus region to tarsal segments of the leg.

We also expressed *UAS-tj-RNAi* another driver, *Dll-Gal4*, which directs Gal4 expression from the distal half of the tibia to more distal leg segments. However, we did not see any detectable phenotypes with this driver. The results with *Dll*–Gal4 driver was unexpected and could suggest that the effect observed with *rn-Gal4* is driver-specific. However, different phenotypes are observed with *rn-Gal4* and other *UAS-RNAi* transgenes (as shown above). A target gene can be knocked down and produce weak or no phenotype. Potential reasons this might happen include ineffective RNAi stock lines or a driver that is not expressed at optimal time points [122]. In addition, Gal4 is known to be temperature sensitive. The progeny from the cross with *Dll-Gal4* were raised at room temperature. It is plausible that it will be necessary to raise progeny of the cross at higher temperatures to observe a phenotype with this driver. Finally, in other experiments we have observed phenotypes with the *rn-Gal4* driver, but not the *Dll-Gal4* driver.

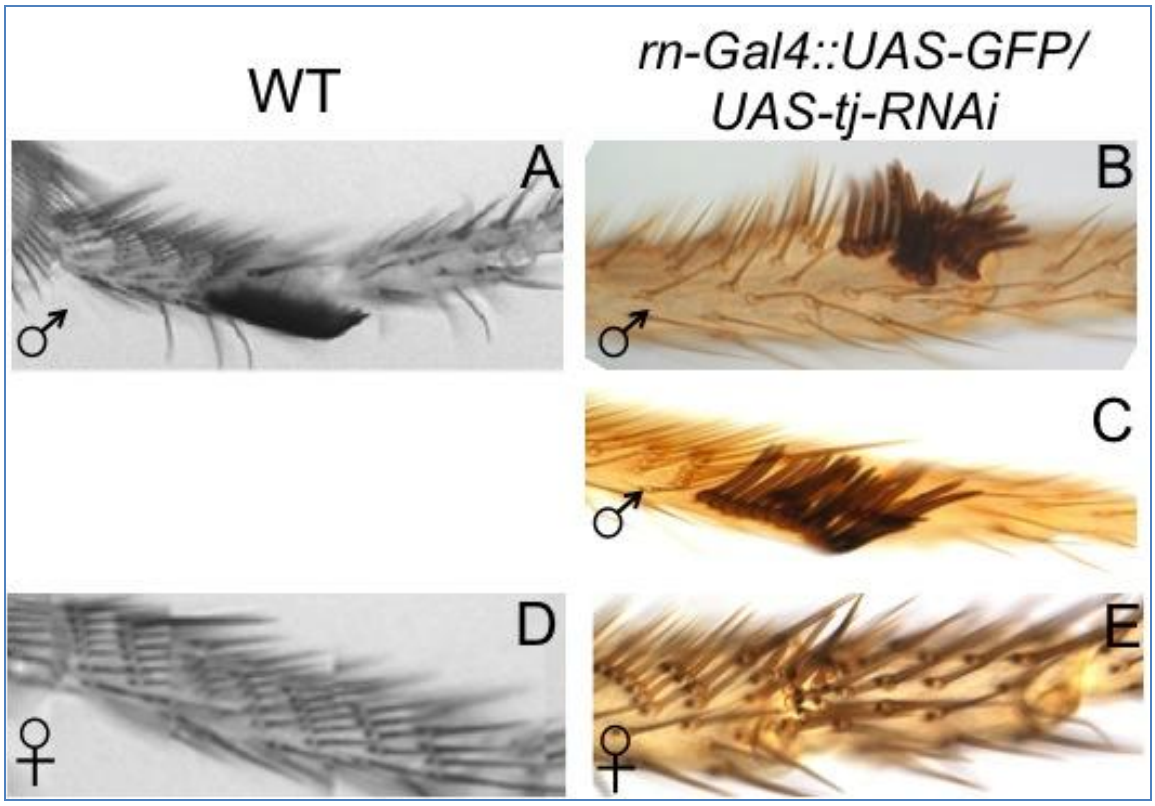

Figure 9: Traffic jam inhibits sex comb development

Tj knockdown in males flies results in the production of supernumerary sex combs at the expense of T-rows (B, C), suggesting that Tj functions to specify a T-row vs. a sex comb fate. Compare to wild type leg in panel A. In female legs, T-rows appear disorganized (E); compare to wild type female legs in panel D.

For example, expression of *UAS-Scr* under the control of *rn-Gal4*, but not *Dll-Gal4*, causes development of ectopic T-rows and sex combs on the T2 legs.

