uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Tatiana Morozova

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Cellular and Molecular Medicine)

GRADE / DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Jing Zinc Finger Protein:
Its Transcriptional Regulation by bHLH-PAS, POU and ETS-domain Transcription Factors and its Role in Tracheal Cell Migration During Drosophila Embryogenesis

TITRE DE LA THÈSE / TITLE OF THESIS

Margaret Sonnefeld

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

David Lohnes

Marie-Andrée Akimenko

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

The Jing zinc finger protein: its transcriptional regulation by bHLH-PAS, POU, and ETS-domain transcription factors and its role in tracheal cell migration during *Drosophila* embryogenesis

Tatiana Morozova

This thesis is submitted as partial fulfillment of the M.Sc. program in

Cellular and Molecular Medicine

March 1st, 2010

Faculty of Medicine

Faculty of Graduate and Postdoctoral Studies

University of Ottawa

Ottawa, Ontario, Canada

Your file Votre référence
ISBN: 978-0-494-73826-9
Our file Notre référence
ISBN: 978-0-494-73826-9

Acknowledgements

I would like to thank:

Drs.C.Pratt, A.Colavita and D.Lohnes for being my advisory committee members and for their helpful advice.

Dr.M.Sonnenfeld for supervision, helpful support during experimental work and during preparation of talks, poster and committee reports, for helpful discussions, for proof-reading of my thesis and its critical revision, and especially for help with confocal microscopy and light microscopy, embryo analysis, and fly genetics.

My co-workers M'omena Dawood, Danya Alhayuri, Xuetao Sun, and Lunde Huang for help with my experimental work and for the nice atmosphere in the lab.

Table of contents **Page**

Abbreviations

AEBP2	AE-binding protein/adipocyte enhancer-binding protein
Ago	Archipelago
Aop	Yarn/Anterior open
Argo	Argos
bHLH	basic helix-loop-helix motif
Bnl	branchless
Btl	breathless
C/EBP	CCAAT enhancer binding protein
CME	Central Midline Element
CNS	central nervous system
DB	dorsal branch
Dfr/ Vvl	Drifter/ Ventral veinless
dml	dorsal midline
Dpp	Decapentaplegic
dp-MAPK	diphospho-mitogen-activated protein kinase
DTa	dorsal trunk anterior
DTp	dorsal trunk posterior
Dys	Dysfusion
EGF	Epidermal Growth Factor
EGFR (DER)	Epidermal Growth Factor Receptor
En	Engrailed
Esg	Escargot
EST	expressed sequence tag

ETS	E-twenty six
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
GB	ganglionic branch
GTFs	General transcription factors
hth	*homothorax*
HTH	helix-turn-helix motif
LTa	lateral trunk anterior
LTp	lateral trunk posterior
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
ml	midline
MNB	interneuronal and motoneuronal progeny of median neuroblast
MP1	midline precursor 1 interneurons
Odd	odd-skipped
TC	transverse connective
Tgo	Tango
Trh	Trachealess
PBS	phosphate buffered saline
PD axis	proximo-distal axis
pl	placoid
PNS	Peripheral Nervous System
Pnt	Pointed
PntP1	Pointed P1

PntP2	Pointed P2
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
RAS	Rat Sarcoma protein
Rho	Rhomboid
Robo	Roundabout
RTK	Receptor tyrosine kinase
Shg	Shortgun
Slbo	Slow border cells
Sal	Spalt
Spry	Sprouty
Sspitz	secreted Spitz
Sim	Single-minded
Sli	Slit
TF	transcription factor
TGF-α	transforming growth factor alpha
tsh	*teashirt*
UAS	Upstream Activating Sequence
UMI	unpaired median interneurons
VC	visceral branch
VNC	ventral nerve cord
VUM	ventral unpaired median motoneurons and interneurons

Abstract

Tyrosine kinase signaling through the Epidermal growth factor receptor (EGFR) and Fibroblast growth factor receptor (FGFR) controls tracheal development during *Drosophila* embryogenesis. Directed cell migration in the trachea is guided by the FGF-like chemoattractant Branchless through its receptor Breathless (Btl). Branchless is expressed in the ectodermal cells surrounding the trachea while Btl is restricted to tracheal cells.

Jing is a C_2H_2-type zinc finger containing protein that controls expression in embryonic and adult tissues including wings, legs, ovaries, embryonic CNS and trachea. During embryogenesis, *jing* is expressed in the CNS midline, lateral CNS, trachea, and segmental ectodermal stripes. Loss-of-function *jing* alleles result in aberrant expression of all CNS midline and tracheal markers tested. *jing* exhibits dominant genetic interactions with genes controlling tracheal cell migration, such as Trh:Tgo and *btl*. It also regulates tracheal MAPK activity.

The basic helix-loop-helix (bHLH) transcription factors, Trachealess (Trh) and Tango (Tgo) bind as heterodimers to the *btl* 5'regulatory region to activate *btl* expression therefore initiating cell migration. Evidence gathered from *in vivo* experiments suggests that Trh/Tgo function combinatorially with POU- and ETS-domain containing transcription factors to activate *btl* in the trachea. Trh/Tgo bind to consensus sites called CNS midline elements (CMEs).

In this research project we investigated a regulatory region upstream of the *jing* open reading frame containing bHLH-PAS consensus DNA binding sites in order to identify control elements conferring tracheal specificity. Three transgenic fly strains were generated that carried LacZ fusions with genomic sequences from the *jing*5' regulatory region. Two overlapping fragments (1.5 kb and 2.8 kb) from the *jing* 5'*cis*-regulatory region were fused to a *lacZ* reporter and *in vivo* expression patterns studied. The 1.5 kb fragment was chosen because it contains three CMEs DNA binding sites for Trh::Tgo and consensus binding sites for the Pou domain transcription factor Drifter and ETS domain transcription factor Pointed.

Transgenic embryos carrying the *jing1.5-lacZ* reporter show expression in the CNS midline, lateral CNS, and in the fusion cells of the trachea. During the early stages of tracheal development (stage 11), *jing1.5-lacZ* is expressed in the cells adjacent to the tracheal pits. However, despite the widespread distribution of Trh in trachea, *jing1.5-lacZ* expression was restricted to the fusion cells as confirmed by double labeling of the embryos with anti-β-gal and the fusion cell-specific antibody, anti-Dysfusion (anti-Dys) and was dependent on *trh*. *trh* ectopically activated *jing1.5-lacZ* in *Drosophila* embryos in combination with Drifter. Furthermore, the CME sites were required for tracheal *jing1.5-lacZ* activation.

A larger 2.8 kb fragment, encompassing *jing1.5* and with no additional CMEs, drove *lacZ* expression in a larger subset of tracheal cells. *jing2.8-lacZ* is expressed in all tracheal branches, as early as stage 11. These results suggest that activation of *jing* in the trachea requires regulators in addition to Trh::Tgo. *jing2.8-lacZ* reporter is also expressed in the CNS midline, tracheal fusion cells, segmental ectodermal stripes, and posterior spiracles consistent with endogenous *jing* expression.

Previous studies showed that the Jing zinc finger protein is required for both EGFR- and FGFR-dependent tyrosine kinase signaling during tracheal development. Consistent with this, Jing protein is present in the nuclei of most tracheal cells throughout development. We studied its relationship with bHLH-PAS, POU and ETS-domain transcription factors which control FGFR expression during branching in the embryonic *Drosophila* trachea. The influence of tyrosine kinase signaling on *jing1.5-lacZ* expression was determined by ectopic expression and mutant analysis. EGFR-mediated signaling activates *jing1.5-lacZ* expression in surviving midline glia and was required for *jing1.5-lacZ* tracheal fusion cell expression in *rhomboid*^{7M} (*rho*^{7M}) mutant embryos. In addition, genes known to regulate tracheal FGFR expression were required for *jing1.5-lacZ* activation in the fusion cells. Loss of *pointed* function and deletion of putative Pnt DNA binding sites in the *jing1.5-lacZ* reporter was associated with significant reductions in *jing1.5-lacZ* expression in fusion cells. In combination with reduced *jing* mRNA in *pointed* and *drifter* mutants, these

results reveal that *jing* is regulated by Pnt and Dfr. This is consistent with a reduction in *jing1.5-lacZ* expression in *breathless* and *branchless* mutants which are known to regulate *pointed.*

Furthermore, we show that *jing* is required for maximal levels of *btl* expression. *In situ* hybridization on whole mount embryos revealed that *btl* transcript levels are significantly reduced in *jing* loss-of-function backgrounds. Reduction of *jing* mRNA by RNA interference, specifically in tracheal cells, was associated with reductions in *btl* mRNA. Finally, we show that expression of Jing protein with truncated N-terminal transactivation domains specifically in tracheal cells was associated with reduced *btl* mRNA. Together, these results suggest that *jing* is an integral component of the positive feedback mechanism that maintains expression of itself and *btl* downstream of Trh/Tgo during primary branching.

Introduction

1. Development of the *Drosophila* trachea.

The *Drosophila* tracheal system is a composite of seamless tubes that carries air to target tissues and in which gas exchange occurs. The trachea can be visualized by antibody staining of whole mount embryos using anti-lumen 2A12 antibody (Fig.1B and 1C.) (Beitel GJ, Krasnow MA, 2000). Each tracheal metamere contains the following parts as shown in Fig.1A: dorsal trunk anterior (DTa), dorsal trunk posterior (DTp), dorsal branches (DB), transverse connective (TC), visceral branch (VB), lateral trunk anterior (LTa), lateral trunk posterior (LTp), and ganglionic branches (GB) reaching out to the central nervous system of the embryo (Fig.1A) (Beitel GJ, Krasnow MA, 2000).

The trachea arises from clusters of ectodermal cells (stage 10, placode, 80 cells) that invaginate to form epithelial sacs (pits, stage10-11) from which starts outgrowth of tracheal branches (Fig.2). The last mitotic cell division occurs at stage 10 in the placode. The following stages involve extensive directed cell migrations.

2. Breathless/FGFR

Tracheal cell migration is guided by the chemoattractant Branchless (fibroblast growth factor, FGF-like molecule) expressed in the ectodermal cells surrounding the placode at 5 points where future primary branching will occur (Fig.3) (Ohshiro T et al., 2002). The receptor of Branchless is Breathless (fibroblast growth factor receptor tyrosine kinase, FGFR). Fig. 3 illustrates the process of tracheal branching involving Bnl and Btl. Bnl acts as a chemoattractant for tracheal cells by activating Btl and inducing the formation of filopodia (Ribeiro C, Ebner A, Affolter M, 2002) at the tips of budding primary branches.

There are two functionally distinct classes of cells in budding tracheal branches: cells at the tip that respond directly to Branchless FGF and lead branch outgrowth (tip cells), and trailing cells that receive a secondary signal to follow the lead cells and form a tube. These roles are not pre-specified. There is

competition between cells: those with the highest FGF receptor activity take the lead positions, whereas those with less FGF receptor activity assume subsidiary positions and form the branch stalk (Ghabrial A.S., Krasnow M.A., 2006). Tip cells are characterized by the highest level of activated mitogen-activated protein kinase (MAPK) presumably due to closest proximity to the Bnl source.

The receptor tyrosine kinase signaling pathway known to control tracheal cell migration is shown in Fig. 4. It illustrates a positive feedback loop of FGFR (Ohshiro T et.al., 2002). Btl is activated by its ligand Bnl which in turn activates universal cytoplasmic cascade components resulting in the activation of MAPK. Expression of *btl* itself is regulated by several transcription factors including Trachealess::Tango heterodimers (Trh::Tgo), Drifter or Veinless (Dfr/Vvl), Spalt (Sal), Pointed (Pnt), and Yan/Anterior open (Aop) (Ohshiro T. et al, 2002).

3. Fusion cells

Primary branches contain 3-20 cells that organize into a tube as they migrate out from the tracheal pit (stage10-11, Fig.2). The tip cells branch out in response to Bnl. Other cells at the ends of primary branches become fusion cells that connect with neighboring branches from an adjacent or contralateral metamere. Positions of the fusion cells (red) and tip cells (blue) are shown in Fig.5A (Ikeya T. and Hayashi S., 1999). Tip cell and fusion cell fate decisions are influenced by the Notch/Delta, Dpp and Wingless signaling pathways (Ghabrial A.S., Krasnow M.A., 2006; Ikeya T. and Hayashi S., 1999). In particular, it has been shown that Notch signaling plays a crucial role in the singling out process of the fusion cell. Notch is activated in tracheal cells by Branchless signaling through stimulation of Delta expression and activated Notch represses fusion cell fate (Ikeya T. and Hayashi S., 1999). Fusion cells have relatively less FGFR activity than tip cells due to Notch-mediated lateral inhibition (Ghabrial and Krasnow, 2006).

Fusion cells express *escargot* (*esg*), the zinc finger transcription factor (Fuse et al, 1994) and the major transcriptional regulator in those cells is the bHLH-PAS Dysfusion::Tango heterodimer (see section

3). Fusion cells join tracheal branches by forming cadherin-dependent intercellular adhesion and by undergoing a unique cell-shape change to form doughnut-like cells (Tanaka-Matakatsu et al., 1996; Samakovlis et al., 1996). During fusion of the branches they change their shape to a seamless ring to allow formation of lumen. The cell adhesion molecule DE-cadherin accumulates at the site of contact to form a ring that marks the site of lumen entry and is essential for the fusion (Fig.5A-B).

The tracheal primordium in each metamere extends branches in six directions. All of these branches except the visceral branch will fuse with tracheal branches from other primordia. The dorsal branches extend over the dorsal side of the embryo and fuse along the dorsal midline with their contralateral partner from the identical hemisegment. The ganglionic branches migrate ventrally and join at the ventral midline. There are 11 fusion points on the dorsal midline (one of cerebral branch and ten of dorsal branch), 18 on each side (nine each on dorsal trunk and lateral trunk), and 3 on the ventral midline in the thorax (Tanaka-Matakatsu M. et. al., 1996).

4. Embryonic midline and central nervous system (CNS)

The *Drosophila* embryonic CNS (Goodman and Doe, 1993) consists of a brain and ventral nerve cord (VNC). The VNC is comprised of 14 fused ganglia. Each ganglia consists of 400 neurons and glia. CNS neurons extend axons that join together to form axon bundles. Longitudinal axon bundles connect the ganglia and run along the anterior-posterior axis of VNC. In each ganglion, two commissural axon bundles cross the midline connecting each side of the CNS. Each hemiganglion is separated by CNS midline cells which are distinct from the rest of the CNS (Nambu et. al., 1993).

Midline cells have a different from the rest of the CNS developmental origin and are specified by distinct regulatory genes. In *Drosophila*, the ventral midline and trachea are ectodermal derivatives patterned by positional cues present in the embryo. Development of the CNS midline is illustrated in Figure 6. The major transcription regulator of the midline development is bHLH-PAS transcription factor Single-

minded (Sim). Sim is active as a heterodimer with Tgo (Sim::Tgo). The Sim::Tgo heterodimer binds CME consensus sequence of the sequence ACGTG (Crews S.T., 1998). The midline is patterned by the combinatorial actions of dorsal/ventral and neurogenic genes that confine expression of Sim to the mesectoderm (Crews, 1998). At blastoderm stage transcription factors Twist and Snail are activated (Nambu et.al., 1990). These transcription factors activate expression of Sim resulting in the molecular specification of midline precursors (Nambu et.al., 1990; Crews et.al., 1988). Development of the midline requires the regulatory functions of Sim and in the absence of the *sim* function midline cells take on lateral neuroectodermal cell fates (Crews, 1998). The midline-inducing capabilities of *sim* were shown by ectopic expression experiments (Nambu et.al., 1991). Subsequent CNS midline gene regulation requires the combinatorial functions of the three different transcription factors including bHLH-PAS, SOX and POU domain-containing proteins (Ma et.al., 2000). CNS midline precursors give rise to midline glia and various interneuron and motoneuron lineages including two MP1 neurons, two UM1 neurons, the MNB and VUMs (Fig. 6).

Midline cells control the formation of adjacent tissues and guide the commissure formation (Crews S.T., 1998). In particular, axon growth cones are guided by attractive and repellent signals. Both midline neurons and glia provide specific cues to growth cones (Jacobs, 2000). Midline glia provides the attractive guidance cue Netrin. In *Drosophila*, midline glial cells express Netrin A and B guiding first pioneer axons during the formation of the longitudinal and commissural tracts (Jacobs, 2000). Netrins bind to their receptor Frazzled and attracts commissural axons to the midline. Midline glia also secrets Slit (Sli), a repellent guidance cue. Slit binds to its receptor Roundabout (Robo) expressed by ipsilaterally and contralaterally projecting axons. Slit-Robo complex prevents ipsilateral axons from crossing and contralaterally projecting axons from re-crossing (Kidd, 1999).

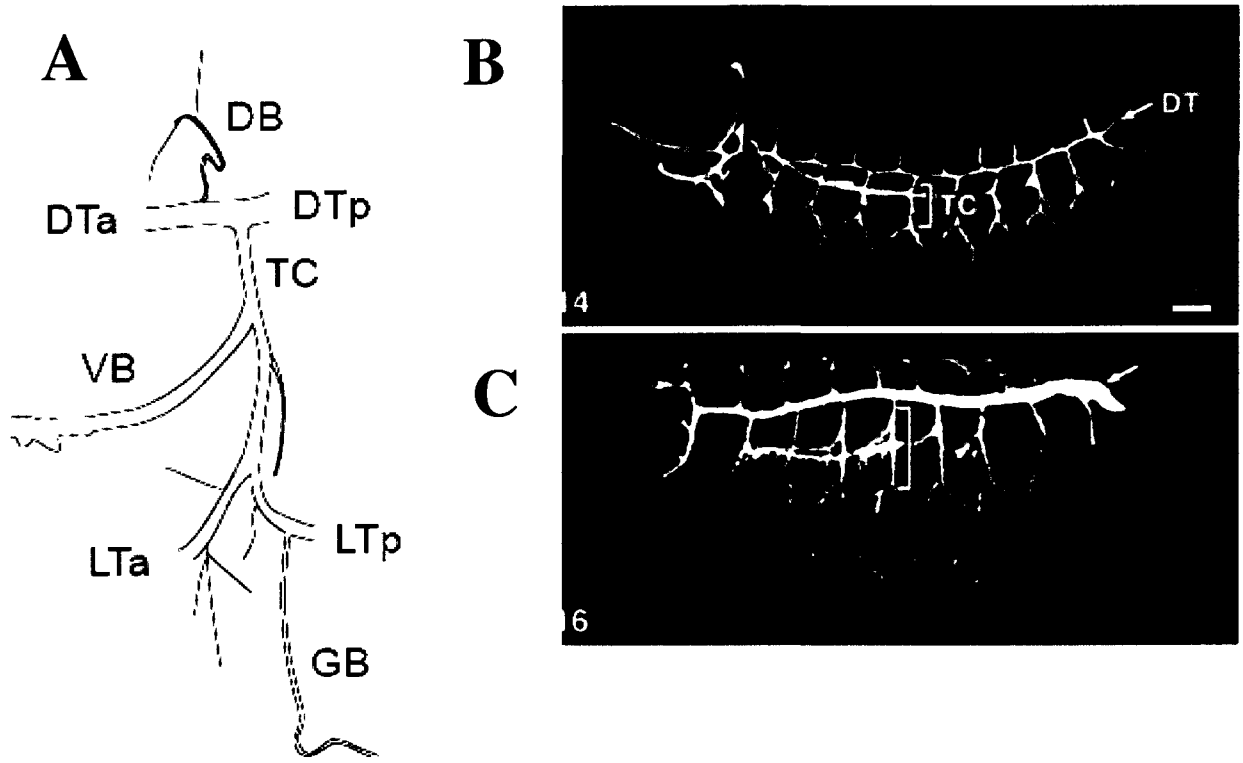


Fig.1. (A) Schematic representation of a tracheal metamere. Adapted from Ohshiro T. et al 2002. (B, C) Anti-lumen 2A12 staining of the embryonic trachea at stage 14 (B) and stage 16 (C). DTa – dorsal trunk anterior, DTp – dorsal trunk posterior, VC – visceral branch, DB – dorsal branch, TC – transverse connective, LTa – lateral trunk anterior, LTp – lateral trunk posterior, GB – ganglionic branches. Adapted from Beitel G. J, Krasnow M. A, 2000.