3.3.2 DE-cadherin

One of the reasons we selected DE-Cad as a candidate regulator of T-row/sex comb development is that this protein has been shown to be important for patterning and cell polarization; two processes involved in generating the unique T-rows on Drosophila T1 legs [95]. Additionally, we hypothesize that the tandem arrangement of the T-rows in the adult leg might involve adhesion proteins. Cadherins are classical adhesion molecules that have been shown to be involved in wing bristle morphogenesis. There is evidence that Cadherins are involved in the cell migration of specific structures that ultimately generate a pattern or the final morphology [96]. For instance under the regulation of cell adhesion molecules in the developing pupal eye undergoes a series of coordinated epithelial movement that gives rise to the precise epithelial pattern seen in the adult eye [97,126,127]. Cell migration is an important phenomenon in generating the sex combs in male fly legs. The sex combs originate as a transverse bristle row that eventually rotates 90° to form a single longitudinal row [98]. These observations suggested *DE-cad* as a candidate gene that functions in T-row/sex comb development. Hence, we decided to test it in our RNAi screen.

The phenotype we observed with knockdown of the *shg* gene, which encodes DE-cad, was disorganization of T-rows in both males and females and sex combs in males (Figure 10). The number and morphology of T-row and sex comb bristles appear similar to those on wild type legs (Figure 10 A, B), but the arrangement of bristle into T-rows, in which bristles are directly adjacent to their neighbors, is disrupted.

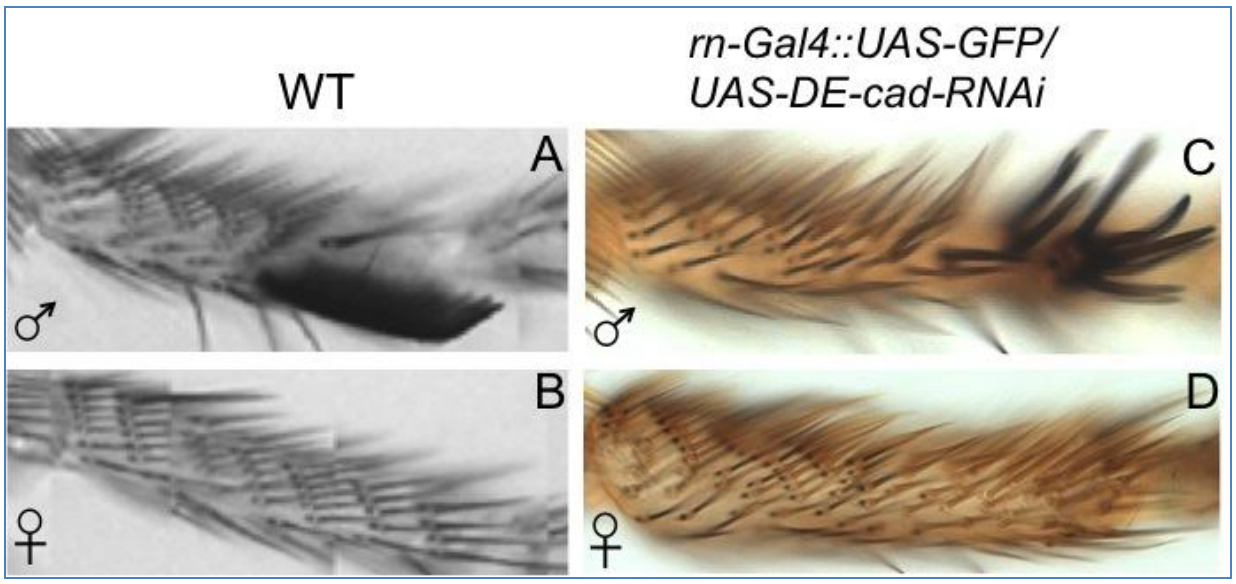

Figure 10: DE-cadherin is required for tandem arrangement of T-row mCs

Knockdown of dE-cad function causes disruption of sex comb and T-row organization in males and female legs (C, D). While the number and morphology of T-row and sex comb bristles appear similar to those on wild type legs (A, B), the arrangement of bristle into rows in which bristles abut their neighbors is disrupted. This suggests that DE-cad affects the organization of T-row/sex comb bristles, but not specification of fate.

This would suggest that DE-cad is required for proper morphogenesis of T-row rows and sex combs but does affect specification of their fate. We also found that the T-rows on T3 legs are disorganized in leg with *tj* knocked-down. On the other hand, T-rows in the tibia and proximal basitarsus appear wild type.

3.3.3 diminutive (dm)

dm encodes the drosophila Myc, (dMyc) homolog and belongs to Myc family of evolutionarily conserved proto-oncogenes[129]. These proteins are classic regulators of cell growth, cell cycle progression, apoptosis and terminal differentiation. Drosophila Myc is a basic helix loop helix zipper proteins that binds E-boxes on DNA [103] and controls organismal size and shape by regulating the growth of mitotic and endoreplicating cells [102]. It has also been shown to be necessary and sufficient for rRNA synthesis and ribosome biogenesis. At a molecular level dMyc has been shown to recruit Groucho as a co-repressor. Hairy mediated repression that is responsible for SOP formation also recruits Groucho. It has also been shown that dMyc along with Groucho is responsible for neuronal fate and mitosis [104].

We observe an unexpected phenotype with knock-down of *dm*: about half of the bristles in the distal T-row fail to acquire a sex comb bristle fate and those that diffentiate as sex comb bristles fail to undergo rotation (Figure 11).

3.3.4 nejire

nej encodes the Drosophila protein CBP or CREB binding protein (dCBP). dCBP belongs to CBP/p300 family of proteins and acts as a transcriptional co-activator proteins [124-125]. It

functions by acting as a bridge and linking basal transcription machinery with other specific DNA binding proteins [126,130]. It has also been shown that dCBP binds acetylated histones and participates in chromatin remodeling [131,132].

We chose *nej* because dCBP has been shown to bind Hox proteins and modify their activity. For instance dCBP can Deformed (Dfd) and Ubx and regulatestheir homeotic activity [133]. It has also been shown to physically interact with members of signaling cascades [131]. It functions as a co-activator of Ci in Hedgehog signaling acting as a transcriptional co-activator of downstream target genes [134]. CBP has been shown thus to regulate expression of the Hedgehog target genes, *patched*, *dpp* and *wg* signaling [135]. By acting as a scaffolding protein linking signaling cascades to transcriptional machinery, CBP is considered as an all important factor in development [136,137].

Knockdown of *nej*, results in truncation of tarsal segments 2-4 of legs from males and females. In the male, we observed a lack of or fewer sex comb bristles (Fig. 12B). The loss of sex combs could be a result of cell deatth of distal basitarsal cells, or it could refect a requirement for nej in specifying a sex comb fate. In addition to these phenotype we also wing defects.

We also examined legs carrying an antimorphic allele of *nej*, nej^{Q7} [145]. Figure 12B shows a leg from a nej^{Q7}/*Dp(1;Y)FF1* male. Normally nejQ7/Y males die as embryos, but *Dp(1;Y)FF1* is a Y chromosome with a partial duplication of the X chromosome onto the Y chromosome. The duplicated region includes the nej gene, which allows males to survive to adulthood. In legs from a nej^{Q7}/*Dp(1;Y)FF1* males, the T-rows in the basitarsus often appear to be disorganized and spaced apart, suggesting that they may be transformed toward an L-row fate. In addition, the sex comb often has 5-8 bristles, which is fewer than the wildtype number of 10-12.

The phenotype observed with knock-down of nej function, could be due to reduced *Scr* expression. To determine if this is the case, we assayed *Scr* expression in *UAS-nej-RNAi/UAS-GFP; rn-Gal4/+* prepupal legs. Scr protein was detected with an antibody against Scr, and GFP marks the cells expressing nej-RNAi. As shown in Figure 13, we find that there is no Scr expression in GFP-expressing cells. On the other hand, in wild type legs expressing *UAS-GFP* with *rn-Gal4* there was substantial overlap of GFP and Scr expression. This observation is consistent with the suggestion that nej function is required for *Scr* expression.