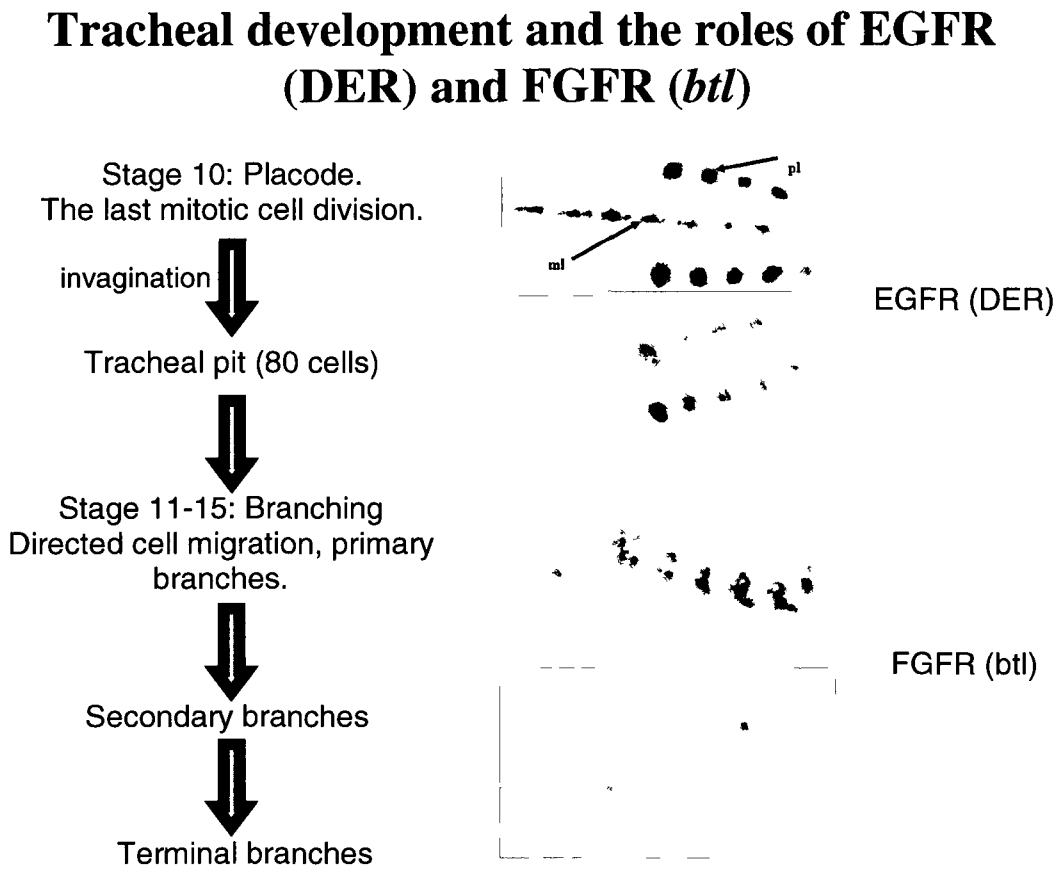


Fig.2. *In situ* hybridization with a digoxigenin-labelled *btl* probe illustrating the stages of tracheal development (tracheal placodes - pl and midline - ml).

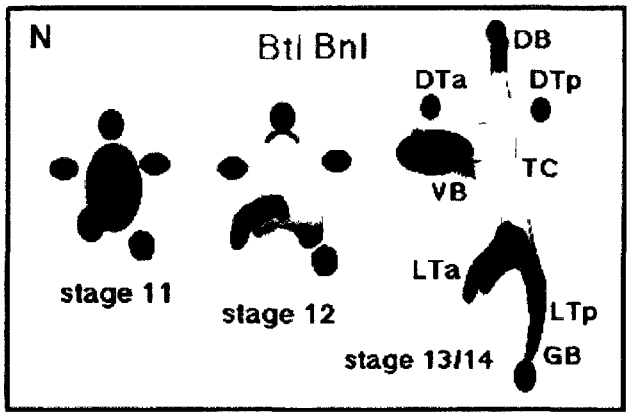


Fig.3. Relationship between Btl and Bnl expression during tracheal branching. The Branchless FGFR ligand (shown in red) guides tracheal tip cells expressing Btl (shown in green). The Bnl ligand (red) is present at five future branching points. Primary branching occurs when Btl-positive cells grow out towards the Bnl ligand. Six branches are formed as a result of the directed outgrowth. In *Drosophila*, each tracheal branch is genetically distinct and has a defined identity that specifies tube size and the subsequent determination of specialized cell fates at precise positions and in the appropriate number (Ikeya T. and Hayashi S., 1999). Adapted from Ohshiro T. et al 2002.

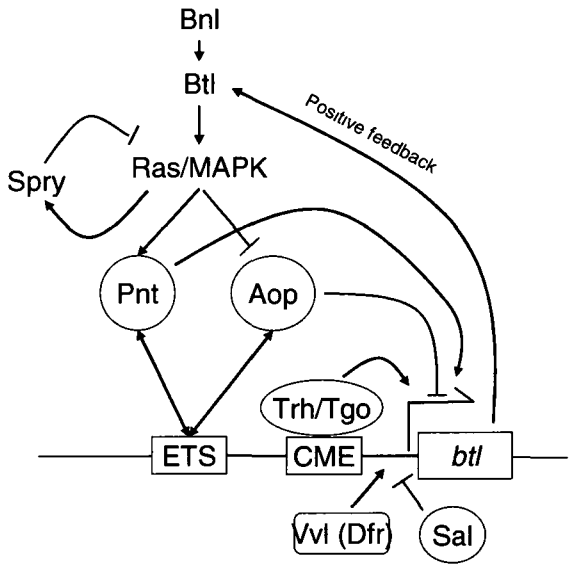


Fig.4. A model of positive feedback regulation of *btl* expression. Bnl/Btl signals activate RAS/MAPK pathway, which then inactivates the Aop repressor through phosphorylation. Trh/Tgo, Vvl, and Sal are also involved in the regulation of *btl* expression. Adapted from Ohshiro T. et al, 2002.

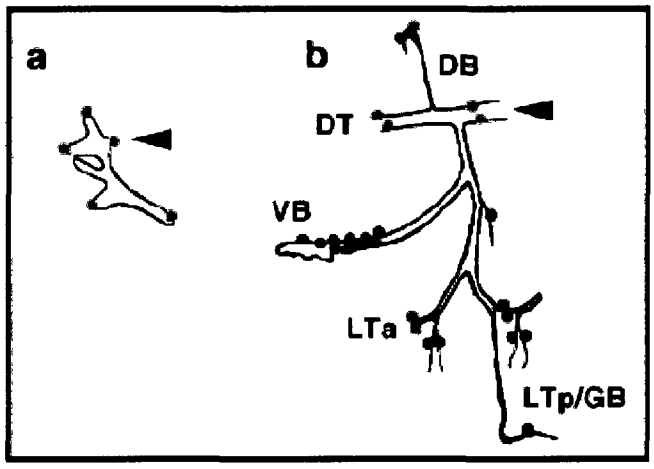


Fig.5A. Schematic representation of the branching pattern of the trachea (modified from Ikea T. and Hayashi S., 1999). (a) At stage 12, primary branches migrate in a stereotypical pattern. Position of fusion cells is indicated by a red dot. (b) At stage 15, DT (dorsal trunk) and LT (lateral trunk) have finished fusion. DB (dorsal branch), VB (visceral branch), and GB (ganglionic branch) are still migrating. Terminal branches have begun to sprout from tip cells (blue).

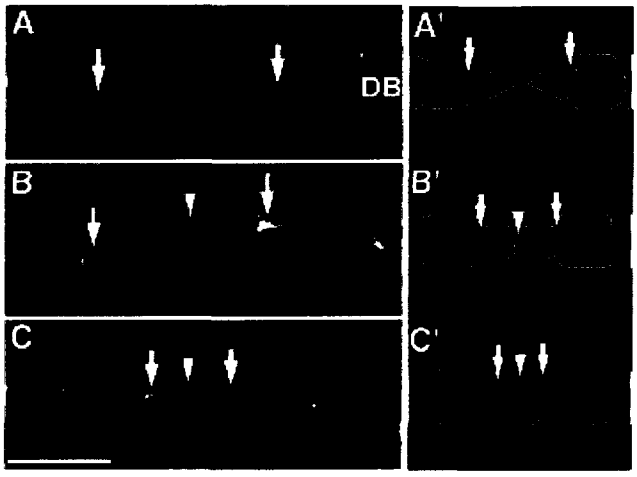


Fig.5B. A ring of *DE-cadherin* (green) protects the site of lumen fusion. Dorsal branch stained for tracheal lumen (red) and *DE-cadherin* (green). Tracheal lumen was not detected within fusion cell while dorsal branch was migrating (A) or when the fusion cells have just contacted each other (B). When *DE*-cadherin forms the ring (C), the lumen rapidly forms in the ring. (A'-C') Schematic diagrams of fusion cells during fusion. Rings of DE-cadherin (green) separate lumen (red) from hemolymph (dark background). In this figures, arrows indicate the boundary between a fusion cell and the adjacent tracheal cell. Arrowheads indicate contact sites between fusion cells. Scale bar 10μm; anterior is to the top. Adapted from Tanaka-Matakatsu M. et al, 1996.

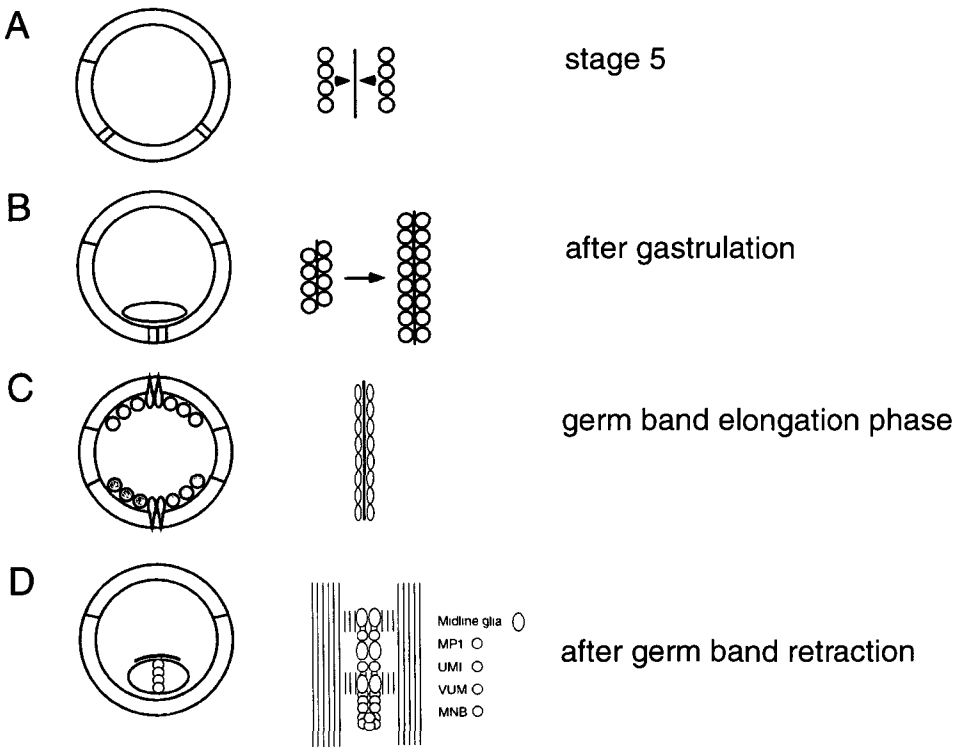


Figure 6. Development and identity of the CNS midline cells. (Left)Cross sections of the embryo with the dorsal side up. (Right) A horizontal view of the midline development. (A) Stage 5 blastoderm embryo showing the primordial of the mesoderm (lavendar), mesectoderm (red), ventrolateral neuroectoderm (blue), dorsal ectoderm and extraembryonic membranes (green). Midline precursors form single cell wide strips of cells on either side of the mesoderm. Each hemisegment has four precursor cells. At gastrulation the midline cells migrate towards the ventral midline (line). (B) After gastrulation, midline precursor cells (red) are joined at the ventral midline and the midline precursors undergo a synchronous cell division. (C) During the germband elongation stage, the midline cells (red) send their nuclei internally and maintain a cytoplasmic projection at the surface of the embryo. The lateral neuroblasts (blue circles) emerge from ventrolateral ectoderm and form a neural precursor layer. (D) After germband retraction, the midline and lateral neural precursor cells differentiate into neurons and glia and form the mature VNC. The midline cells (colored circles) and the lateral neurons of the VNC (blue). Axon bundles form above the VNC. (D, right) A single ganglion illustrating the three pair of midline glia and the four groups of midline neurons. The vertical-line structure represents the orthogonal axon scaffold showing two longitudinal connectives joined by the anterior and posterior commissures that cross the midline. MP1 - midline precursor 1 interneurons; UMI -unpaired median interneurons; VUM - ventral unpaired median motoneurons and interneurons; MNB - interneuronal and motoneuronal progeny of median neuroblast. Adapted from Crews S.T., 1998.

5. General transcriptional regulation.

5A. Background

5A1 RNA polymerase II and transcription factors

Initiation of messenger RNA (mRNA) synthesis is the primary control point in the regulation of gene expression (Lehninger et al., 1998). Cells respond to intra- and extracellular cues by turning certain genes on or off and by changing the extent of transcription of active genes.

Eukaryotes have three different RNA polymerases, (Pol I, II, and III). Each has a specific function and binds to a different promoter sequence. **RNA polymerase I (Pol I)** is responsible for the synthesis of only one type of RNA, a preribosomal RNA transcript that contains the precursor for the 18S, 5.8S, and 28S rRNAs. **RNA polymerase II (Pol II)** has the central function of synthesizing mRNAs, as well as some special function RNAs. It recognizes thousands of promoters, many of which share some key sequence similarities. These sequences are binding sites for proteins called **transcription factors** (**TFs**), which modulate the binding of RNA polymerase to the promoter. **RNA polymerase III (Pol III)** makes tRNAs, the 5S rRNA, and some other small specialized RNAs.

RNA polymerase II promoters and regulatory regions have some important sequence elements (Lehninger, Nelson, Cox, 1998). The first of these regulatory elements is a TATA box (consensus sequence TATAAAA). In higher eukaryotes, TATA boxes are located 25 to 30 base pairs from the mRNA initiation site. **TATA boxes** are binding sites for transcription factor **TFIID** that is required for RNA Pol II binding. Although TATA boxes are common, many genes are expressed without them. A variety of other short sequence elements that function in the regulation of a given promoter are often found within a few hundred base pairs of the transcription start site.

Additional regulatory sequence elements with more complex sequence structures are called **upstream activator sequences** (UASs) in yeast and **enhancers** in higher eukaryotes. For enhancer (and UAS) sequences, the location and orientation of the sequences relative to the transcription start site are

relatively unimportant; they exert their regulatory effects even when moved experimentally, and may naturally occur thousands of base pairs away from the gene being regulated.

Each of these sequence elements is recognized and bound specifically by one or more TFs. Transcriptional regulation involves protein-protein interactions between different TFs bound at different sites and/or between TFs and RNA polymerase. Because the DNA sites bound by TFs are often hundreds and thousands of base pairs away from each other or from the transcriptional start site, the protein-protein contact often requires DNA looping.

A number of general TFs bind to RNA polymerase II and are required for recognition of the TATA box and initiation of transcription at most RNA polymerase II promoters. These are the so called general transcription factors (**GTFs**) **TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH** (Naar, Lemon, and Tjian, 2001). The first two proteins to bind in the initiation process are TFIIA and TFIID. TFIID is the protein that specifically recognizes the TATA box. RNA polymerase II binds to the TFIIA-TFIID complex on the DNA, and the other TFs then bind to complete the complex (Fig.7). Pol II and the GTFs, together called basal transcriptional machinery, are both structurally and functionally conserved among eukaryotes (Mitchell and Tjian, 1989).

The other regulatory sequences are generally bound by transcriptional activator/repressor proteins (TFs). These proteins typically have a distinct structural domain for specific DNA binding and one or more additional domains required for activation/repression or interaction with other regulatory proteins (Lehninger, Nelson, Cox, 1998). Dimerization of TFs is often mediated by domains containing the leucine zipper or helix-loop-helix structural motifs.

TFs can be classified according to the type of their DNA binding domain. Some of the DNA-binding motifs found in TFs include helix-turn-helix (HTH), zinc finger, basic-helix-loop-helix (bHLH), and the MADS-box.

Transcription activation domains identified thus far are characterized by three different primary sequence motifs: acidic, glutamine-rich, and proline-rich. They are likely to represent regions that function by contacting other proteins (Mitchell P.J. and Tjian R., 1989).

5A2 Chromatin structure and transcriptional control. Chromatin-remodeling or –modifying enzymes

The minimal unit of chromatin is the nucleosome. It consists of 146 base pairs of DNA wrapped around an octamer of histone proteins composed of a $(H3\text{-}H4)_2$ heterotetramer and two H2A-H2B dimers. Linker histones, such as H1, and other chromatin-associated proteins, including members of the high-mobility group (HMG) and silent information regulator (SIR) proteins, further package DNA into higher-order structures from **euchromatin**, where genomic regions are decondensed and actively transcribed, to **heterochromatin**, the most highly ordered, condensed, and silenced genomic regions (Lehninger, Nelson, Cox, 1998).

The activation of transcription is associated with multiple changes in the structure of the chromatin in the transcribed region. Actively transcribed regions have increased sensitivity of DNA to nuclease-mediated degradation (hypersensitive sites). They are also usually undermethylated. Finally, transcriptionally active chromatin tends to be deficient in histone H1, and the other core histones have a greater tendency to be modified by acetylation or by attachment of ubiquitin. In some cases, nucleosomes are completely absent in regions very active in transcription (such as rRNA genes) (Lehninger, Nelson, Cox, 1998).

Several types of chromatin-directed activities play important roles in facilitating the binding of activators and the general transcriptional machinery to chromosomal DNA. In addition, many coactivators recruited by activators harbor histone-modifying activities and are directly involved in altering local chromatin structure to facilitate transcriptional activation.