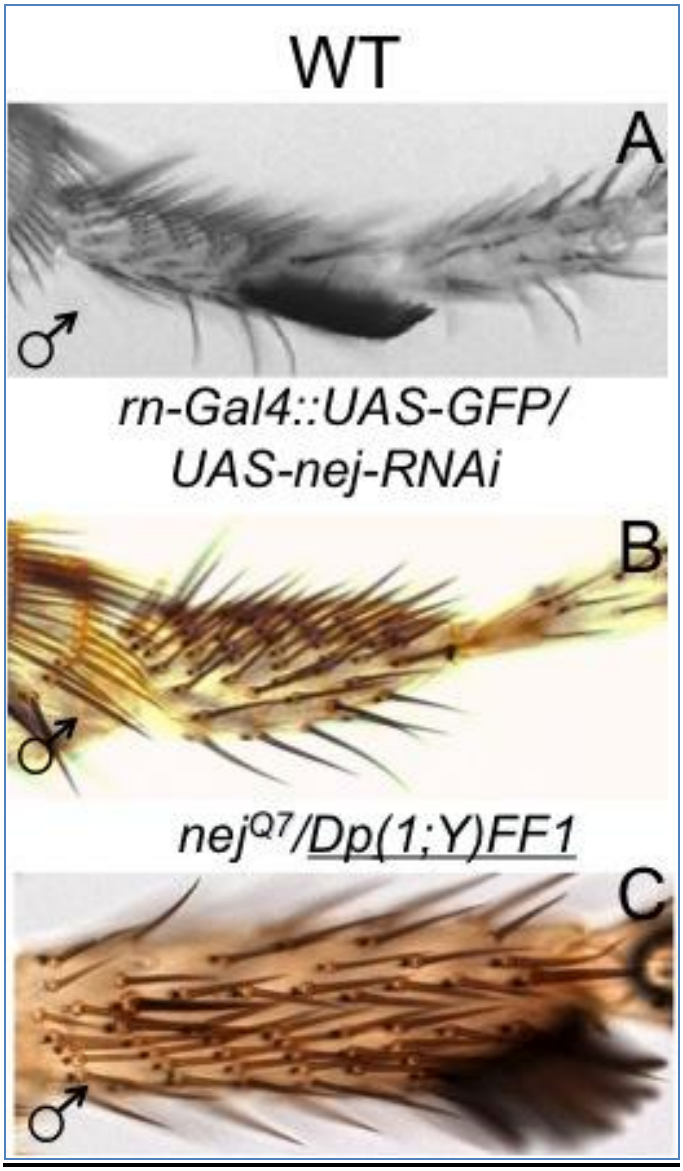

Figure 11: Nejire is required for sex comb formation

Knock-down of nej function results in truncation of tarsal segments 2-4 and loss of the sex comb in the basitarsus (B). Legs from nejQ7/Dp(1:Y)FF1. males have fewer sex comb bristle and disorganized T-row bristles. nejQ7 is an antimorphic allele and the m ales shown in panel C, carry a duplication of the nej gene on the Y chromosome.

3.3.5 Generation of a line for screening in a sensitized background

An important consideration when perfuming RNAi screens is that expression of hairpin RNAs generally results in partial reduction rather than complete loss of gene function. If the reduction of gene function is mild, this can lead to a false negative result and failure to identify genes that function in a pathway of interest. One approach to address this problem is to perform the screen in a sensitized background [118]. This can be done by incorporating a mutant allele of a gene known to function in the pathway of interest [119] into the screen. We, therefore, generated a line, in which the Dll-Gal4 driver was combined with a driver line with an amorphic allele of *Scr*, Scr^2. Scr is known to exhibit haploinsufficient phenotypes in the sex comb; there are fewer sex comb bristles in males heterozygous Scr^2. We, therefore reasoned that knock-down of genes involved in sex comb development would either enhance or suppress this phenotype. Indeed, we have observed that a mutant allele of nej enhances the sex comb phenotype of Scr^2/+ males (Eksi and Orenic, unpublished).

To increase the success of the screen in the future, I generated a line of the genotype: *+/+; Dll-Gal4, UAS-GFP/ CyO;* Scr^2*/ Tb.*, which will be used to cross to UAS-RNAi.

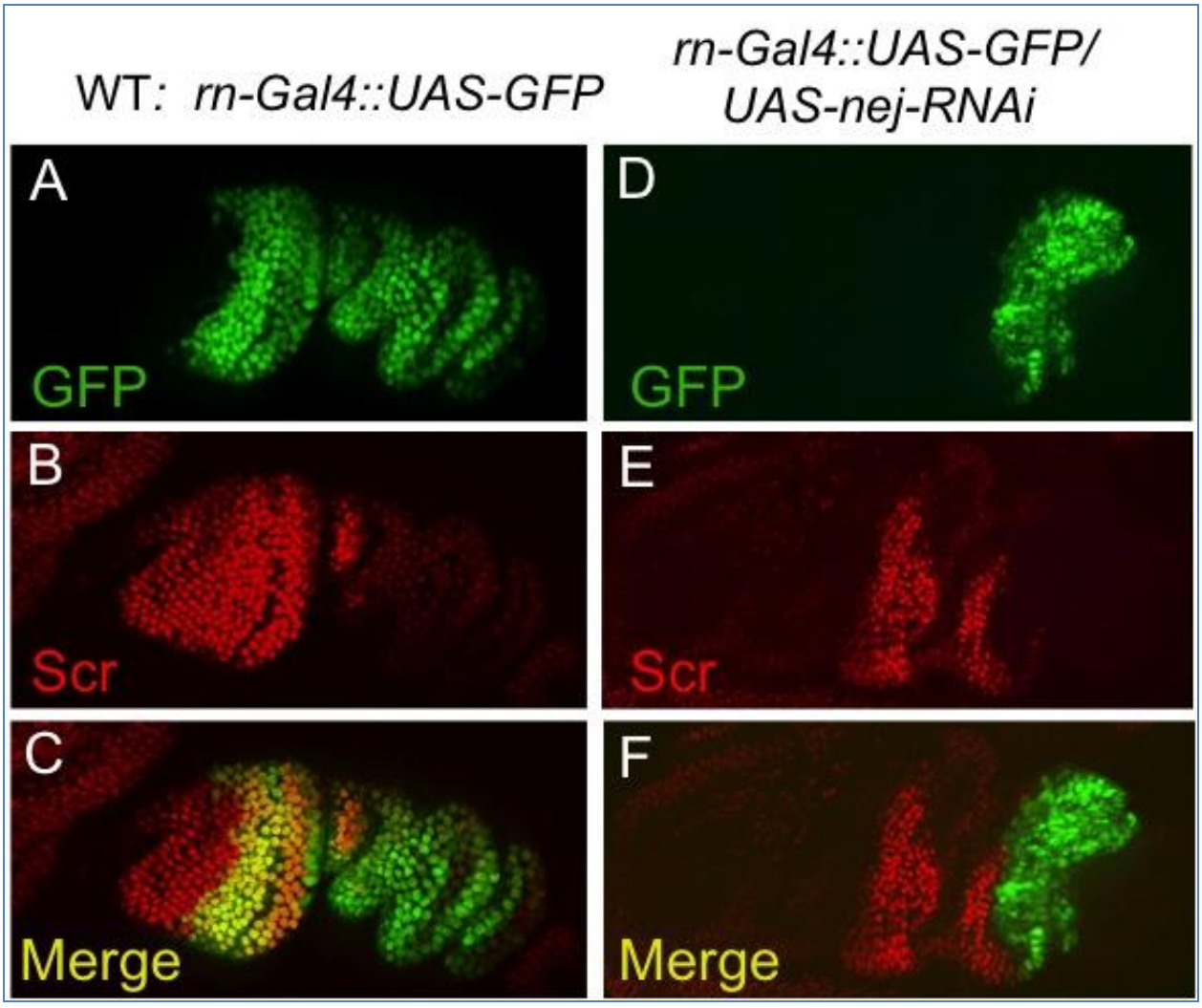

Figure 12: Nejire is required for Scr expression in the T-row/sex comb primordia

Knock-down of nej function results in compromised Scr expression in the T-row/sex comb primordia of prepupal legs. (A-C) Wild type prepupal legs expressing UAS-GFP (green in A,C) under control of rn-Gal4 and stained with anti-Scr (red in B,C). Note that Scr and GFP expression overlap in the distal half of the basitarsus. (D-F) Expression of UAS-nej-RNAi under control of rn-Gal4 results in reduced Scr expression (red in E,F) in prepupal legs. UAS-nej-RNAi expression is marked with GFP (green in D,E). Note that most GFP-expressing cells (green in E, fail to express Scr. Compare D-F to the wild type control leg in A-C.

4. DISCUSSION

4.1 traffic jam

The phenotype observed with *tj* knockdown suggests that it promotes a T-row vs. sex comb fate. A potential explanation for this phenotype is that TJ regulates expression of genes involved in sex comb vs. T-row specification, such as *Scr*. Differential expression of *Scr* in the sex comb vs. T-row primordia is thought to important for sex comb development. *Scr* expression is up regulated in the tibia and basitarsus during prepupal stages, and this domain corresponds to the T-row primordia [7]. In pupal legs, between 20-24h APF, *Scr* is expressed at low levels throughout most of the tissue, at medium levels throughout the T-row primordium and, in males, at high levels in the sex comb primordium, which is in the distal basitarsus (7,116)]. This high-level, male-specific, *Scr* expression in the sex comb primordium is thought to be required for sex comb fate specification. Regulation of high-level *Scr* expression in the sex comb primordium is poorly understood. A candidate regulator of *Scr* expression in the sex comb primordium is *doublesex* (*dsx*), which encodes a transcription factor involved in sex determination in Drosophila somatic tissues [114]. Dsx controls many conspicuously seen sexually dimorphic traits, such as pigmentation and bristle patterns in both sexes [115]. An example of a sexually dimorphic trait is the sex comb, seen only in male flies. Dsx-M, the male isoform of the gene is required for development of sex combs [116]. Dsx-M, is expressed in the sex comb primordium and is thought to stimulate male-specific high-level *Scr* expression [116].