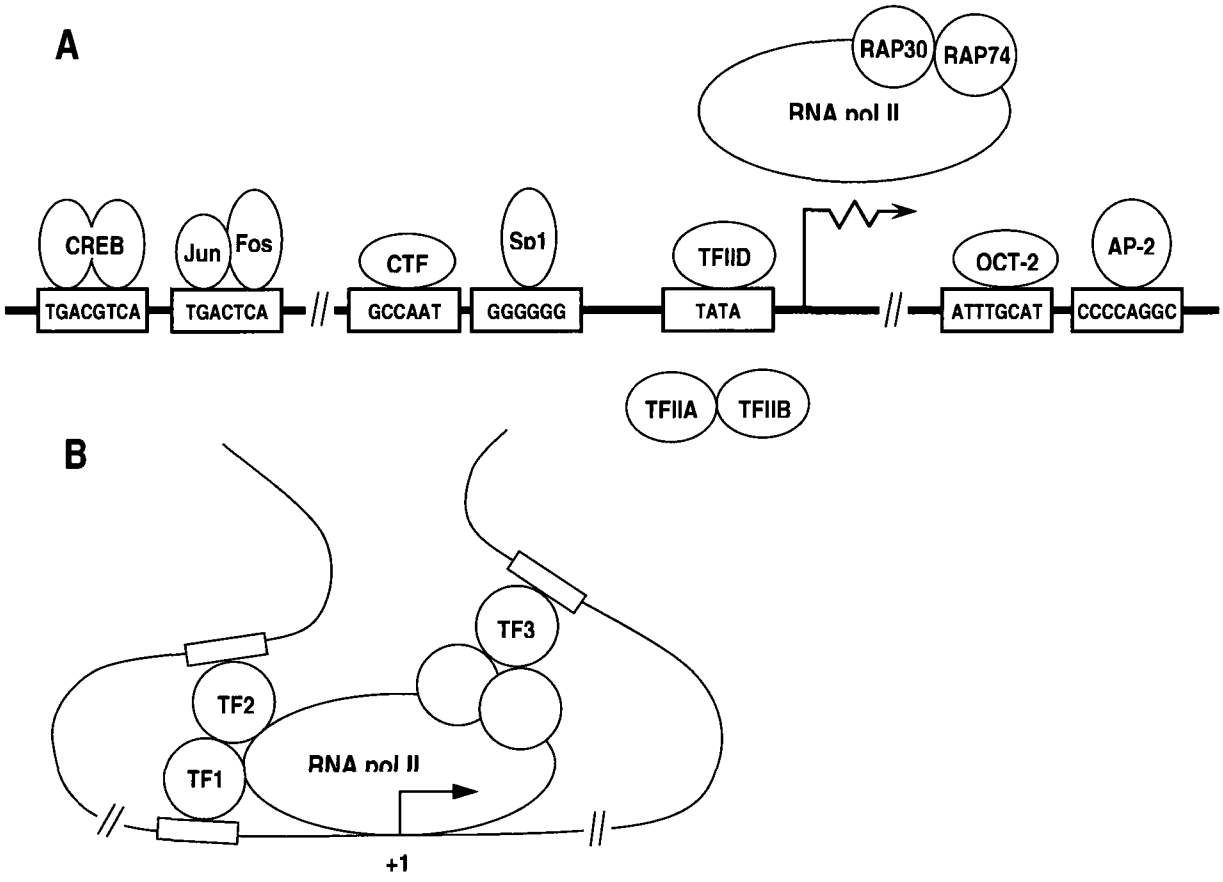


Fig. 7. Features of the transcriptional control region for a mammalian protein-coding gene. **(A)** A hypothetical array of cis elements that constitute the promoter and enhancer regions of a gene transcribed by Pol II. Proteins that associate at these control regions are symbolically represented and include Pol II, TFIIA, TFIIB, TFIID, RAP30, and RAP74 of the general transcriptional machinery, and various DNA binding proteins (CREB, Jun, Fos, CTF, Sp1, OCT-2, and AP-2) that activate through specific sequence elements. The transcription initiation site is indicated by an arrow. **(B)** The mechanism or mechanisms by which cis elements activate transcription from a distance are unknown, but specific protein-protein interactions between certain TFs bound to distal elements and factors associated with transcription initiation complexes at RNA start sites may be required so that some distally bound factors can participate directly in the initiation process. Adapted from Mitchell and Tjian, 1989.

Eukaryotic protein complexes that use the energy from ATP hydrolysis to reorganize chromatin structure by destabilization and displacement of histone-DNA contacts have been identified (Naar, Lemon, and Tjian, 2001). Such proteins act as coactivators by removing or altering repressive chromatin structures to allow the binding of activators and the transcriptional apparatus to enhancer and promoter regions.

The prototypical member of this family of chromatin remodeling complexes, SWI/SNF, was first identified in yeast (Naar, Lemon, and Tjian, 2001). Its hallmark subunit has intrinsic DNA-stimulated ATPase activity and a number of other eukaryotic ATP-dependent remodeling complexes contain homologous subunits. Brahma (Brm), a gene first identified in genetic screens as required for activation of homeotic genes, is the *Drosophila* homolog of SWI2/SNF2. Mononucleosome core disruption by the SWI/SNF family of cofactors is catalytic and induces the reversible formation of an altered dimeric form of the mononucleosome. SWI/SNF complexes may be directed by activators to specific sets of genes. For example, C/EBP β activator and the c-Myc proto-oncoprotein bind directly to subunits of mammalian SWI/SNF complexes (Naar, Lemon, and Tjian, 2001).

A second family of ATP-dependent remodeling complexes contains homologs of the *Drosophila* initiation switch (ISWI) ATPase. The ISWI-containing nucleosome remodeling factor (NURF) was purified based on its ability to enhance sequence-specific DNA binding by GAGA TF to nucleosomal templates in an ATP-dependent manner. Other ISWI-containing complexes including *Drosophila* ATP-utilizing chromatin assembly and remodeling factor (ACF), *Drosophila* chromatin-accessibility complex (CHRAC), human remodeling and spacing factor (RSF), and yeast ISWI1-containing complexes, exhibit the ability to reorganize and space nucleosomes, and ACF is also capable of facilitating nucleosome assembly. CHARC catalytically remodels chromatin via an octamer-sliding mechanism, promoting redistribution of nucleosomes (Naar, Lemon, and Tjian, 2001).

Actively transcribed genes are characterized by increased histone acetylation whereas silenced genes (heterochromatin) are associated with hypoacetylated histones. Many transcriptional coactivators and

corepressors are found in complexes harboring subunits that have histone acetylase (HAT) or histone deacetylase (HDAC) activities, respectively. Interestingly, TFIID coactivator complexes also harbor a subunit that possesses histone acetyltransferase activity.

Another family of proteins that can remodel chromatin such that TFs cannot bind to promoters in DNA is Polycomb-group proteins. They were first discovered in fruit flies (Schwartz, Pirrotta, 2007). Polycomb-group proteins play a role in silencing HOX genes through modulation of chromatin structure. In *Drosophila*, the Trithorax-group (trxG) and Polycomb-group (PcG) proteins act antagonistically and interact with chromosomal elements, termed Cellular Memory Modules (CMMs). TrxG proteins maintain the active state of gene expression while the Polycomb-group (PcG) proteins counteract this activation with a repressive function that is stable over many cell generations and can only be overcome by germline differentiation processes (Schwartz, Pirrotta, 2007).

5B. Combinatorial transcriptional regulation in the *Drosophila* trachea

5B1 Trachealess::Tango heterodimer (Trh::Tgo)

There is a master transcription factor controlling all *Drosophila* embryonic tracheal development called Trachealess (Trh) (Isaac DD, Andrew DJ., Genes Dev., 1996). Trh is a basic helix-loop-helix PAS domain transcription factor and is active only as a heterodimer with another bHLH-PAS transcription factor called Tango (Trh::Tgo) (Sonnenfeld M., et.al., 1997; OhshiroT., Saigo K., 1997). Trh is expressed in the tracheal placode cells and maintains expression in almost every cell of the trachea throughout all stages of embryogenesis excluding tracheal fusion cells. On the other hand, Tango is expressed in every cell of the *Drosophila* embryo and is localized in the cytoplasm of the cell. When Tango encounters its partners such as Trh, Single-minded, Spineless, or Dysfusion, it forms heterodimers and moves to the nucleus (Crews S.T., 1998; Ward M.P. et al, 1998; Jiang L., Crews S.T., 2003; Ohshiro T., Saigo K., 1997; Emmons R.B. et al., 1999). The consensus DNA binding site of these bHLH-PAS heterodimers is called the Central Midline

Element (CME) of the consensus sequence ACGTG. Trh is not only required for tracheal development but can induce tracheal cell fates in the cells competent to become trachea (Boube M. et al, 2000; Zelzer and Shilo, 2000). The capacity of Trh to induce tracheal cell fates is spatially restricted to the cells expressing the Pou-domain TF Drifter/Ventral veinless (e.g. in *sal-Gal4* domain). Combined ectopic overexpression of both Trh and Dfr is sufficient to induce some downstream genes, but not others (Boube M., et. al., 2000). To conclude, not a single master regulator is responsible for the appropriate expression of many tracheal genes but the co-operation of several factors (Boube M., et. al., 2000).

Trh is also required for the development of the salivary ducts and posterior spiracles (Boube M., et. al., 2000).

A hallmark of Trh is the PAS domain, which includes a duplicated sequence of 50a.a. The function of the PAS domain is protein-protein interaction where association between PAS domains and additional DNA binding proteins is essential for target specificity (Crews ST, 1998; Zelzer E. et al., 1997). The diagram below is a schematic representation of Trh and its interaction with the POU domain transcription factor Drifter/Ventral veinless (Anderson M.G. et. al., 1996; Zelzer E. and Shilo B-Z., 2000).

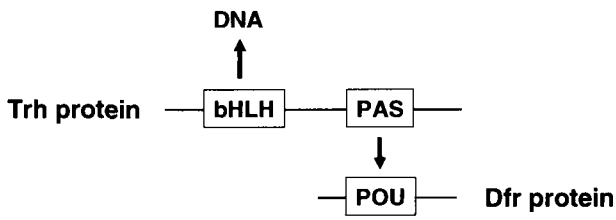


Fig.8. Schematic representation of Trh and its interaction with the POU domain transcription factor Drifter (Anderson M.G. et. al., 1996)

Until stage 12, Trh expression is regulated by signals from anterior-posterior and dorsal-ventral axes (AP, DV), while after stage 12, Trh expression is autoregulated in combination with Dfr/Vvl (Boube M., et. al., 2000; Anderson M.G. et. al., 1996).

5B2 Drifter/Ventral Veinless (Dfr/Vvl)

Drifter (Dfr) or Ventral veinless (Vvl) is a POU domain DNA binding transcription factor that is expressed in tracheal precursor cells and in all tracheal cells throughout all the stages of embryogenesis in *Drosophila melanogaster*. *dfr* mutations cause reduced expression of tracheal specific genes (*breathless*, *rhomboid*) and cell migration defects after stage 11/12 (Anderson M.G. et. al., 1996). However, Dfr is not required for the initiation of *btl* expression or for *btl* expression until primary branching (Anderson M.G. et. al., 1996). In particular, homozygous dfr^{E82} (loss-of-function mutation) embryos are characterized by a severely disrupted tracheal phenotype with limited tracheal cell migration and the absence of primary branch formation. In *dfr* mutant backgrounds, initial stage 10 *btl* expression is unaffected but *btl* transcript levels are significantly reduced beginning in stage 12 and rapidly fade to undetectable levels as tracheal differentiation proceeds (Anderson M.G. et. al., 1996). Interestingly, in *dfr* mutant embryos the first tracheal metamere retains high levels of *btl* expression significantly longer than other tracheal metameres. This phenomenon may reflect tracheal metamere-specific differences in *dfr*-independent upstream regulators involved in the initiation of *btl* expression (Anderson M.G. et. al., 1996). Ubiquitous *btl* expression was shown to rescue the *dfr* tracheal mutant phenotype (Anderson M.G. et. al., 1996).

Dfr physically interacts with Trh through POU-PAS domain binding, which has been shown by GST pull down experiments (Zelzer E, Shilo B-Z., 2000; Fig.7).

Until stage 12, Dfr expression (like Trh) is regulated by signals from major developmental axes (A/P and D/V). After stage 12, expression of Dfr is autoregulated and Trh and Dfr are required simultaneously for the autoregulation of Trh and Dfr themselves (Anderson M.G. et. al., 1996; Zelzer E. and Shilo B-Z., 2000).

Ectopic combined expression of Trh and Dfr was shown to trigger ectopic *breathless* expression and the formation of extra tracheal pits (Anderson M.G. et. al., 1996; Boube M. et al, 2000; Zelzer E. and Shilo B-Z., 2000). Dfr is required in extra pits for Trh to induce them (Boube M. et al, 2000). In addition, *btl*

regulatory DNA contains seven high affinity Drifter binding sites which were shown to bind GST-Dfr (Anderson M.G. et. al., 1996).

5B3 Pointed and Yan/Anterior open (Pnt and Aop)

Another group of transcription factors regulating tracheal development are the ETS-domain containing transcription factors (Pointed (Pnt) and Yan/Anterior open (Aop). ETS-domain containing transcription factors bind to DNA via conserved ETS domains. The canonical sequence of their targets is 5'GGA (Ohshiro T. et al., 2002; Nye J.A. et al., 1992). Pointed activates transcription of *btl* and *Argos* (an inhibitory ligand of EGFR, section 6A1) whereas Aop represses transcription of *btl* and *Argos* (Gabay L. et. al., 1996). Pnt and Aop compete for the DNA binding sites on their targets (Gabay L. et. al., 1996).

Aop is inactivated through phosphorylation by Bnl/Btl or Spitz/DER-activated MAPK. Inactivation causes degradation of Aop and release of Aop target genes from repression. Aop is expressed in most developing tracheal cells and is localized mainly in the nucleus except for the tip cells with the highest MAPK activity where it is localized in the cytoplasm.

The *pnt* gene harbors two promoters separated by 50kb, which generate 2 transcripts encoding Pointed P1 and Pointed P2 transcription factors. Pointed P1 is a constitutive transcriptional activator, while Pointed P2 requires phosphorylation by MAPK to activate transcription. Pointed P1 is expressed in the ventral ectoderm and tracheal cells (Gabay L. et. al., 1996). High levels of activated MAPK trigger expression of Pointed P1 (Gabay L. et. al., 1996).

5B4 Dysfusion::Tango heterodimer (Dys::Tgo)

The Dysfusion protein is a basic helix-loop-helix (bHLH)-PAS transcription factor and it is active only as a heterodimer with Tango (Dys::Tgo). The binding site of Dys::Tgo is the Central Midline Element with consensus sequence ACGTG. Dysfusion controls tracheal fusion events (Jiang L. and Crews S.T.,

2003) and is expressed in a variety of embryonic cell types, including tracheal fusion cells, leading-edge and foregut atrium cells, nervous system, hindgut, and anal pad cells. The *Drosophila dysfusion* gene is conserved between *Caenorhabditis elegans*, insects and humans. The *escargot* gene, a zinc finger transcription factor that is also expressed in fusion cells and is required for tracheal fusion, precedes *dysfusion* expression. Early in tracheal development, Trh is present at uniformly high levels in all tracheal cells, but since the levels of Dysfusion rise (onwards from stage 12) in wild-type fusion cells, the levels of Trachealess in fusion cells decline. The down regulation of Trachealess is dependent on *dysfusion* function (Jiang L. and Crews S.T., 2003). At a molecular level, another protein called Archipelago (Ago) is required for proteasome-dependent elimination of Trh in response to expression of Dysfusion protein (Mortimer N.T. and Moberg K.H., 2007). *ago* encodes a subunit of ubiquitin ligase which binds to Trh and stimulates its ubiquitin-dependent proteasomal degradation (Mortimer N.T. and Moberg K.H., 2007). In non-fusion tracheal cells, *ago* is required to limit overall levels of Trh. In tracheal fusion cells, the *ago* degradation mechanism is strongly potentiated by the signal generated by Dys, such that Trh is completely eliminated from Dys-expressing cells (Mortimer N.T. and Moberg K.H., 2007).

6. Background for the project

In my research project, I examined the role of the Jing zinc finger protein (Sedaghat Y. et al., 2002) in tracheal development. The *Drosophila jing* gene encodes a nuclear protein with three C_2H_2-type zinc fingers in the C-terminus similar to those of the Glioblastoma (Gli)-Kruppel family of transcription factors (see Fig. 9B). In vertebrates, mutations in the Gli genes cause absence of larynx, trachea and lung among other defects. Jing zinc fingers are most homologous (50% amino acid identity within the zinc finger motifs) to those of the mouse protein AEBP2 which has been shown to bind DNA (He G.P., et. al., 1999). In the amino terminus, Jing protein has multiple putative transactivation domains (Sedaghat Y. et al., 2002). Genetic interactions with chromatin remodeling genes and transcription factors support a regulatory role for

jing (Sun X. et al., 2006). The *jing* locus is located on the second chromosome at cytological location 2R 42B (FlyBase).

During embryogenesis, *jing* is expressed in the CNS midline, lateral CNS, trachea and segmental ectodermal stripes (Fig.9 C-H). Throughout embryogenesis, the expression pattern of *jing* was studied with a *jing-lacZ* enhancer trap line ($jing^{01094}$), digoxigenin-labeled *jing* DNA probes and a rat anti-JING antibody (Sedaghat Y. et al., 2002). *jing* mRNA and protein are first detected during precellular blastoderm stages, suggesting that *Drosophila* embryos contain a maternal supply of *jing* (Sedaghat Y. et al., 2002). A detectable *jing* expression pattern is apparent from stage 9 as *jing* transcripts accumulate in the CNS midline (Fig.9 F, G, H), neuroectoderm (Fig.9 G), trachea (Fig.9 C-E), and segmental stripes (Fig.9 F-H). In the wild-type stage 9 CNS, *jing* mRNA is distributed in a dorsoventral pattern not continuous between segments. During stage 9, embryos carrying the *jing-lacZ* enhancer trap and stained with anti-β-gal and anti-SIM show co-localization in subsets of CNS midline cells, confirming the midline expression of *jing* (Crews S., 1998) (Fig.9G). During stage 9, *jing* expression also occurs in the neuroectoderm and in the supraoesophageal ganglion (Sedaghat Y. et al., 2002; Sedaghat Y. and Sonnenfeld M., 2005). During stage 12/3, *jing* transcripts and protein product are present in CNS midline cells and segmental ectodermal stripes (Fig.9 F-H) (Sedaghat Y. et al., 2002). During stage 14, the *jing-lacZ* enhancer trap shows strong expression in CNS midline cells and weaker expression in lateral CNS cells (Sedaghat Y. et al., 2002). By stage 14, *jing* is strongly expressed in the midline glia occupying dorsal positions in the ventral nerve cord, and weaker *jing* expression is detected in ventrally positioned midline neurons (Sedaghat Y. et al., 2002). JING protein can be detected in the nuclei of the midline glia and to a lesser degree in midline neurons (Sedaghat Y. et al., 2002).

During stage 10, JING protein and *jing-lacZ* enhancer trap line expression are present in tracheal placodes (Sedaghat Y., et al., 2002) (Fig.9C, D). A pair of JING positive cells flank the tracheal placodes dorsally (Fig.9C, small arrowhead). The *jing-lacZ* enhancer trap is also expressed in TRH-positive tracheal

cells in the anterior of each placode (Fig.9D). From stage 10 until stage 16 of embryogenesis the *jing-lacZ* enhancer trap is co-expressed with *trh* and *tgo*. JING protein is detected in all tracheal branches throughout embryonic tracheal development, consistent with a role for *jing* throughout embryogenesis (Fig.9E).

Loss-of-function mutations in *jing* cause reductions apoptosis of CNS midline and tracheal precursors (Sedaghat Y., et. al., 2002; Sedaghat Y., Sonnenfeld M., 2002; Sonnenfeld M. et al, 2004).

CNS axon and tracheal tubule development was assessed in *jing* homozygous mutant embryos. At stage 12, in $jing^3$ homozygous mutant embryos, commissural growth cones are often absent in the midline compared with the wild type. By stage 14, homozygous $jing^3$ mutants show losses of longitudinal connections and reduced commissures compared with wild type. The GAL4/UAS system was used to determine the effects of overexpressing *jing* in the CNS midline. The GAL4/UAS system for targeted gene expression allows selective activation of any cloned gene in a tissue-specific patterns (Brand and Perrimon, 1993). It requires a cross between the driver and responder lines. The driver line expresses yeast Gal4 transcriptional activator under control of the tissue specific promoter. Responder line carries the cloned gene fused to the yeast Upstream Activating Sequence. Expression of one copy of P[UAS-*jing*] in the CNS midline is sufficient to inhibit commissural and longitudinal axon formation. Thus, appropriate *jing* dose is a requirement for proper CNS axon development in the CNS midline (Sedaghat Y. et al., 2002).

The homozygous *jing* CNS phenotype suggests an alteration in the mechanisms that guide CNS axons. In 95% of $jing^3$ mutant segments longitudinal fascicles stall within segment boundaries causing breaks in the longitudinal tracts (Sedaghat Y. et al., 2002). A subset of normally ipsilateral axons of the most medial fascicle projects instead contralaterally in $jing^3$ mutants. Ipsilateral fascicles are prevented from crossing the midline in wild type embryos, suggesting midline repulsive mechanisms are perturbed in *jing* mutant embryos (Sedaghat Y. et al., 2002).

Early differentiation of midline lineages requires *jing* function. During stage 9 and 11 in homozygous $jing^3$ mutants there are reductions in the number of SIM-positive midline cells and *sli-lacZ*

expressing midline glia cells compared with the wild-type. SIM (Single-minded) and SLI (Slit) immunoreactivity is drastically reduced in *jing* mutant nerve cords compared with the wild-type. There is evidence that midline lineages are lost by cell death in *jing* loss-of-function backgrounds as shown by TUNEL assays (Sedaghat Y. et al., 2002). The midline of *jing*^{3} mutant embryos exhibits increased occurance of apoptotic glia which correlates with reductions in SLI immunoreactivity. To conclude, *jing* function is required for midline glial survival (Sedaghat Y. et al., 2002).

jing loss-of-function mutations are associated with reductions in the expression of all neuronal markers tested. In *jing*^{3} mutant embryos there are absences of immunoreactivity in the VUMs, MNB and MP1 neuronal lineages in some VNC (Ventral Nervous System) segments compared with wild-type (Sedaghat Y. et.al., 2002). During stage 10 in homozygous *jing*^{3} mutant embryos, there is a loss of odd-skipped (odd) immunoreactivity in MP neurons and similar reductions in the number of 22C10 immunoreactive vMP2 and dMP2 neurons. Within a particular VNC segment in *jing*^{3} mutants, there is a loss of Engrailed (EN)-positive neurons.

Data strongly suggest that the primary site of *jing* CNS function is at the midline (Sedaghat Y. et al., 2002). In summary, these results demonstrate that midline neuronal and glial populations do not differentiate without proper *jing* function and suggest a positive role for *jing* in promoting CNS midline cell development (Sedaghat Y. et al., 2002).

In the trachea, embryos homozygous for *jing*^{3} allele (strong hypomorph) are associated with losses of the dorsal trunk, severely disrupted transverse connectives and absences of the visceral branch compared with the wild type (Sedaghat Y. et al., 2002). Overexpression of *jing* in the trachea is associated with defects in dorsal trunk fusion, as well as improper formation of the transverse connective, dorsal branch and visceral branch.

In embryos homozygous mutant for *jing*^{3} allele (strong hypomorph) initial defects in tracheal morphogenesis occur during tracheal placode stages. In stage 10 homozygous *jing*^{3} mutant embryos the

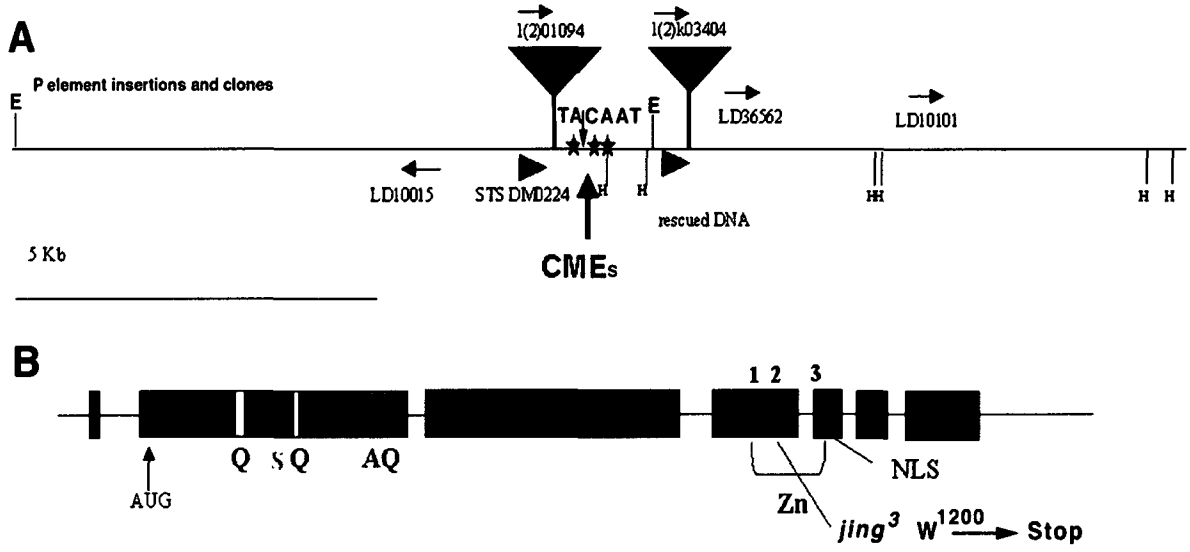
number of TRH-positive precursors is approximately 22% of the expected number of wild-type cells. The positioning of tracheal placodes in *jing*^{3} mutants is not altered from that of wild-type embryos (Sedaghat Y. et al., 2002). In stage 11 *jing*^{3} mutant embryos cell death is observed in TRH-positive precursors. There is also an increase in the number of apoptotic profiles surrounding the tracheal pits in *jing*^{3} compared with wild-type embryos. Throughout embryogenesis in homozygous *jing*^{3} mutant embryos cell death is also observed in all tracheal branches suggesting that *jing* function is required and the requirement is not branch specific (Sedaghat Y. et al., 2002). In *jing*^{3} homozygous mutant embryos, tracheal cells invaginate but the tracheal branches do not migrate properly anteriorly across EN-positive (Engrailed-positive) stripes as in the wild-type embryos. By stage 15 in *jing*^{3} mutant embryos, parts of the dorsal trunk, the dorsal branch and transverse connectives are missing and correlate with a loss of cells by apoptosis (Sedaghat Y. et.al., 2002). The visceral branch does not form in *jing*^{3} mutant embryos.

Genetic interaction studies show that in *jing*^{01094}; *tgo*^{1} *sim*^{H9} triple heterozygotes, midline repulsion mechanisms are affected which causes ventral displacement of midline cells (Sedaghat Y. et al., 2002, *sim*^{H9} null allele, *tgo*^{1} hypomorph). Due to the alteration in the repulsive signaling mechanisms in triple heterozygotes, longitudinal axons collapse into a single tract along the midline (Sedaghat Y. et al., 2002). In *jing* and *sli*^{1} double heterozygotes (*sli*^{1} null allele), embryonic midline cells are present, but they do not adequately express *sli* (*slit*). These results imply that *jing* dosage may be important for the regulation of *sli* (Sedaghat Y. et al., 2002).

Tracheal tubule formation is defective after both *trh* and *jing* are reduced by only one copy each. By stage 15, 51% of embryos double heterozygous for *jing*^{01094} and *trh*^{1} show a significant loss of most tracheal branches (Sedaghat Y. et al., 2002). In triple heterozygotes *jing*^{01094}; *trh*^{1} *tgo*^{1}, 69% of embryos show tracheal phenotypes. In addition, 98% of *jing*^{01094} and *btl*$^{H82\Delta3}$ double heterozygotes show tracheal phenotypes that affect the formation of transverse connectives and visceral branches (Sedaghat Y. et al.,

2002). In summary, genetic analysis provides strong evidence that proper dose of *jing* in combination with that of *trh* or *btl* is important for tracheal tubule patterning (Sedaghat y. et al., 2002).

In conclusion, *jing* is required in the CNS midline and trachea for patterning of CNS axons and tracheal tubules, respectively. Loss-of-function *jing* alleles result in aberrant expression of all CNS midline and tracheal markers tested. The loss of CNS midline and tracheal cells in homozygous *jing* mutant embryos is at least partially mediated by cell death.


A
P element insertions and clones
E
1(2)01094
1(2)k03404
TACAAT
E
LD36562
LD10101
LD10015
STS DMD224
H
H
HH
H H
rescued DNA
5 Kb
CMEs

B
1 2 3
Q SQ AQ
AUG
NLS
Zn
jing3 W1200 Stop

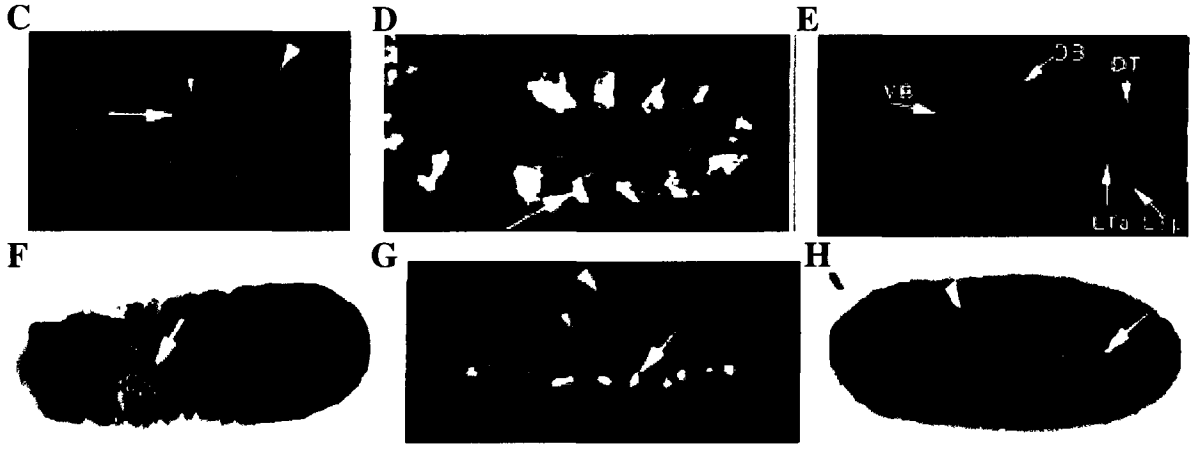

C
D
E
VB
DT
F
G
H

Fig. 9. *jing* genomic structure and expression pattern (Sedaghat Y. et. al., 2002). (A) Genomic interval containing *jing* ESTs (expressed sequence tags), lethal P element insertions (l(2)*01094* and l(2)*k03404*) and adjacent gene (LD10015). *jing* gene is designated as LD36562. l(2)*01094* is an embryonic lethal *lacZ* insertion into the *jing* 5' regulatory region (enhancer trap strain), l(2)k03404 is a second embryonic lethal insertion of the P[*lacW*] P-element into the *jing* gene. Putative DNA binding sites of bHLH-PAS (CMEs) and SOX HMG protein Dichaete (TACAAT) are present in the *jing5'* regulatory region. (B) *jing* exon/intron structure and point mutation. Exons (filled boxes), introns (lines) and protein motifs (colored boxes). Exon 2 in the N-terminus contains repeats of poly-glutamine (Q), poly-serine (S) and alanine-glutamine (AQ) characteristic to transactivation domains. C-terminus contains three C_2H_2-type zinc fingers (Zn, shown in green). The nuclear localization signal in C-terminus is shown in red. The $jing^3$ mutation is a G-to-A change at nucleotide 3806 of cDNA LD36562 converting W^{1200} to a stop codon. (C-H) Embryonic expression pattern of *jing*. Wild-type embryos are shown with anterior to the left. (C, E, H) Anti-JING immunostaining of wild-type embryos. (F) *In situ* hybridization of *jing* digoxigenin-labeled DNA probes to wild-type embryos. (D, G) *jing-lacZ* enhancer trap expression detected via anti-β-gal staining and confocal microscopy. (C) Stage 10 embryo in sagittal-ventral view showing JING localization in tracheal placodes (arrow) and two adjacent cells (small arrowhead) that maybe Branchless expressing cells. In this focal plane, JING is observed in three MP neurons in the CNS (larger arrowhead). (D) Merged confocal image showing co-localization of *jing-lacZ* product (anti-β-gal, green) and TRH (red) in tracheal placodes (arrow) detected via double-label immunostaining of wild-type stage 10 embryos. Sagittal view of the wild-type embryo. (E) Localization of JING to all tracheal branches in a stage 15 wild-type embryo. Sagittal view of the wild-type embryo. (F-H) At stage 12/3, *jing* transcription occurs in the CNS midline (arrows) and segmental ectodermal stripes (arrowheads). Small arrowhead in G indicates Sim+ muscle cells. (F-H) Ventral views of the wild-type embryos stage 12/3. Adapted from Sedaghat Y. et.al., 2002.

6A. RTK (Receptor Tyrosine Kinase) activity in the trachea

Two RTKs, the Epidermal Growth factor receptor (EGFR) and Fibroblast Growth Factor Receptor (FGFR) play critical roles during the differentiation of the CNS midline (Sonnenfeld M et al, 2004; Stemerdink C. and Jacobs J.R., 1997) and tracheal cells (Sonnenfeld M. et al, 2004; Llimargas M. and Casanova J., 1999; Wappner P. et al, 1997; Glazer L. and Shilo B.Z., 2001; Gabay L. et al, 1997; Ohshiro T. et al, 2002). RTKs activate a universal cytoplasmic signaling pathway (Gabay L. et al, 1997). Receptors are activated by a ligand and trigger the activation of RAS. Activated RAS induces activation of protein kinases, including RAF, MEK, MAPK (ERK) and some others, collectively known as the MAPK signaling cascade. The central step of the cascade is the activation of the MAPK (mitogen-activated protein kinase, ERK) by dual phosphorylation of threonine and tyrosine residues by MEK. Both threonine and tyrosine lie adjacent to each other in the unique TEY sequence motif (activation loop). The activation loop of ERK is highly conserved between species. Although MEK is a dual specificity kinase, dp-MAPK has a wide range of substrates, both in the cytoplasm and in the nucleus (Gabay L. et al, 1997). The function of the whole signal transduction pathway is an amplification of the signal (Lehninger, Nelson, Cox, 1998).

6A1 Epidermal Growth Factor Receptor

In *Drosophila*, the EGFR has multiple functions during embryogenesis (e.g. pattern formation in the ventral ectoderm) and is active in different types of cells (tracheal placodes, CNS, PNS, midline glia, etc.). EGFR has two ligands: activating ligand Spitz (Gabay L. et al., 1997) and inhibitory ligand Argos (Gabay L. et al., 1997). The activating ligand Spitz requires processing for activation, which is regulated by two transmembrane proteins, Rhomboid (Rho) and Star. The inhibitory ligand Argos is part of the EGFR negative feedback loop. The diagram below (Fig.10) illustrates the EGFR signaling cascade in the *Drosophila* trachea (Llimargas M. and Casanova J., 1999; Brodu V. and Casanova J., 2006)

Induction of ventralmost cell fates by the EGFR pathway in the cellular blastoderm involves activation of the transcription of Pointed P1 (Pnt P1), as well as phosphorylation by EGFR and subsequent inactivation of Yan/Anterior open (Aop). As a result, secondary EGFR targets (Argos, etc.) are induced by Pnt P1 (Klambt, 1993; Gabay L. et. al., 1996; Wappner P. et al, 1997).

In vertebrates, EGFR signaling is involved in branching morphogenesis in the lung, control over highly migratory cells during lung development and invasive processes such as tumor formation (Warburton D. et al, 2000; Risau W., 1997). In mammals, mutations that lead to EGFR up regulation have been associated with a number of cancers, including lung cancer and glioblastoma multiforme.

In humans, the EGFR family comprises four members designated EGFR, ErbB2, ErbB3, and ErbB4. Although certain members of the EGFR family can be activated by several EGF family members (e.g., EGFR), others do not directly bind any ligand (e. g., ErbB2) or are devoid of intrinsic RTK activity (e. g., ErbB3). Therefore, the action of ErbB3 and ErbB2 is dependent upon combinatorial interactions with other members of the EGFR family that are stimulated by EGF, transforming growth factor α (TGF- α), heparin-bound EGF (HB-EGF), or other EGF family members (Schlessinger J., 2004). Null mutations in the EGFR in humans cause 50% reductions in branching and neonatal lethal failure of lung maturation (Miettinen P.J. et al, 1997; Miettinen P.J. et al, 1995).

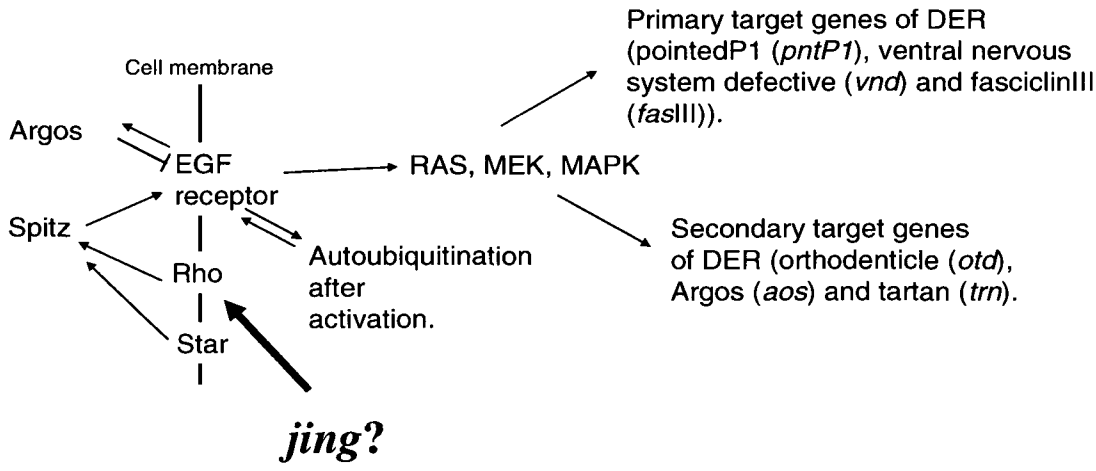


Fig. 10. The EGFR signal transduction cascade. Spitz is an activating ligand of EGFR which requires processing by Rho and Star. Activated EGFR triggers phosphorylation of MAPK at tyrosine and threonine residues by MEK. dp-MAPK activates or represses downstream targets of EGFR. *jing* may be involved in the regulation of *rho* expression. (Gabay L. et al., 1997).