These observations combined with our preliminary results on the phenotype of *tj* knock-down suggest a potential model for *tj* function in T-row/sex comb development, shown in Fig. 9. We propose that Tj is expressed specifically in the T-row primordium and that its function is

required in these cells to specify a T-row vs. a sex comb fate. In addition, we hypothesize that *tj* promotes a T-row fate, at the expense of sex comb development, by spatially defining Dsx-M and/or *Scr* expression through repression of *dsx-M* and/or *Scr* expression in the T-row primordium. *tj* could be expressed in the T-row primordia, which normally express medium levels of *Scr* and it might function to inhibit very high levels of *Scr* directly or through repression of *Dsx-M* expression. This would then restrict high *Scr* levels to the sex comb primordia, restricting sex comb fate specification to the distal basitarsus. The Drosophila small MAF has been shown to interact with CncB a basic leucine zipper protein, to cause suppression of *Dfd* expression. Hence it is plausible that Mafs like TJ regulates other Hox genes, such as *Scr* [108]. This model, although speculative, is consistent with the observed phenotypes. To test this model, it will be necessary to determine where *tj* is expressed relative to *dsx* and assay *dsx-M* expression in prepupal and pupal legs with reduced *tj* function. If we observe proximal expansion of *dsx-M* expression, this would be consistent with our model.

Tj has been shown to regulate the levels of homophilic and heterophilic adhesion molecules. Through its regulation of the expression of adhesion molecules in the embryo, TJ enables the interaction of soma and the germ line cells. These adhesion proteins play an important role in mediating specific cell binding, cell rearrangement and cell sorting [121].
The leg T-row mC bristles are tandemly arranged within the rows. It is possible that adhesion molecules are responsible for the generation of the tandem arrangement of bristle cells in the T-rows. [121], and that Tj regulates expression of *DE-cad* or other genes that mediate cell-cell adhesion. If this is the case, it would explain the disorganization of T-row bristles observed in knock-down experiments.

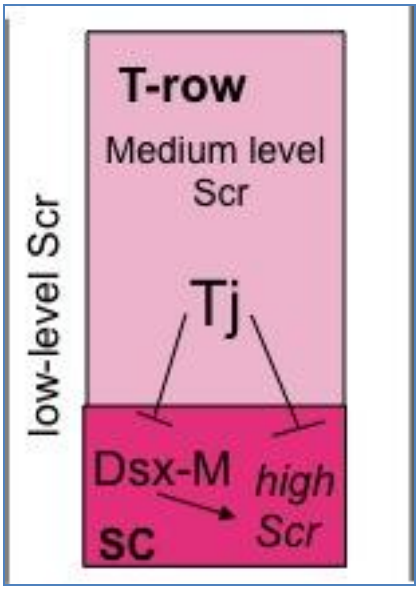

Figure 13: Model for Traffic Jam function in T-row vs. sex comb fate specification

Sex comb specification requires expression of dsx-M and elevated expression of Scr [115-118] in the sex comb primordium of pupal legs, while T-rows lack dsx-M expression and have medium levels of Scr expression. We hypothesize that Tj function is required in the T-row primordium to inhibit dsx-M expression and/or high-level Scr expression.

4.2 DE-cadherin

Cadherins are calcium dependent homophilic adhesion molecules that mediate adhesion between cells. Cadherins are important proteins required for many cellular processes and provide stability to epithelial cells, aid movement of epithelial cells, mesenchymal cells and developing neurites [93]. Classical Cadherins are seven pass transmembrane glycoproteins that mediate cell adhesion with an extracellular domain composed of Cadherin repeats. The cytoplasmic tail binds to catenin and forms a Catenin-Cadherin complex or adherens junction. It has been shown that adherens junctions are dynamically regulated and facilitate many processes ranging from cell signaling, cell sorting, cell polarization a1rnd cell migration [94]. There are three known Drosophila cadherins, of which DE-Cad is encoded by the *shg* gene and is expressed in all epithelia.