6A2 Fibroblast Growth Factor Receptor

In *Drosophila*, FGFR is expressed in all tracheal cells and in the CNS midline (see section 2) (Ohshiro T. et al, 1997; Ohshiro T. et al, 2002). The FGFR signaling transduction cascade is summarized in Fig.11. The activating ligand of Breathless is Branchless. Activated FGFR triggers activation of RAS resulting in dual phosphorylation of MAPK by MEK. dp-MAPK activates or represses downstream cytoplasmic and nuclear targets of the FGFR.

In vertebrates FGFR is involved in branching morphogenesis in the lung. In the lung bud, FGFR type 2 isoform IIIb and its high affinity mesenchymal ligands (FGF10, FGF7 and FGF1) are essential for patterning and morphogenesis of the respiratory tract (Bellusci S. et al, 1997). Furthermore, induction of a liver versus lung fate is controlled by different concentrations of FGFs. FGFR mutations are implicated as well in invasive processes such as cancer (Bellusci et al., 1997; Peters et al., 1994; Celli et al., 1998).

Defects in the Sonic Hedgehog-Gli and FGF pathway are involved in adriamycin-mediated teratogenic model tracheoesophageal anomalies (SpildeT.L. et al., 2004; Spilde T.L. et al., 2003).

During *Drosophila* tracheal development EGFR and FGFR are active at different stages (Fig.2) (Gabay L. et al, 1997). EGFR is essential during the early stages of tracheal morphogenesis (stage10 placode formation and following invagination). FGFR guides directed cell migration during branching morphogenesis at later stages (Gabay L. et al, 1997).

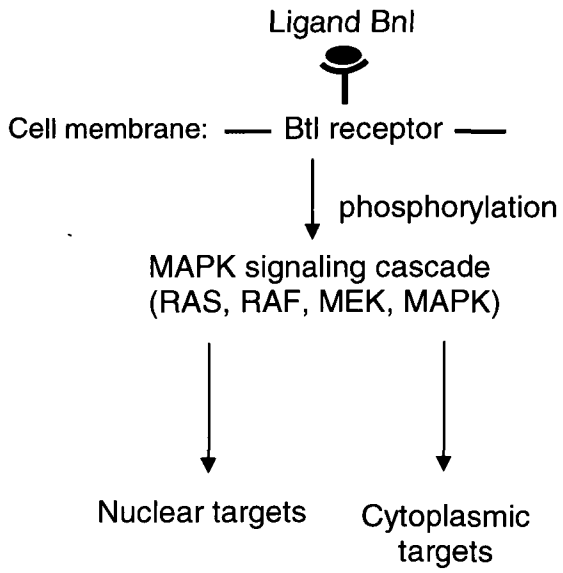


Fig.11. FGFR signaling cascade (Ohshiro T. et al, 2002)

6B. Role of *jing* in tyrosine kinase signaling

It was shown that *jing* is required for both EGFR and FGFR-dependent MAPK activity (Sonnenfeld M., et al, 2004). Homozygous *jing* mutants show reductions in the levels of activated MAPK in the CNS midline and in the trachea (placodes and pits). In particular, EGFR-dependent MAPK activity in the tracheal placodes (stage 10) and FGFR-dependent MAPK activity in the tracheal pits (stage 11) are reduced in homozygous *jing* mutant embryos (Sonnenfeld M., et al, 2004).

jing can also induce the EGFR pathway and is required for EGFR signaling and expression of EGFR in midline glia (Sonnenfeld M., et al, 2004). *jing* over-expression in the CNS midline induces extra *Egfr* expressing midline glia and resembles the over-expression phenotype of *Egfr* pathway genes. Midline *Egfr* expression is reduced in homozygous $jing^{01094}$ and $jing^{3}$ embryos compared to controls (Sonnenfeld M. et al., 2004).

Together, these results suggested that *jing* may play a regulatory role in EGFR and FGFR signaling cascades.

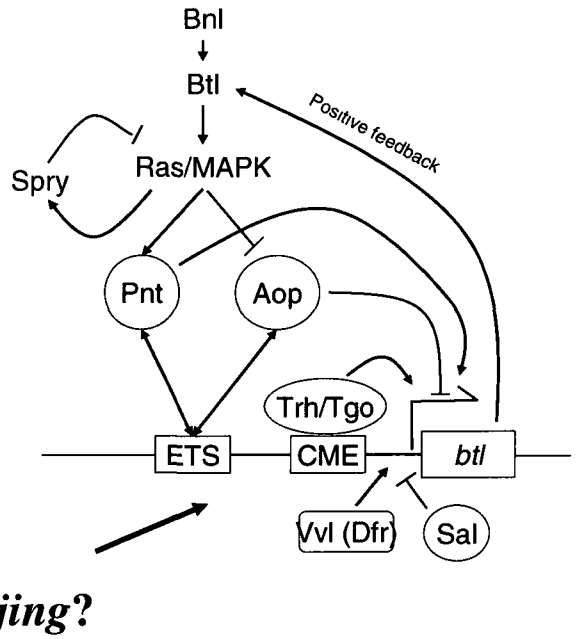


Fig.12. Possible function of *jing* in *btl* positive feedback loop.

6C. Other functions of Jing

5C1 Jing is required for border cell migration in *Drosophila* ovaries

In *Drosophila* ovaries, *jing* is required for the initiation of border cell migration. The *jing* locus was first identified in a screen for mutations that cause border cell migration defects in mosaic clones in *Drosophila* ovaries (Liu Y. and Montell D.J., 2001). In the ovaries, the *jing* locus functions downstream of

slow border cells (*slbo*) in the transcription activation pathway. The *slbo* locus encodes the *Drosophila* homolog of the CCAAT enhancer binding protein (C/EBP). In *Drosophila* ovaries, Jing is suggested to cooperate with SLBO in activating transcription of SLBO downstream genes (Liu Y. and Montell D.J., 2001). One of the critical downstream targets of SLBO is the *shotgun* (*shg*), gene which encodes *Drosophila DE-cadherin* (Niewiadomska et al, 1999). The central function in the guidance of the border cell migration in *Drosophila* ovaries belongs to EGFR and FGFR signaling (Duchek P. et. al., 2001; Lee T. et.al., 1996; Murphy A.M. et.al., 1995) linking together RTK signaling and *jing* function.

6C2 Jing is required for wing development and to establish the proximo-distal axis of the leg in *Drosophila melanogaster*

The establishment of the proximo-distal (PD) axis in the legs of *Drosophila melanogaster* requires expression of several TFs activated in discrete domains by secreted signaling molecules. The precise regulation of these TFs is essential for generating morphological characteristics along the PD axis, such as positioning of specific bristle types and leg joints. *jing* is critical for PD axis formation in *Drosophila* leg (Culi J., Aroca P., Modolell J, Mann R.S., 2006). In particular, *jing* represses transcription of the proximal gene *homothorax* (*hth*) and is necessary to keep *hth* repressed in the medial domain of the PD axis in the leg. Clones of cells homozygous for $jing^{22F3}$ and $jing^{47H6}$ alleles (strong, possibly null) have profound effects on leg development. $jing^{-}$ clones located in the most proximal segments of the leg do not have an observable phenotype. However, clones located in the femur, tibia, or first tarsal segment display the loss of bracts located at the base of the bristles. This suggests a transformation toward a more proximal fate, because bracted bristles are characteristic of the distal regions of the leg. $jing^{--}$ clones located in the distal regions also show expression of the *hth* gene, which is normally repressed in this area (Culi J., Aroca P., Modolell J, Mann R.S., 2006).

In the adult wing, *jing* is required for alula and vein development. In the wing, *jing* represses another proximal gene, *teashirt* (*tsh*), in a small domain that will give rise to the alula. The *jing*⁻ clones located in the alula region show strong derepression of the *tsh* gene. Resulting alulae are small or reduced, suggesting that repression of the *tsh* in this region of the wing disc is essential to allow the development of alula (Culi J., Aroca P., Modolell J, Mann R.S., 2006).

jing also interacts with several members of the polycomb (Pc) group of genes during development. Data suggest that *jing* encodes a transcriptional repressor that may participate in a subset of Pc-dependent activities during *Drosophila* appendage development (Culi J., Aroca P., Modolell J, Mann R.S., 2006).

6D. Mammalian homolog of *jing* is the transcriptional repressor AEBP2

The Jing protein is most closely related to a mouse transcription factor, AEBP2, which also has three tandem C_2H_2-type zinc fingers (H-S Ro and D.A.K. Roncari, 1991; Liu Y. and Montell D.J., 2001). Jing exhibits 50% amino acid identity with AEBP2 within the zinc finger motifs and 20% identity C-terminal to the zinc fingers. After AEBP2, the most similar proteins are several members of the GLI family of zinc finger transcription factors. However GLI proteins typically contain five zinc finger motifs. The GLI family proteins are only 25% identical to Jing within zinc fingers and do not exhibit homology outside of these motifs (Liu Y. and Montell D.J., 2001).

AEBP2 was identified on the basis of its ability to bind a small regulatory sequence (AE1) within the mouse adipocyte aP2 gene proximal promoter region (nucleotides -159 to -125) to which mammalian C/EBP also binds (Liu Y and Montell D.J.). The AE1 sequence is shown in Fig.12 (H-S Ro and D.A.K. Roncari, 1991).

In mouse preadipocytes, the aP2 gene encodes adipose fatty acid binding protein involved in triglyceride metabolism during adipocyte differentiation. AEBP2 (AE-binding protein/adipocyte enhancer-binding protein) is a transcriptional repressor of the aP2 gene whereas C/EBP is a transcriptional activator

of the aP2 gene. AEBP2 was found in a chromatin remodeling complex (Kim H., et.al., 2009). Two transcripts (4.5 and 3.5 kb) of AEBP2 are ubiquitously expressed in every adult mouse tissue examined.

AE-1

GATCCAGGGAGAACCAAA**GTTGAGAAATT**TCTATTAAA
-159 **-125**

AE-1 agttgagaaat

C/EBP aattgggcaat

Fig.13. DNA sequences of the aP2 promoter region and C/EBP-binding site. The AE-1 binding sequence represents nucleotides -159 to -125 relative to the transcription start site of the aP2 gene. Bottom part: comparison of C/EBP-binding sites in the AE-1 sequence and the consensus C/EBP-binding sequence. Adapted from Ro H-S. and D.A.K.Roncari, 1991.

7. Rationale

In this project, I studied the relationship between transcriptional regulation and signal transduction during cell migration and cytoskeletal remodeling of epithelial cells. This model will provide information that is essential to understand what controls the behavior of highly migratory cells during lung development and invasive processes such as tumor formation. I continue to investigate the relationship between the *Drosophila* Jing zinc finger protein and tyrosine kinase signaling through the EGFR and FGFR in the *Drosophila* embryonic trachea and midline. I also studied the transcriptional regulation of *jing*.

7.1 Hypothesis

Since *jing* is required for both EGFR and FGFR signal transduction in the trachea and CNS midline (Sonnenfeld M. et al, 2004), I first proposed that *jing* is responsive to tyrosine kinase signaling through EGFR and FGFR in the trachea and in the CNS midline. Since genetic interactions of Trh::Tgo and *jing* were demonstrated in trachea (Sedaghat Y. et.al., 2002), and because Trh::Tgo and Dfr and Pnt often regulate tracheal gene expression in combinatorial manner (Boube M. et.al., 2000), I next proposed that *jing* expression is regulated by Trh::Tgo, Pou-domain transcription factor Drifter/Vvl, and ETS-domain transcription factor Pointed, *in vivo*. Finally, I proposed that *jing* is required for maximal levels of *breathless (FGFR)* expression in the embryonic trachea.

7.2 Objectives

1. To generate *lacZ* reporter constructs containing 1.5kb, 1.3kb and 2.8kb of the *jing5'* regulatory sequence to identify cis-regulatory elements and to study transcriptional regulation of *jing in vivo.*

2. To investigate transcriptional regulation of *jing* in the trachea by Trh::Tgo, Pou-domain TF Drifter, and ETS-domain transcription factor Pointed using *lacZ* reporter constructs containing 1.5kb and of the *jing5'* regulatory sequence.

3. To determine if *jing* expression is regulated by the Pointed ETS-domain TF in the trachea by binding site deletion analysis.

4. To show that *jing* is responsive to tyrosine kinase signaling.

5. To show that *jing* is required for maximal levels of *btl* (FGFR) expression in the embryonic trachea by using in situ hybridization with *btl* antisense digoxigenin labeled RNA probes.

Materials and methods

1. *Drosophila* strains

Flies were raised on cornmeal molasses/yeast/agar medium with both 1.6% Tegosept and 0.6% propionic acid at room temperature. The following stocks were obtained from the Bloomington *Drosophila* Stock Center at Indiana University: w^{1118}, *armadillo (arm)-Gal4*, *paired (prd)-Gal4*, *breathless (btl)-Gal4*, *single-minded (sim)-Gal4*. Gal4 responsive UAS lines were P[*UAS-trh*], P[*UAS-trhUAS-vvl*], P[*UAS-jingRNAi*] (Sun X. et al, 2006), P[*UAS-jingE*] (Sedaghat Y. et al., 2002), P[*UAS-jingΔ5N*]. P[*UAS-rhomboid*] and P[*UAS-sspitz*] were a gift of J.R.Jacobs. Mutations used were: $jing^{3}$ (a strong hypomorph) (Sedaghat Y. et al., 2002); $drifter^{B129}$ (dfr^{B129}) (a null allele) and $drifter^{E82}$ (a strong hypomorph) (Anderson et al., 1995, 1996; Certel and Thor, 2004); $pointed^{316}$ (pnt^{316}) and $pointed^{E039}$ (pnt^{E039}) (obtained from C.Klambt), $pnt^{\Delta 88}$ (a null allele); trh^{2} (a mild hypomorph) (Issac and Andrew, 1996; Wilk et al., 1996); $breathless^{dev1}$ (btl^{dev1}) (a hypomorphic allele) (Gindhart and Kaufman, 1995); and *branchless* (bnl^{1}). trh^{2}, btl^{dev1}, rho^{7M43} (a null allele) and bnl^{1} were obtained from the Bloomington *Drosophila* stock center (Indiana University).

2. Construction of riboprobes for *in situ* hybridization (*btl* and *jing* antisense dig labeled RNA probes)

To determine *btl* or *jing* mRNA expression levels in the embryo I synthesized *btl* and *jing* antisense dig labeled RNA probes.

btl cDNA, cloned at BstXI/KpnI sites in pBluescriptSK (-) vector, was a gift of the Ohshiro lab (Ohshiro et al., 2002). The DNA plasmid construct was linearized with BstXI restriction enzyme. Digested DNA plasmids were purified using Qiagen PCR purification kit. The sticky DNA ends were blunted using Klenow enzyme followed by purification using Qiagen PCR purification kit. To synthesize the RNA strands, (RNase-free conditions), 1 μg of DNA was mixed with the following reagents provided in Genius 4

RNA labeling Kit (Roche): 2µl NTP 10X labeling mixture, 2µl 10X transcription buffer, 2µl RNase inhibitor, 2µl T3 RNA polymerase for antisense probe, and DEPC treated water up to 20µl total volume. The T3 reaction mixture was incubated at 37^0C for 2 hours.

The transcription reaction was terminated by adding 2µl of 200mM EDTA solution. The RNA was precipitated with 0.1 volume of 4M LiCl and 2.5-3.0 volume of 100% chilled ethanol. Following 2 hours incubation at -70^0C the mixture was centrifuged at 13,000 rpm for 15 minutes at 4^0C for precipitation. Pellets were washed with 100µl of 70% ethanol and centrifuged at 13,000 rpm for 15 minutes at 4^0C. Finally, the pellet was air dried at room temperature (RT) and resuspended in 150µl of Hybridization A solution [50% (v/v) formamide; 25% (v/v) 20XSSC; 10 mg/ml sonicated salmon sperm DNA; 20 mg/ml tRNA; 100 mg/ml heparin, pH 5.0]. Riboprobes were stored at -80^0C.

Full-length *jing* cDNA (Sedaghat Y. et al., 2002) was cloned at EcoRI/XhoI restriction sites in pOT_2 vector. The DNA plasmid construct was linearized with EcoRI restriction enzyme. *jing* antisense probe was synthesized as described above using SP6 RNA polymerase.

3. Embryo collection and fixation for *in situ* hybridization

Embryos were collected on apple juice agar plates. Embryos were dechorionated in 25% bleach (Chlorox) in water for 2-3 minutes and transferred onto a nitex sieve. Then embryos were rinsed with distilled water and transferred into a glass scintillation vial containing 6 ml heptane and 1 ml fixation solution (4% formaldehyde in 1X PBS pH 7.4). The vial with the embryos was shaken on a nutator for 15-20 minutes. The lower phase was removed with a glass pipette and replaced with 8ml of methanol. The vial was shaken vigorously for 10 seconds to burst the vitelline membrane and release devitellinized embryos to the bottom. Embryos were transferred into a reaction vial and washed three times with methanol. Devitellinized embryos were stored for a few weeks at -20^0C or processed immediately for *in situ* hybridization.

4. *In situ* hybridization of DIG-labeled riboprobes to tissues in whole-mount embryos

The following protocol was modified from the Nusslein-Volhard lab. All materials used during the making of the RNA probes were cleaned with RNase free solution to avoid contamination with RNase. All solutions were made in DEPC water.

Embryos stored at -20^{0}C were thawed at room temperature, washed once in fresh methanol, then washed three times for 10 minutes in PBT [998.5 ml 1X PBS; 500µl DEPC; 0.1% (v/v) Tween 20]. Detergent (Tween 20) was used to permeabilize the tissue. Embryos were post-fixed in 1 ml PBT, 4% formaldehyde for 15 minutes then washed three times for 5 minutes in PBT and incubated in 0.05% Proteinase K in PBT for 5 minutes to remove proteins surrounding the target sequence. Proteinase K digestion was stopped by washing embryos once in 2mg/ml glycine solution in PBT and embryos were washed twice for 5 minutes in PBT. Embryos were again fixed in 1 ml PBT, 4% formaldehyde for 15 minutes and were washed again five times for 5 minutes in PBT. Then embryos were washed in hybridization B solution [Hyb B: 50% (v/v) formamide; 25% (v/v) 20X SSC; 25% (v/v) DEPC in water] for 5 minutes and prehybridized in Hybridization A solution [50% (v/v) formamide; 25% (v/v) 20X SSC; 10 mg/ml sonicated salmon sperm DNA; 20 mg/ml tRNA; 100 mg/ml heparin, pH 5.0] for 2 hours in a water bath at 70^{0}C. Then hybridization A solution was replaced with 180µl of the fresh hybridization A solution containing 6µl of the riboprobe. Hybridization was carried out in a 70^{0}C water bath overnight. The next day, embryos were incubated 2X for 15 minutes in hybridization B solution at 70^{0}C followed by incubation in 50% hybridization B solution in PBT for 5 minutes at room temperature, which was followed by three washes in PBT for 10 minutes.

The anti-DIG antibody conjugate was prepared freshly and was preabsorbed against fixed embryos in order to remove any unspecific binding material. The final working dilution of the antibody conjugate was 1:2000 in PBT. Embryos were incubated for 2 hours at room temperature in 1.2 ml of the diluted, preabsorbed anti-DIG antibody complex. Embryos were washed three times 20 minutes each in PBT, and

then washed again twice for 20 minutes each in staining buffer [1M $MgCl_2$; 5M NaCl; 1M Levamisol; 20% Tween 20; 0.1M Tris-HCl, pH 9.5]. Then staining buffer containing [2.56% (v/v) NBT/BCIP: Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt in DMSO] was prepared freshly and 1 ml of the staining solution was added to the embryos. The color was allowed to develop in the dark by covering the tube containing the embryos with aluminum foil. The staining reaction was allowed to proceed until sufficient staining was observed under a dissecting microscope. The staining reaction was stopped by washing with PBT. Embryos were washed twice 10 minutes each in PBT, and twice 10 minutes each in water on the nutator. Finally, the embryos were dehydrated through a series of washes with increasing ethanol concentrations starting with 70% ethanol. After 70% ethanol was removed, the embryos were washed with a mix of 70% ethanol and 100% ethanol. Then the embryos were washed twice in 100% ethanol with the 5 minutes incubation at room temperature. Embryos were stored in 80% glycerol at room temperature.

5. Immunohistochemistry

5.1 Embryo fixation

Embryos from various stages of embryonic development were collected overnight at room temperature on apple agar-filled Petri plates containing a small smear of live yeast paste (Fleischmann's Dry). The plates were kept in the fridge for up to 3 days. Before fixation, embryos were allowed to recover at RT for 2 hour to restore axon morphology and to allow microtubule re-polymerization. Embryos were dechorionated for 7 minutes with 50% bleach. Dechorionated embryos were collected onto a nitex sieve and rinsed with distilled water. Then embryos were transferred into a scintillation vial containing a fixation solution [500µl of 37% formaldehyde, 4.5ml of 1X PBS, and 5ml of heptane]. Embryos were fixed for 30 minutes at the interface between the heptane and bottom aqueous layer containing the fixative. The heptane at this interface ensures permeabilization of embryos to allow entry of the fixative and subsequent

antibodies. The bottom aqueous layer was removed, replaced with fresh methanol and the vial containing the embryos was shaken vigorously for approximately 20 seconds in order to remove the vitelline layer surrounding the embryos. Devitellinized embryos in the bottom methanol layer were transferred to a disposable plastic tube using a Pasteur pipette. Traces of heptane were removed by three methanol washes. Embryos were stored in -20^0C for a maximum of two weeks.

5.2 Antibody staining

The following protocol was modified from Dr.M.Sonnenfeld's lab. To proceed with antibody staining, the methanol in the samples was replaced with PBT (1X PBS containing 0.1% triton X-100). Embryos were washed three times in PBT for 20 minutes each time. Then, to prevent unspecific binding, embryos were incubated in 100µl of PBT containing 10µl of normal goat serum (NGS, Vector Laboratories) for 30 minutes. Following the blocking, a primary antibody with specific dilution was added to the embryos in the blocking solution and was incubated at 4^0C overnight. Residual antibody was washed away with PBT 3X for 1 hour. The washed embryos were treated again in PBT containing NGS for 30 minutes. The secondary antibody (goat anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase or the fluorescent analog) was added to the embryos in blocking solution and was incubated for 2 hours at RT. Then the secondary antibody was washed out 3 times with PBT for an hour on the rotator.

The staining was visualized by incubating embryos in 1000µl PBT containing 500µl 0.3 mg/ml diaminobenzadine (DAB). 3µl of 3% hydrogen peroxidase was added to catalyze the reaction. The reaction was allowed to proceed until appropriate staining was achieved. The reaction was terminated by addition of PBT. Embryos were dehydrated through a series of increasing ethanol concentrations (50%, 70%, 95%, and 100%). Embryos were stored at room temperature in methyl salicylate to preserve the staining. Embryo staging was according to Campos-Ortega and Hartenstein, 1985.

5.3 Antibodies

The following primary antibodies were used: mouse anti-fasciclin 1D4 (Developmental Studies Hybridoma Bank, DSHB, dilution 1:10) to visualize embryonic CNS axons; monoclonal mouse anti-β-galactosidase (Promega, dilution 1:100) and polyclonal rabbit anti-β-galactosidase (Rockland, Cedarlane, dilution 1:100) to visualize expression of *lacZ* reporter constructs. Anti-dioxigenin-AP (alkaline phosphatase) primary antibody was used for *in situ* hybridization. Polyclonal rabbit anti-Dysfusion antibody was used to visualize the tracheal fusion cells (gift from Crews S., dilution 1:500). Monoclonal mouse anti-Tango antibody was used as a tracheal cell marker (Developmental Studies Hybridoma Bank, DSHB, dilution 1:1). Monoclonal mouse anti-Engrailed antibody was used as a marker for segmental ectodermal stripes (Developmental Studies Hybridoma Bank, DSHB, dilution 1:3). The secondary antibodies were HRP- and Texas Red-conjugated goat anti-mouse IgG (Jackson), and HRP- and FITC-conjugated goat anti-rabbit IgG (Jackson). We also used FITC-conjugated goat anti-rabbit IgG AlexaFluor 488 (Invitrogen), and Texas Red-conjugated goat anti-mouse IgG AlexaFluor 594 (Invitrogen).

5.4 Microscopy

A Zeiss Axioskop with Nomarski optics was used for light microscopy. Images were captured on a Nikon DXM1200 digital camera and processed using Adobe Photoshop 7.0 software. Fluorescently-labeled embryos were analyzed on a Zeiss LSM5 Pascal confocal microscope.

6. Purification of genomic DNA from *Drosophila melanogaster*

25mg of tissue was cut up into small pieces, placed in a 1.5 ml microcentrifuge tube, and resuspended in a 180 μg Buffer ATL (Qiagen DNeasy Tissue kit). 20 μl of proteinase K was added, mixed by vortexing, and the tube was incubated at 55^0C overnight until the tissue was completely lysed. Following the incubation, the tube was vortexed for 15 s., and 200 μl Buffer AL (Qiagen DNeasy kit) was added to the

sample, mixed thoroughly by vortexing, and incubated at 70^0C for 10 min. Then 200 µl of ethanol (96-100%) was added to the sample and mixed by vortexing. The mixture was pipeted into the DNeasy Mini spin column placed in a 2 ml collection tube (provided) and centrifuged at 8000rpm for 1 min. The flow-through and the collection tube were discarded, and the DNeasy spin column was placed in a new 2 ml collection tube (provided), 500 µl Buffer AW1 (Qiagen DNeasy Tissue kit) was added, and the column was centrifuged at 8000rpm for 1 min. The flow-through and the collection tube were discarded, and the DNeasy Mini spin column was placed in a 2 ml collection tube (provided), 500 µl Buffer AW2 (Qiagen DNeasy Tissue kit) was added, and the column was centrifuged at 14000rpm for 3 min to dry the DNeasy membrane. The flow-through and the collection tube were discarded. The DNeasy mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and 200 µl Buffer AE (Qiagen DNeasy Tissue kit) was pipeted directly onto the DNeasy membrane. The column was incubated at room temperature for 1 min, and then it was centrifuged at 8000rpm for 1 min to elute genomic DNA. Elution was repeated second time with 200 µl Buffer AE.

7. Construction of *lacZ* reporter fusions

The original *jing5′* regulatory sequence (Sedaghat et. al, 2002, Fig.14) was entered into an NCBI Blast program (a Basic Local Alignment Search Tool. Altschul et al, 1990). It was aligned with *Drosophila melanogaster* chromosome 2R, section 6 complete sequence (AE003789, *jing* complete sequence (FlyBase)). The search resulted in 1.5kb fragment of *jing* 5′ genomic regulatory sequence and we also extended it to 2.8kb of the *jing* 5′ genomic *cis*-regulatory sequence.

7.1 Generation of the *jing1.5-lacZ* reporter

A 1.5 kilobase *jing5′lacZ* transgene was constructed by inserting the 1.5kb *jing*5′ regulatory sequence into the multiple cloning site upstream of the *lacZ* gene of the transposable-based

pCaSpeRhs43βgal vector. The 1.5 kb fragment was PCR amplified from genomic DNA isolated from wild-type w^{1118} flies. Genomic DNA was purified using the Qiagen DNeasy kit. Primers used were NotIforward 5'TAGCGGCCGCCGTAGAAACAGAAACGTCAGTG3' and KpnIreverse 5'GGGGTACCCAGATGCGGATTCATACTCAAA3' (underlined in red in Fig.14). The 1.5kb fragment was generated, gel extracted and ligated into the pCasper vector at NotI/KpnI sites. The ligation reaction continued for 2 hours at room temperature as in the T4 ligase instruction manual (Invitrogen). Transformation of the construct was completed by adding 2.5μl of the ligation product to 50μl of thawed competent DH5α cells. The cells were incubated on ice for 30 minutes followed by heat shock for 20 seconds in a 37^0C water bath, and finally 2 minutes on ice. Then, 950μl of LB broth was added to the transformed mix which was incubated for 1 hour at 37^0C on a rotating incubator (250 rpm) to allow for the expression of antibiotic resistance markers. 250 μl and 150 μl of each transformation reaction were added to LB + ampicillin plates and a bent glass rod was used to spread the cells. Plates were placed inverted at 37^0C for at least 18 hours. QIA prep Spin Miniprep Kit (Qiagen) was used to perform minipreps. The minipreped DNA was stored at -20^0C. The constructs were confirmed by restriction digest with BamHI enzyme to release the 1.5kb fragment (Fig.20). DNA from the positive colonies was sequenced and aligned using Blast NCBI against genomic regulatory sequence 5' to the *jing* open reading frame obtained from the FlyBase. Maxipreps (Qiagen maxiprep kit) were required in order to obtain higher concentration and larger amounts of the constructs to send for micro-injection (see section 7.5).

7.2 Generation of the *jing2.8-lacZ* reporter

The 2.8kb *jing* regulatory sequence is shown in Fig. 15. The *jing2.8-lacZ* transgene was constructed by inserting 2.8kb of *jing*5' genomic sequence into the multiple cloning site upstream of the *lacZ* gene in the pCaSpeRhs43βgal vector. The 2.8 kilobase fragment was PCR amplified from genomic DNA of w^{1118} flies. Primers used were NotIforward 5'TAGCGGCCGCCGTAGAAACAGAAACGTCAGTG3' and

KpnIreverse 5'GGGGTACCGAACAAGAGAAGGAGAAGAGCT3' (primers are shown in bold and underlined in green in Fig. 15). The 2.8kb fragment was ligated into pCaSpeRhs43βgal vector at NotI/KpnI sites. After transformation into DH5α *E.coli* cells colonies were selected and digested with BamHI enzyme to release the 2.8kb fragment (Fig.20). DNA from positive colonies was sequenced (OHRI, Ottawa, Canada) and aligned using Blast NCBI against *jing* 5' genomic sequence obtained from the FlyBase database (see section 7.5 for generation of *jing2.8-lacZ* transgenic flies).

7.3 Generation of the *jing1.3-lacZ* reporter

jing1.3-lacZ transgene was constructed by inserting the 1.3kb of *jing* genomic sequence into the multiple cloning site upstream of *lacZ* gene of pCaSpeRhs43βgal vector. The 1.3 kilobase fragment was PCR amplified from genomic DNA of w^{1118} flies. Primers used were NotIforward 5'TAGCGGCCGCTTTGAGTATGAATCCGCATCTG3' and
KpnIreverse 5'GGGGTACCGAACAAGAGAAGGAGAAGAGCT3' (primers are shown in bold and underlined in black in Fig. 15). The 1.3kb fragment was ligated into pCaSpeRhs43βgal vector at NotI/KpnI sites. After transformation into DH5α *E.coli* cells colonies were selected and digested with BamHI enzyme to release the 1.3kb fragment (Fig.20). DNA from positive colonies was sequenced (OHRI, Ottawa, Canada) and aligned using Blast NCBI against *jing* 5' genomic sequence obtained from the FlyBase database (see section 7.5 for generation of *jing2.8-lacZ* transgenic flies).

7.4 Generation of the *jing1.5Δpnt-lacZ* reporter

Pnt binds to the consensus 5'GGA DNA binding site in the *btl* regulatory region (Nye J.A. et. al., 1992; Ohshiro T. et. al., 2002). Pnt and Aop/Yan compete for the common ETS binding target sites, 5'GGA, in order to regulate gene expression (Gabay et al., 1996). Seventeen 5'GGA sites are present in the 1.5kb sequence but only one region is associated with an inverted repeat (TCC..GGA) similar to the site bound by

Aop/Yan (Fig.14) (Ohshiro et al., 2002). Consensus site, TCCCTGGA, in the 5′ *cis*-regulatory region of *jing*1.5 is conserved in the *Drosophila* sibling species, *Drosophila ere*. The sites, GGA and TCCCTGGA, were removed from the 1.5kb *jing* regulatory region to generate the *jing1.5ΔPnt-lacZ* reporter construct. The deleted fragment is underlined in green in Fig.14.

To delete the chosen sites we used the Stratagene Quik Change XL kit (Fig.18). Primers used were deletion forward 5′CAACGGACGAAG$^{\Delta}$ATGGCCGTCGGC3′ and deletion reverse 5′GTTTGCCGACGGCCAT$^{\Delta}$CTTCGTCCGTTG3′. The DNA fragment with deleted Pnt/Aop sites was amplified using DNA of the *jing1.5-lacZ* construct in pCaSpeRhs43βgal vector as a template. PCR reactions were carried out according to the instruction manual (Stratagene). The colonies showing blue color on Amp^{+}/IPTG/X-gal plates were sequenced and aligned using Blast NCBI against the 2.8kb from *jing* 5′ genomic sequence (Fig.17), maxipreped (Fig.19) and sent for micro-injection (see section 7.5). Eight resulting transgenic lines were bred to homozygosity.

7.5 Generation of transgenic flies carrying the *jing1.5-lacZ*, *jing2.8-lacZ*, *jing1.3-lacZ*, and *jing1.5Δpnt-lacZ* reporters

50μg of the recombinant plasmid DNA dissolved in sterile water was sent for micro-injection into stage 1 or 2 embryos (Fig.16). The injection procedure was carried out by transgenic services (Genetic Services Inc., Cambridge, USA). White eyed flies (w^{1118}) were injected so that genomic integration could be scored using the eye color marker on the plasmid (Spradling A.C. and Rubin G.M., 1982). Five transgenic lines for the *jing1.5-lacZ* construct, 4 transgenic lines for the *jing2.8-lacZ* construct, and 10 transgenic lines for the *jing1.3-lacZ* construct were bred to homozygosity.

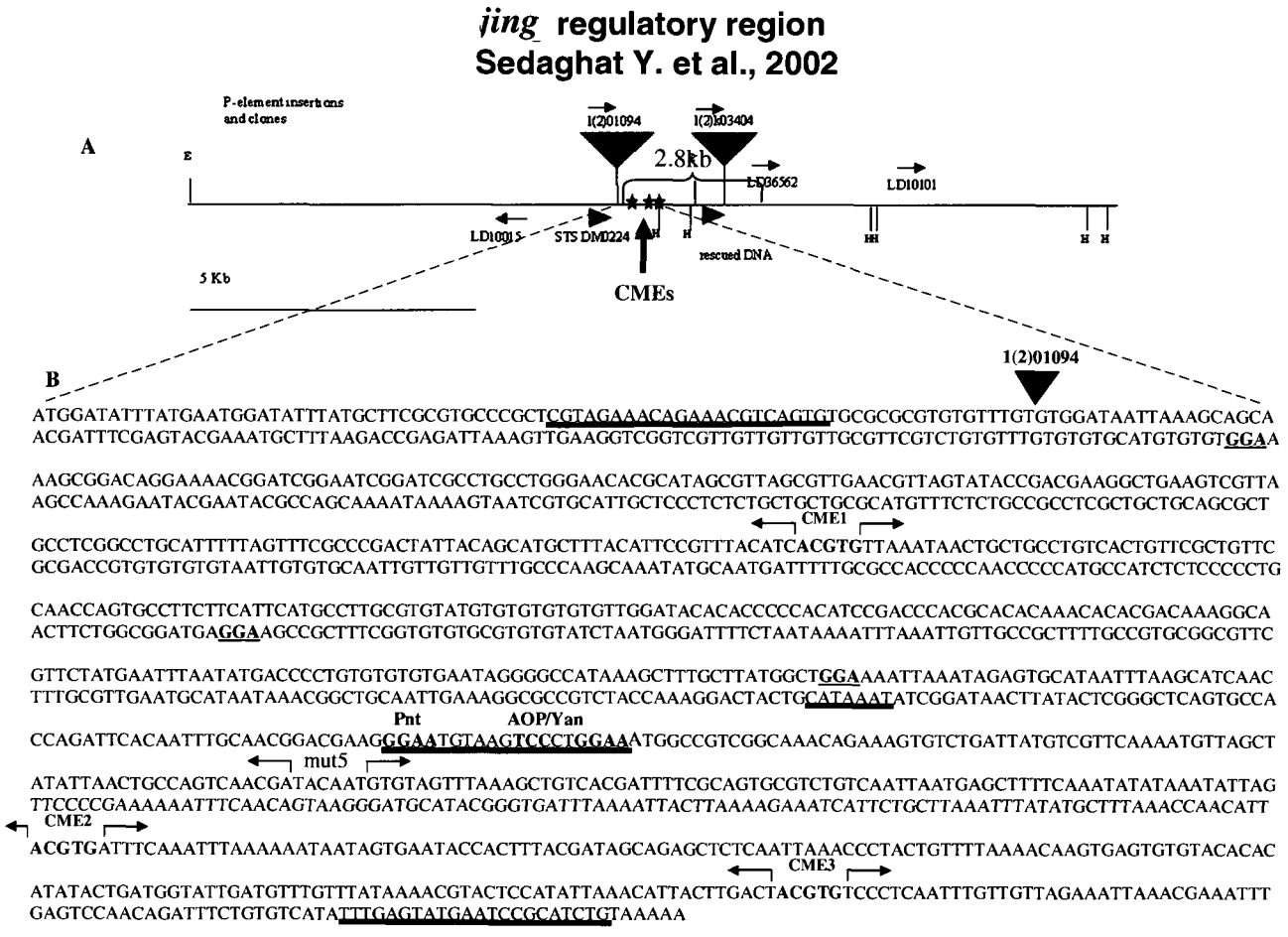


Fig. 14. (A) The extent of the *jing* 2.8kb element is shown. (B) The *jing* 1.5 regulatory region (Sedaghat Y. et. al., 2002). DNA binding sites of bHLH-PAS (CMEs), SOX HMG protein Dichaete (TACAAT), and Pnt/Aop (5'GGA; underlined in black) are present in the *jing* 5' regulatory region. The fragment containing Pnt and Aop binding sites with inverted repeat is underlined in green. Consensus binding site CATAAAT of the transcription factor Drifter is underlined in blue. Sequences used to create reverse and forward primers for the amplification of the *jing* 1.5kb fragment are underlined in red.

Fig.15. 2.8kb of *jing* 5′ *cis*-regulatory sequence. Sequences used to create primers for 1.5kb fragment are underlined in red. Sequences used to create primers for 2.8kb fragment are underlined in green. Sequences used to create primers for 1.3kb fragment are underlined in black.