Knockdown of the *shg* gene resulted in disruption of the arrangement of bristles into T-rows and sex combs, but the number and morphology of T-row and sex comb bristles appear similar to those on wild type legs (Figure 10 A, B). This would suggest that DE-cad is necessary for morphogenesis of T-row rows and sex combs but does affect specification of their fate.. We also found the T-rows on T3 legs became disorganized. It is possible that cell-cell adhesion between neighboring cells in the T-row primordial gives rise to the tandem arrangement that we see in the adult legs. When *shg* is knocked down there is no adhesion molecule to hold neighboring cells and hence individual bristle cells lose contacts with each other giving rise to the disorganized structure. It is also possible that other adhesion molecule like Dachsous, Neuroglian, Neurotactin or Echinoid are also responsible for T-row formation [100,128].

4.3 nejire

Our results suggest that Nej is a positive regulator of *Scr* expression or a transcriptional co-activator for Scr. This conclusion is supported by the observation that reduced *nej* function results in loss or reduction of sex combs and disorganization of T-rows. In addition, the finding that *Scr* expression is compromised in prepupal legs expressing *UAS-tj-RNAi*. Further analysis is necessary to confirm that Nej is indeed a regulator of *Scr* expression in the T-row and/or Sex comb pathway. For example, analysis of *Scr* expression and T-row/sex comb phenotypes in prepupal and adult legs, respectively, carrying mutations in the endogenous *nej* gene should exhibit similar, but likely more severe phenotypes. In addition, ChiP analysis to determine wether Nej is associated with the *Scr* promoter or T-row/sex comb enhancer would provide insight into the mechanisms of Nej regulation of *Scr* expression.

4.4 Other potential regulators of T-row/sex comb development

On legs, the mCs all point distally, indicating that these bristles have polarity. This shared polarity within a of group of cells enables diverse processes like differentiation, vectorial transport of molecules, morphogens and signaling molecules across cell layers ; cell migration and localized membrane growth [121,111] . It is likely, therefore, that establishment of proper cells polarity is essential for generating the tandem arrangement of the T-rows. Also, in the adult legs, we observe spacing of T-rows along the P/D axis, and not along the leg circumference. For most adult sense organs, lateral inhibitory signals are emitted by the SOP in all directions. On the other hand, it appears that, if lateral inhibition is involved in spacing of T-rows, it acts only along the P/D axis of the leg. This implies that T-row bristle polarity is important for spacing of T-rows

at precise intervals along the P/D axis. Therefore, regulators of planar cell polarity are plausible candidate regulators of T-row/sex comb morphogenesis.

Additional potential regulators of T-row/sex comb patterning are Notch and/or EGFR signaling, both pathways which have been implicated in controlling the spacing of T-row bristles along the P/D axis [7].

4.5 Potential problems associated with analysis of RNAi phenotypes

Knock-down of gene function by RNAi involves binding of short single-stranded RNAs, 21-23 bp in length, to target endogenous mRNA via sequence complementarity [77, 78]. One problem with this approach is that a particular mRNA might recognize more than one target based on partial sequence complementarity, resulting in off-target effects. Therefore, it is essential to verify that phenotypes observed in RNAi screens are due to bona fide knock-down of the targeted gene.

One approach to control for off-target effects would be to test additional RNAi lines that produce RNAis that have a sequence that differs from the originally tested RNAi. It is unlikely, that RNAi transgenes that recognize different sequences will have the same off-target effects. Therefore, if similar phenotypes were observed with additional RNAi lines, this would suggest that compromised function of the gene of interest underlies the defect.

If mutant alleles of the gene of interest are available, a second approach would be to analyze the phenotype of legs homozygous for mutant alleles of genes of interest. However, it is important to keep in mind that mutant alleles might cause a stronger phenotype than that caused

by RNAi expression, which often does not completely knock down gene function. For example, if the gene of interest is required during multiple stages of leg development, strong mutations in the gene could result in compromised growth or loss of tissue in the leg.

A third approach would be to test for rescue of the RNAi phenotype by co-expression of the gene of interest, gene X, with the RNAi against gene X under UAS/Gal4 control. In this case, a UAS-gene-X that is impervious to the RNAi will be used. Incorporating a modified target gene that has a different nucleotide sequence but still encodes the same protein is possible by exploiting the redundancy of the genetic code. Alternatively sequence changes can also be made in the 3' UTR.

In addition to tests to determine whether phenotypes are caused by off-target effects, it is also important to determine if expression of the gene of interest is knocked down by expression of an RNAi against the gene. For genes that encode proteins for which antibodies are available, one can examine expression of the protein in negative control (see above) and mutant legs by immunofluorescence.