5′ cttctg ctgccgcctc ttttttggat aaatggatat ttatgcttcg

cgtgcccgct **cgtagaaaca gaaacgtcag tg**tgcgcgcg tgtgtttgtg tggataatta

aagcagcaac gatttcgagt acgaaatgct ttaagaccga gattaaagtt gaaggtcggt

cgttgttgtt gttgcgttcg tctgtgtttg tgtgtgcatg tgtgtggaaa agcggacagg

aaaacggatc ggaatcggat cgcctgcctg ggaacacgca tagcgttagc gttgaacgtt

agtataccga cgaaggctga agtcgttaag ccaaagaata cgaatacgcc agcaaaataa

aagtaatcgt gcattgctcc ctctctgctg ctgcgcatgt ttctctgccg cctcgctgct

gcagcgctgc ctcggcctgc atttttagtt tcgcccgact attacagcat gctttacatt

ccgtttacat cacgtgttaa ataactgctg gctgtcactg ttcgctgttc gcgaccgtgt

gtgtgtaatt gtgtgcaatt gttgttgttt gcccaagcaa atatgcaatg atttttgcgc

caccccaac ccccatgcca tctctccccc tgcaaccagt gccttcttca ttcatgcctt

gcgtgtatgt gtgtgtgtgt tggatacaca cccccacatc cgacccacgc acacaaacac

acgacaaagg caacttctgg cggatgagga agccgctttc ggtgtgtgcg tgtgtatcta

atgggatttt ctaataaaat ttaaattgtt gccgcttttg ccgtgcggcg ttcgttctat

gaatttaata tgacccctgt gtgtgtgaat aggggccata aagctttgct tatggctgga

aaattaaata gagtgcataa tttaagcatc aactttgcgt tgaatgcata ataaacggct

gcaattgaaa ggcgccgtct accaaaggac tactgcataa atatcggata acttatactc

gggctcagtg ccaccagatt cacaatttgc aacggacgaa gggaatgtaa gtccctggaa

atggccgtcg gcaaacagaa agtgtctgat tatgtcgttc aaaatgttag ctatattaac

tgccagtcaa cgatacaatg tgtagtttaa agctgtcacg attttcgcag tgcgtctgtc

aattaatgag cttttcaaat atataaatat tagttccccg aaaaaatttc aacagtaagg

gatgcatacg ggtgatttaa aattacttaa aagaaatcat tctgcttaaa tttatatgct

ttaaaccaac attacgtgat ttcaaattta aaaaataata gtgaatacca ctttacgata

gcagagctct caattaaacc ctactgtttt aaaacaagtg agtgtgtaca cacatatact

gatggtattg atgtttgttt ataaaacgta ctccatatta aacattactt gactacgtgt

ccctcaattt gttgttagaa attaaacgaa atttgagtcc aacagatttc tgtgtcatat

ttgagtatga atccgcatct gtaaaaaaaa agcagcgcta aatgcacgaa aacaaaaatg

gtcggggtcg tccaccccat taaaaaatta cacaaatgca ggagaaaaat ggacgtaata

ggcttatcac aaaagcagtt tggtcgtcgt aatctgtggt atctcccggg gtaggcaaac

ataaaataaa atgtgttttc atttcatgag ttttcttttt gttgtgcttt gtacatagat

ctaaaatttg agtaggtaga acttggatgt gattaaaaat gtttcacgga aggccgacgg

aaaatacaaa accctgaaaa tcgctgtcca caaagatttc gggtctagaa tccaagctcg

actaaacgat ttattttaac gatcgagtaa agcgtctcta tagctaaaga ttaaaaaaaa

tatttttac atatttgttt gcaattgcaa cttctacata tgtacatatg tgaaagacaa

tatgtagaaa gcacgcttgc ttcgttttag ttattcaatt gatggataaa tggcattgta

tttattctgc acttatgtat tgtatattta aagtttttag cgttttaaat tcatttttta

ataagcttgt aaacttttgc gtgttccaaa agtgaaaaag tatcatctat ttgtatatct

tttcgagcac caaaatagca gaccgaattc aaaaataccc tttttgtgta accagaggat

tttttgtata cttgccgacg tttatcccgt ttcacatgta catgtgttta accgaaaccg

cacaaacaca cacgcgactt ggggctacct gaaactgaaa ttttcccgca ttagattgcg

gcccttgcct tccatgcttc gtcttcttct tcttctttct catcgttgtt tctgtaaata

aagaaagatg tggagaggga gtttttttctt cctttacgtt cgctctttct tccagttttt

tttgtttact ttttattact tttcatgttc tcttccacga aaattagtgc gaaacagaaa

cgaattgctt agcgtttatc gacccgtttt gtgttctctt attctgttgc gctcttctcc

ttctcttgtt cgctctcttg aaacaataat ttccctattt ctcgtgtaaa atgaatacat

gtttttttaca gttattcaaa attaaaatat tttgtatcac aaacacggag taaatcgatt

attttaagat taattttaaa aatgttttgc gcgcatgttg ttgaatttaa tttcgcagct

gaatgccttg tgagttctct tattctcttt **agctcttctc cttctcttgt tc**ggtctctt

gcaaccatat tttccttatt tctcgtgtaa aatgaatata tgtttttttа cagtttctct

aaattaaaat aatttctatc acatcatcga gtaaatcgat tttttaaaga ttaattttaa

aattgttgct tgcgcgcctc ttattgaatt tagtttcata gctgcttgcc ttttactgcg

ggttgttttg ggaaatttat cagctgttag ccgcgctgtt agaggtaaca gcgcttgcga

cggacattct ctaacacagc cgggaaataa acatccaaga ataatttgag tggccttcca

acactgccag gcaaataaac 3'

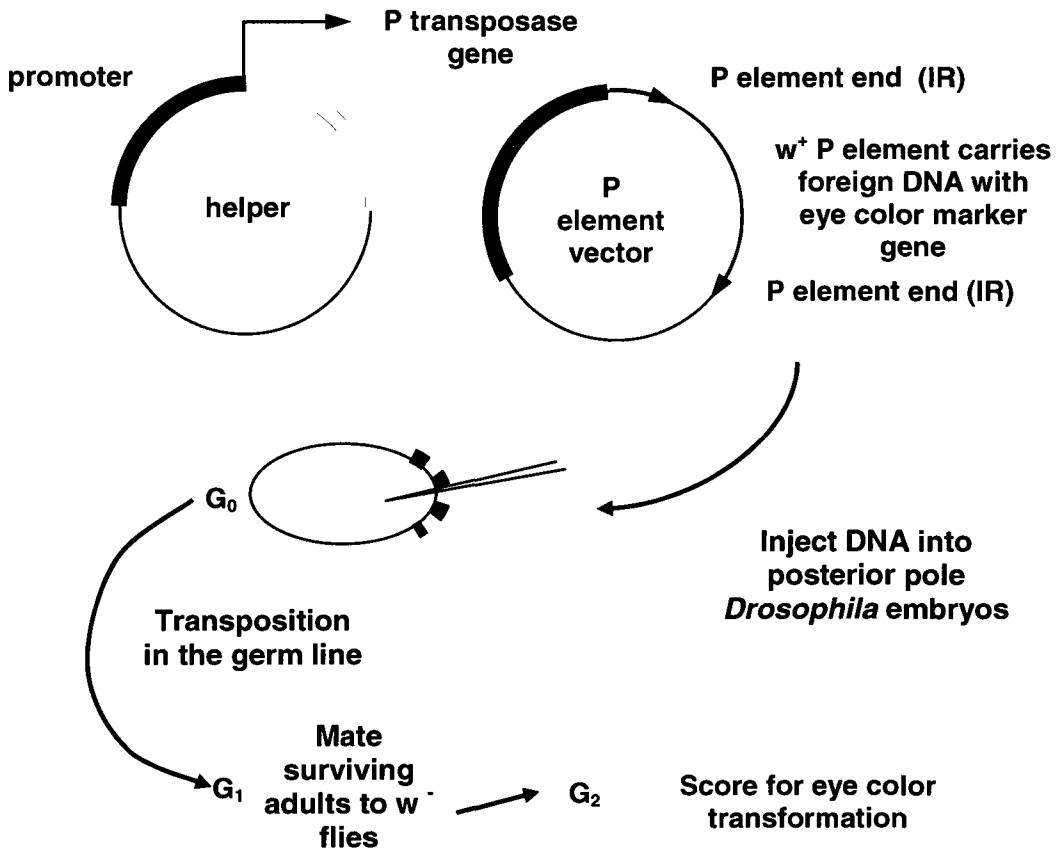


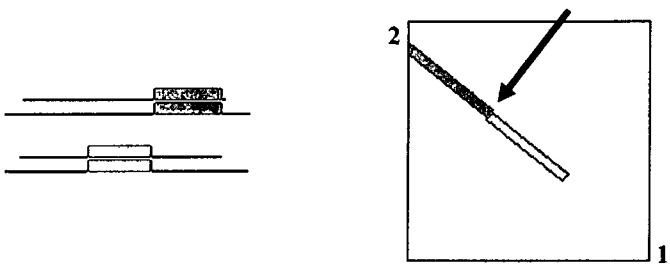
Fig.16. Injection procedure. P element-mediated germ line transformation. Outline of the method for germ line transformation of *Drosophila* with P element vectors. Two plasmids, one encoding the P element transposase protein but lacking P element ends and the second plasmid carrying a foreign DNA segment and an eye color marker gene (w^+) within P element ends, are injected into posterior pole of preblastoderm embryos. The transposase plasmid enters nuclei of germ line cells, is expressed, and leads to transposition of the P element from the second plasmid into *Drosophila* germ line chromosomes. After development of the injected embryos (G_0 generation), the surviving adults are mated to w^- flies (G_1 generation), and the progeny from this cross (G_2 generation) are scored for the red eye color (RubinG.M. and Spradling A.C., 1983).

Fig. 17. Blast results for the deletion cloning experiment. (A) The colonies showing blue color on Amp$^+$/IPTG/X-gal plates were sequenced and aligned using Blast NCBI against the 2.8kb from *jing* 5′ genomic sequence. Arrow points at the site of the deletion. (B) The fragment following the deleted site. (C) The fragment preceding the deletion site. The sequencing reaction done using the reverse KpnI primer used for the PCR amplification of the initial 1.5kb *jing*5' regulatory fragment.

A

Sequence 1: lcl|1_seq_1
Length = 1408 (1 .. 1408)

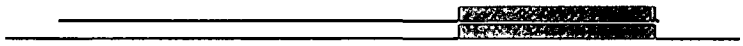
Sequence 2: lcl|2_seq_2
Length = 1726 (1 .. 1726)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE·If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

B

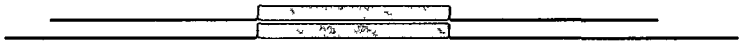


```
Score =  873 bits (454),  Expect = 0.0
Identities = 463/465 (99%), Gaps = 1/465 (0%)
Strand=Plus/Minus
```

```
Query  67    ATGTTTAATATGGAGTACGTTTTATAAACAAACATCAATACCATCAGTATATGTGTGTAC  126
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1471  ATGTTTAATATGGAGTACGTTTTATAAACAAACATCAATACCATCAGTATATGTGTGTAC  1412

Query  127   ACACTCACTTGTTTTAAAACAGTAGGGTTTAATTGAGAGCTCTGCTATCGTAAAGTGGTA  186
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
Sbjct  1411  ACACTCACTTGTTTTAAAACAGTAGGGTTTAATTGAGAGCTCTGCTATCGTAAAGTGGTA  1352

Query  187   TTCACTATTATTTTTTAAATTTGAAATCACGTAATGTTGGTTTAAAGCATATAAATTTAA  246
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1351  TTCACTATTATTTTTTAAATTTGAAATCACGTAATGTTGGTTTAAAGCATATAAATTTAA  1292

Query  247   GCAGAATGATTTCTTTTAAGTAATTTTAAATCACCCGTATGCATCCCTTACTGTTGAAAT  306
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1291  GCAGAATGATTTCTTTTAAGTAATTTTAAATCACCCGTATGCATCCCTTACTGTTGAAAT  1232

Query  307   TTTTTCGGGGAACTAATATTTATATATTTGAAAAGCTCATTAATTGACAGACGCACTGCG  366
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1231  TTTTTCGGGGAACTAATATTTATATATTTGAAAAGCTCATTAATTGACAGACGCACTGCG  1172

Query  367   AAAATCGTGACAGCTTTAAACTACACATTGTATCGTTGACTGGCAGTTAATATAGCTAAC  426
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1171  AAAATCGTGACAGCTTTAAACTACACATTGTATCGTTGACTGGCAGTTAATATAGCTAAC  1112

Query  427   ATTTTGAACGACATAATCAGACACTTTCTGTTTGCCGACGGCCAT  471
              |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1111  ATTTTGAACGACATAATCAGACACTTTCTGTTTGCCGACGGCCAT  1067
```

C



```
 Score =  806 bits (419),  Expect = 0.0
 Identities = 446/447 (99%), Gaps = 0/447 (0%)
 Strand=Plus/Minus

Query  472   CTTCGTCCGTTGCAAATTGTGAATCTGGTGGCACTGAGCCCGAGTATAAGTTATCCGATA  531
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1047  CTTCGTCCGTTGCAAATTGTGAATCTGGTGGCACTGAGCCCGAGTATAAGTTATCCGATA  988

Query  532   TTTATGCAGTAGTCCTTTGGTAGACGGCGCCTTTCAATTGCAGCCGTTTATTATGCATTC  591
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  987   TTTATGCAGTAGTCCTTTGGTAGACGGCGCCTTTCAATTGCAGCCGTTTATTATGCATTC  928

Query  592   AACGCAAAGTTGATGCTTAAATTATGCACTCTATTTAATTTTCCAGCCATAAGCAAAGCT  651
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  927   AACGCAAAGTTGATGCTTAAATTATGCACTCTATTTAATTTTCCAGCCATAAGCAAAGCT  868

Query  652   TTATGGCCCCTATTCACACACACAGGGGTCATATTAAATTCATAGAACGAACGCCGCACG  711
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  867   TTATGGCCCCTATTCACACACACAGGGGTCATATTAAATTCATAGAACGAACGCCGCACG  808

Query  712   GCAAAAGCGGCAACAATTTAAATTTTATTAGAAAATCCCATTAGATACACACGCACACAC  771
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  807   GCAAAAGCGGCAACAATTTAAATTTTATTAGAAAATCCCATTAGATACACACGCACACAC  748

Query  772   CGAAAGCGGCTTCCTCATCCGCCAGAAGTTGCCTTTGTCGTGTGTTTGTGTGCGTGGGTC  831
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  747   CGAAAGCGGCTTCCTCATCCGCCAGAAGTTGCCTTTGTCGTGTGTTTGTGTGCGTGGGTC  688

Query  832   GGATGTGGGGGTGTGTATCCAnnnnnnnnnnnnnnTACACGCAAGGCATGAATGAANAAGG  891
              |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  687   GGATGTGGGGGTGTGTATCCAACACACACACACATACACGCAAGGCATGAATGAAGAAGG  628
```

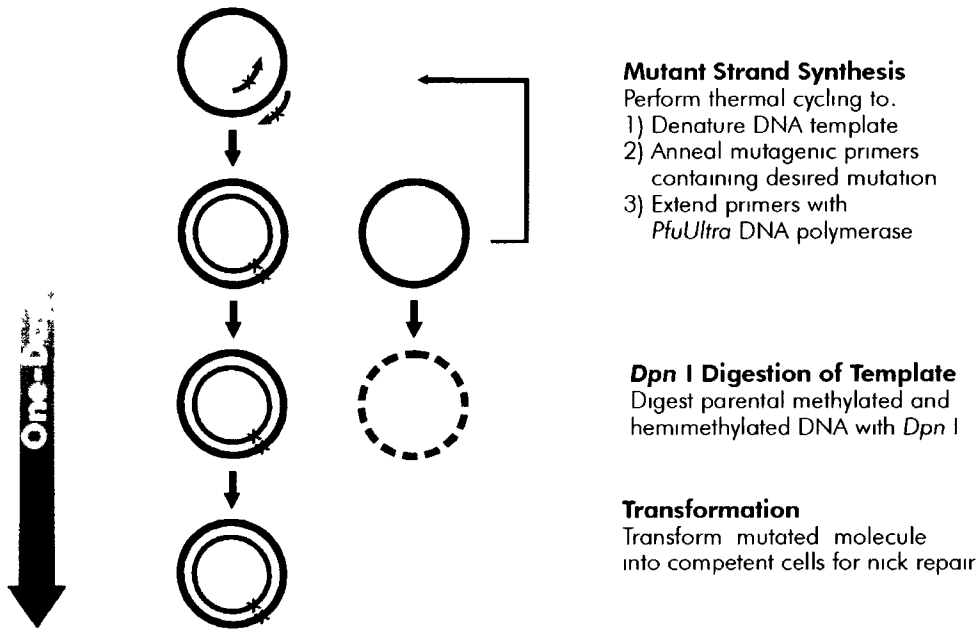


Fig.18. Deletion cloning strategy.

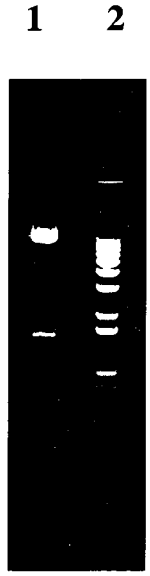


Fig.19. Lane 1 – recombinant Casper1.5kb.fragmentΔPnt/Aop DNA digested with BamHI. Lane 2 – 1kb "+" DNA ladder.

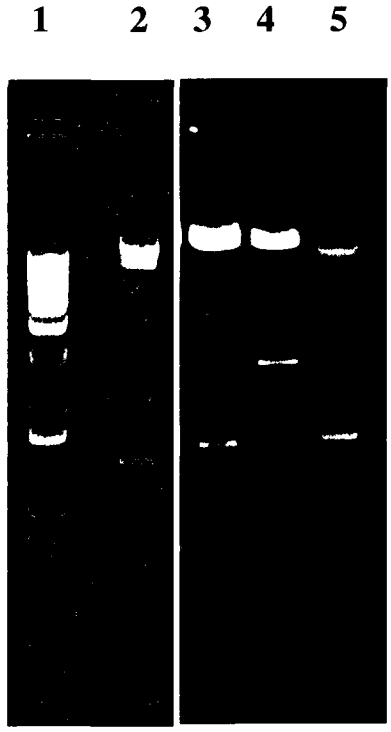


Fig.20. Restriction digest (BamHI) of the recombinant pCasper constructs containing 1.3kb, 1.5kb, and 2.8kb *jing*5′ regulatory sequences. Lane 1 - 1kb "+" DNA ladder, lane 2 - recombinant pCasper1.3kbjing5′ construct digested BamHI, lane 3 – recombinant pCasper1.5kbjing5′ construct digested BamHI; lane 4 - recombinant pCasper2.8kbjing5′ construct digested BamHI; lane 5 – 1kb "+" DNA ladder.

RESULTS

1. Expression pattern of *jing-enhancer-lacZ* reporter constructs

1.1. Embryonic expression pattern of the *jing1.5-lacZ* reporter construct

To investigate the regulation of *jing* expression, we generated a *lacZ* reporter fusion with a 1.5kb fragment from the *jing* 5' *cis*-regulatory region (Fig.14). This fragment of the regulatory region was selected because it includes three putative Trh::Tgo DNA binding sites (CNS midline elements (CMEs, Crews S, 1998; Ohshiro T. and Saigo K., 1997) of the sequence ACGTG) and POU- and ETS-domain binding sites all of which regulate tracheal *btl* expression (Fig.13). Due to the presence of the three CMEs in the *jing* regulatory region it was originally proposed that *jing* is a downstream target of Trh::Tgo (Sedaghat Y. et al, 2002) while Dfr and Pnt are implicated in combinatorial regulation of *btl* expression (Boube M. et.al., 2000) and maybe required for combinatorial regulation of *jing* expression.