4.6 Conclusion

We have initiated an *in vivo* RNAi screen to identify genes involved in generating the T-rows and sex combs on the T1 legs of Drosophila. We identified four genes namely *diminutive, traffic jam, nejire, DE-cad,* that might have a role in T-row and/or sex comb formation. Analysis of *tj* function in T-row vs. sex comb fate specification is likely to provide new insight into this poorly understood process. If our genetic studies suggest that Tj regulates, Scr and Dsx expression, it would be of interest to determine if Tj functions directly to control expression of these genes. We have identified an *Scr* cis-regulatory element that recapitulates endogenous *Scr*

expression in the sex comb primordium. In future studies, we will ask whether TJ functions directly through this CRE. Alternatively, it is plausible that Tj regulates Scr expression indirectly through Dsx-M.

Through the screen in the sensitized background described here, we hope to identify additional Scr targets and potential factors that function in parallel to Scr. Identification of these factors should elucidate the regulatory network that establishes the T-row/sex comb pattern on T1 male legs.

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VITA

AARTHI NARAYAN

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Masters (M.S.) student and researcher with over 8 years of scientific research experience in industry and academics. Experienced in the fields of molecular and cell biology, tissue culture, biochemistry, immunohistochemistry, and genetics. Has over 4 years of experience in teaching college senior students.

- Recombinant DNA technology
- Cell Culture
- Life sciences & Genetics
- Fluorescent Microscopy
- Planning, organization and Team building

RESEARCH EXPERIENCE

University of Illinois at Chicago - Genetics **2008 - Present**

Graduate Researcher

Researched on standardization of a genome wide RNAi screen in Drosophila

- Completed thesis on Identification and characterization of genes involved in segment-specific sensory organ patterning in Drosophila melanogaster adult legs
- Standardized and optimized a genome wide *in vivo* RNAi screen
- Conducted PCR, RNAi, DNA extraction and purifications, recombinant DNA, DNA sequencing, bacterial transformation, molecular cloning, gene mutagenesis and Immunohistochemistry.
- Skilled in Zeiss Axiovert Fluorescent Microscopy, Bright field Microscopy, pH meter, Nanodrop, NCBI blast, Vector NTI, Sequencher, MS office, Adobe reader and Photoshop, PyMol
- Created new driver lines for genetic crosses

University of Illinois at Chicago - Genetics **2008 - Present**

Teaching Assistant

Conducted classes in Bios 221 Genetics and Bios 351 Microbiology to college seniors for 8 semesters

- Independently taught over 20 students each semester Classical and Molecular genetics concepts
- Coached students to learn and perform experiments, collect and analyze data
- Trained students to develop science writing skills and presentation skills
- Helped students to develop independent and team-oriented problem solving skills

ORCHID RESEARCH LABS, Chennai, India **2005 - 07**

Research Associate

Lead Researcher for standardization of protocols in the R& D division of a leading pharmaceutical company headquartered in Chennai, India

- Standardized glucose uptake assay and Adipogenesis assay for screening antidiabetic New Chemical Entities (NCE's)
- Performed cell based assays
- Handled and maintained different cell lines for performing cell based assays
- Carried out genotyping of hundreds of lab mice

RANBAXY RESEARCH LABS, New Delhi, India **2004 – 05**

Research Biologist

Research Technician as a part of an R& D team working for identifying potential drug candidates for Diabetes and Benign prostatic hyperplasia. Ranbaxy Laboratories Limited is India's largest and a top international pharmaceutical company.

- Performed Cloning and sub-cloning of various G-Protein Coupled Receptors (GPCR's) and expressed the GPCRs in mammalian cell lines
- Skilled in SDS PAGE, Western Blotting, pH meter, Handling and maintaining cell lines, spectrophotometer, calorimeter, chromatography, titrations and pipetting.
- Performed Receptor binding assays fo r screening of NCE's, 96-well enzymatic assay for Di-peptidyl peptidase-2 (DPP-2)

EDUCATION

Master of Science in Biological Sciences **2008 - Present**

University of Illinois at Chicago

- Majored in Molecular, Cell and Developmental biology track

Master of Science in Biotechnology **2001 - 03**

University of Madras at Chennai, India Non-GPA, 67%

- Thesis title- Synthesis of a novel peptide against Myocardial Infarction and Rheumatoid arthritis

Bachelor of Science in Biochemistry **1998 - 01**

University of Madras at Chennai, India Non-GPA, 73%

- Minored in Biotechnology

OTHERS

- Published gene sequence Amplification And Cloning Of Serotonin Receptor, 5-Hydroxy tryptamine Receptor 1A From Indian Blood (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=53748103)
- Topped a class of 20 students and ranked top-5 among 200 Masters university students

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OTHERS

- US Permanent Resident
- Interested in adventure sports, traveling and food photography
- Trained in Classical Indian dance, played throwball for school team