The 1.5 kb fragment of the *jing* 5'*cis*-regulatory region was amplified by PCR from *Drosophila* w^{1118} genomic DNA and cloned into the pCasper vector (Fig.20). The pCaSpeRhs43βgal vector was chosen because it contains the *lacZ* reporter gene transcribed from a minimal promoter including the *hsp*70 TATA box allowing for the visualization of *lacZ* expression by anti-β-gal staining. Transgenic lines were generated by injection of the recombinant DNA into *Drosophila* embryos using standard procedures (Rubin G.M. and Spradling A.C., 1982) and whole mount embryos from three resultant independent transgenic lines were tested for *lacZ* expression.

During primary branching in the trachea, *jing1.5-lacZ* is expressed in three rows of cells identified by anti-Tgo as transverse connectives (TC), dorsal trunk (DT) and the tips of the dorsal branches (DB) and anterior lateral trunks (LTa) (Fig.21). Most of *jing1.5-lacZ* expressing cells in these branches were identified as fusion cells by immunostaining with anti-Dysfusion (anti-Dys) and anti-β-gal (Fig.22, 26D-D''). *jing1.5-lacZ* expression in the fusion cells was also detected by their positioning by double labeling

with anti-Tgo and anti-β-gal (Fig.23, dorsal branch fusion cells). The early stages of tracheal development (stage 11) exhibited *jing1.5-lacZ* expression in the cells adjacent to the tracheal pits (Fig.24, 25) similar to endogenous Jing protein (Sedaghat Y. et.al., 2002). These cells may express the chemoattractant Branchless which guide budding tracheal branches (Sutherland D. et al., 1996). However, the absence of *jing1.5-lacZ* expression in the tracheal pits differs from that of endogenous Jing (Sedaghat Y. et al., 2002).

The *jing1.5-lacZ* reporter is also expressed in the CNS midline (Fig. 26A,B; Fig. 27). *jing1.5-lacZ* expression in the CNS midline was detected by immunostaining with anti-β-gal antibody (Fig.26A) or double labeling with anti-Tgo and anti-β-gal antibodies (Fig.27). Our data support previous results indicating *jing-lacZ* enhancer trap expression in the CNS midline, the presence of *jing* transcripts in the CNS midline detected via *in situ* hybridization with a *jing* DNA probe, and anti-JING immunostaining of CNS midline cells (Sedaghat Y. et.al, 2002).

During later stages of development, *jing1.5-lacZ* is strongly expressed in the CNS (Fig.26C, arrow) and CNS neuronal bodies (Fig.28). Double labeling with mouse monoclonal antibody to the anti-fasciclin (1D4) cell adhesion molecule and rabbit polyclonal anti-β-gal shows *jing1.5-lacZ* expression in the CNS neuronal bodies (Fig.28). Fasciclin (FasII) is expressed in axons and neuronal cell body membranes.

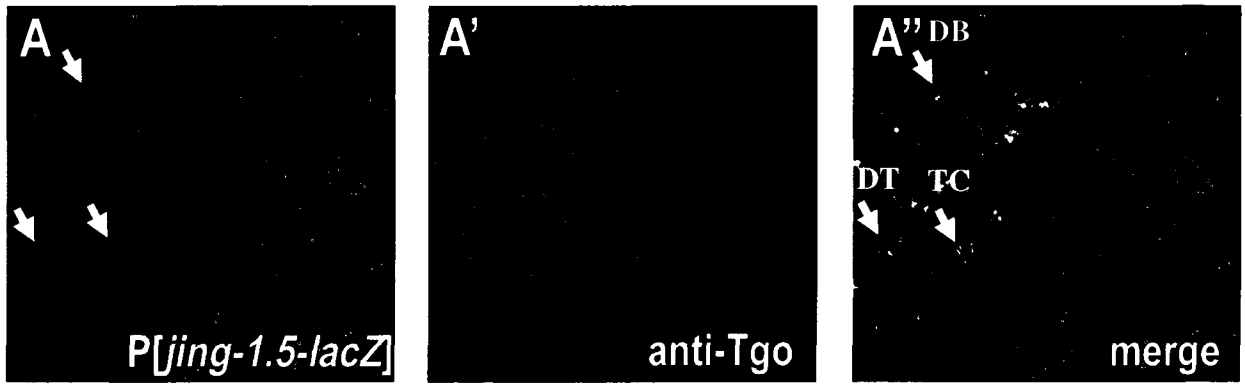


Fig.21. *jing1.5-lacZ* is expressed in three rows of cells in the trachea: dorsal branch (DB), dorsal trunk (DT), and transverse connectives (TC). Panels show confocal images of stage 15 embryos carrying *jing1.5-lacZ* and double labeled with anti-β-gal (AA", green) and anti-Tgo (A'A", red). The embryo is shown in sagittal view with posterior bottom left. The arrows indicate dorsal trunk, dorsal branch, and transverse connectives where *lacZ* expression was detected.

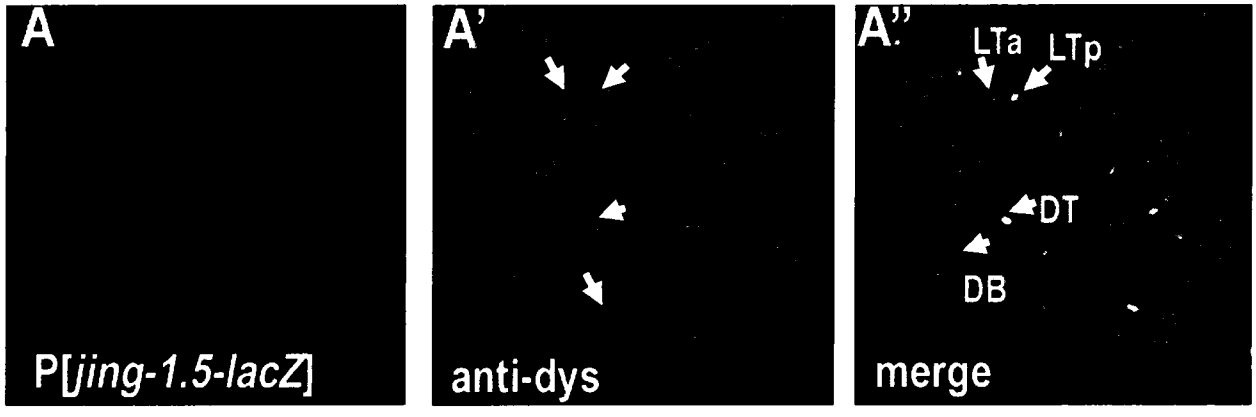


Fig.22. *jing1.5-lacZ* is expressed in all branch specific fusion cells. Panels show confocal images of embryos carrying *jing1.5-lacZ* double labeled with anti-β-gal and anti-dys (anti-dysfusion) (A-A"). An embryo of stage 14 is shown in sagittal view with posterior to the right. Arrows indicate lateral trunk anterior (LTa), lateral trunk posterior (LTp), dorsal trunk (DT) and dorsal branch (DB) fusion cells.

Fig.23. *jing1.5-lacZ* expression in strongest in the dorsal branch fusion cells. Panels show confocal images of embryos carrying *jing1.5-lacZ* double labeled with anti-β-gal (green) and anti-Tgo (red) (A-A″). Embryo is shown in dorsal view with posterior to the right. Arrowhead indicates dorsal branch fusion cell.

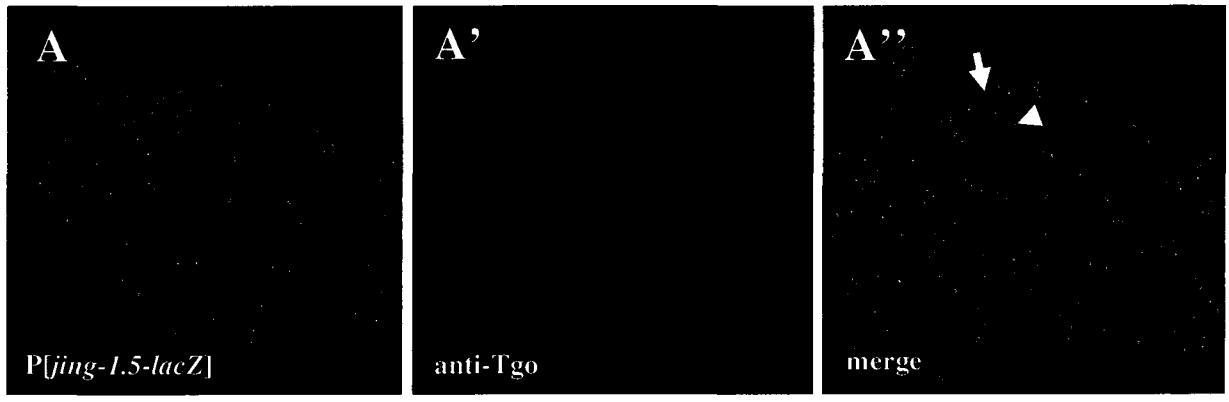


Fig.24. *jing1.5-lacZ* is expressed in the cells adjacent to the tracheal pits during the early stages of tracheal development. Panels show confocal images of embryos carrying *jing1.5-lacZ* double labeled with anti-β-gal (green) and anti-Tgo (red) (A-A″). This stage 12 embryo is shown in sagittal view with posterior to the right. Arrowhead indicates the cells adjacent to the pits expressing *jing1.5-lacZ.* Arrow points at the tracheal pit.

Fig.25. *jing1.5-lacZ* expression during the early stages of tracheal development. A confocal image of the trachea in sagittal view with anterior to the left is shown. Arrowhead indicates cells expressing β-gal (green) and the arrow shows pit cells stained with anti-Tgo (red).

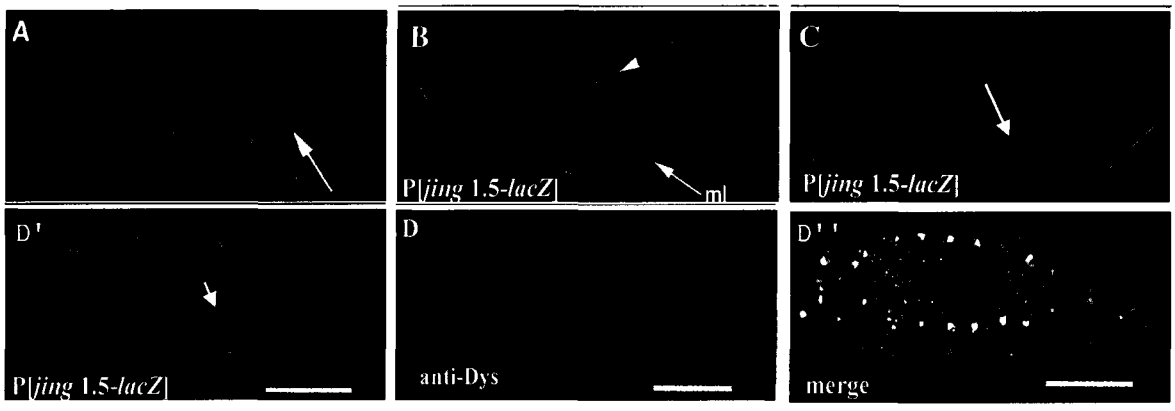


Fig.26. Embryonic expression pattern of *jing1.5-lacZ.* Panels show confocal images of embryos carrying *jing1.5-lacZ* stained with monoclonal mouse anti-β-gal (A, B, C, red) and embryos double labeled with anti-Dys (Dys) (D-D″, green). Embryos are shown with anterior to the left and in sagittal (B, C) and dorsal (A, D-D″) views. (A) *jing1.5-lacZ* is expressed in the CNS midline (arrow) during stage 10. (B) *jing1.5-lacZ* is expressed in the CNS midline (ml, arrow) and lateral ectoderm (arrowhead) during stage 13. (D-D″) *jing1.5-lacZ* is also expressed in fusion cells in the dorsal branch (small arrow). (C) Later stages show strong expression in the CNS (arrow). Scale bars, 50 μm.

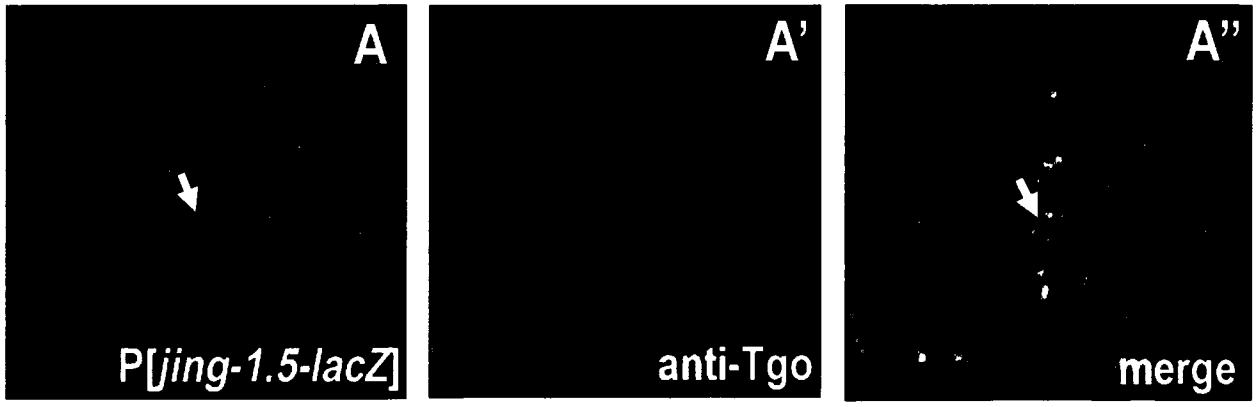


Fig.27. *jing1.5-lacZ* expression in the CNS midline cells. Panels show confocal images of embryos carrying *jing1.5-lacZ* and double labeled with anti-β-gal (A, red) and anti-Tgo (B, green). Stage 14 embryo is shown in ventral view with anterior up. Arrows indicate the CNS midline.

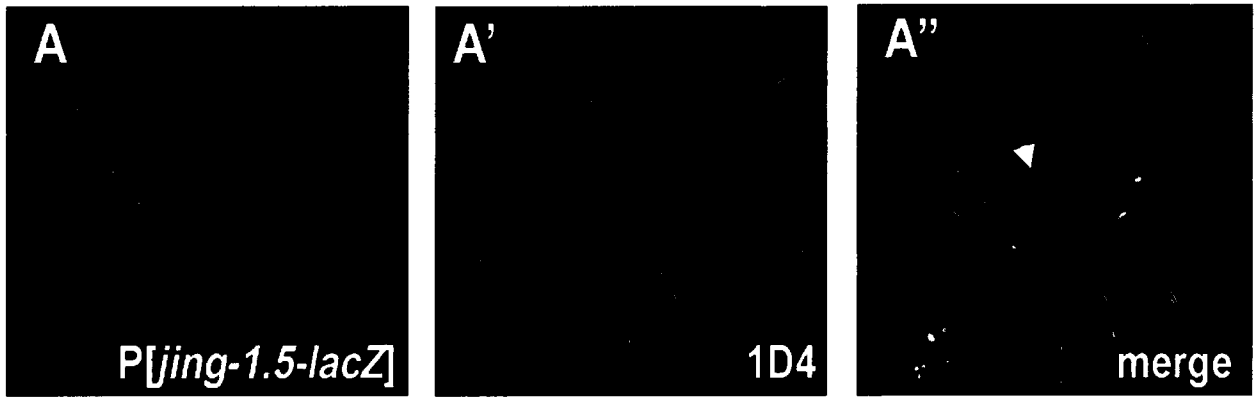


Fig.28. *jing1.5-lacZ* expression in the CNS neuronal bodies. Panels show confocal images of embryos carrying *jing1.5-lacZ* double labeled with anti-β-gal and axonal marker anti-FasII (1D4). Embryo is shown in ventral view (A-A''). Arrowhead points at the CNS neuron.

1.2. Expression pattern of the *jing2.8-lacZ* reporter construct

The early stages of tracheal development (stage 10-12) did not exhibit *jing1.5-lacZ* expression in the tracheal cells. During subsequent branching stages, *jing1.5-lacZ* expression was detected mostly in all branch specific fusion cells. To determine if any additional tracheal enhancers are present in the larger 2.8 kilobase (kb) fragment (see Fig.14 and 15) of the *jing* 5' regulatory region, we studied expression of a *lacZ* reporter under control of the 2.8kb *jing* regulatory region. The 2.8 kb fragment is adjacent to the *jing* open reading frame (Fig.14). We hypothesized that additional sites might be responsible for the activation of *jing* expression during the early stages of embryogenesis, such as in the tracheal placodes and in more lineages during primary branching.

The 2.8 kb sequence from the *jing* 5' regulatory region was amplified by PCR from *Drosophila* w^{1118} genomic DNA and cloned into the pCaSpeRhs43βgal vector (Fig.20). Transgenic lines were generated by injection of recombinant DNA into *Drosophila* embryos using standard procedures (Spradling A.C. and Rubin G.M., 1982). Three independent transgenic lines were tested to minimize experimental error due to random incorporation of the insertion into the fly genome. Embryonic progeny collected from all *jing2.8-lacZ* transgenic lines showed *lacZ* expression in the CNS midline (Fig.29) and in the trachea (Fig.30) as detected by immunostaining with anti-β-gal and anti-Tango.

The *jing2.8-lacZ* expression pattern in the trachea was significantly different from *jing1.5-lacZ* expression and it resembled more closely Jing protein localization, and that of a previously characterized l(2)01094 enhancer trap (Sedaghat Y. et.al., 2002). During tracheal branching stages, *jing2.8-lacZ* expression was detected in all tracheal branches including the transverse connectives, visceral branch, ganglionic branches as well as in the anterior and posterior lateral trunks (Fig.30B-B″, C-C″) as confirmed by anti-Tgo double labeling. *jing2.8-lacZ* expression also included Dys-positive fusion cells (Fig.30). Most interestingly, expression was present during early stages (stage 12) in the tracheal pits (Fig.30A-A″) as confirmed by anti-Tgo double staining. *jing2.8-lacZ* expression in the early tracheal pits is consistent with

endogenous Jing protein localization (Sedaghat Y. et.al., 2002). An additional expression pattern of *jing2.8-lacZ* was observed in the ectodermal stripes (Fig.33) similar to endogenous Jing (Sedaghat Y. et.al., 2002), and in posterior spiracles (Fig.32).

To conclude, the pattern of *jing2.8-lacZ* expression suggests that additional binding sites present in this specific *jing* regulatory region are involved in the early activation of *jing* expression. However, the additional sequences in the 2.8kb fragment do not contain CMEs. Our data therefore suggest that factors other than Trh::Tgo are required to activate early *jing* expression in the trachea. The additional sequences in *jing2.8*, not present in *jing1.5*, are also required for expression in all tracheal branches in non-fusion type cells. Either (1) Trh/Tgo heterodimers regulate *jing* in combination with unidentified transcription factors with DNA binding sites in the additional regulatory sequences or (2) *jing* is activated only by an unidentified transcription factor(s). The combinatorial nature of transcriptional regulation involving CME DNA binding sites has been shown in the *Drosophila* CNS midline and trachea (Ohshiro and Saigo, 1997; Ma et al, 2000).

Fig.29. *jing2.8-lacZ* is expressed in the CNS midline. Panels show confocal images of embryos carrying *jing2.8-lacZ* double labeled with anti-β-gal (green) and anti-Tgo (red) (A-A''). *jing2.8-lacZ* (A, stage 10) is expressed in the CNS midline (arrowhead) of a stage 10 embryo. Anterior is to the right in ventral view.

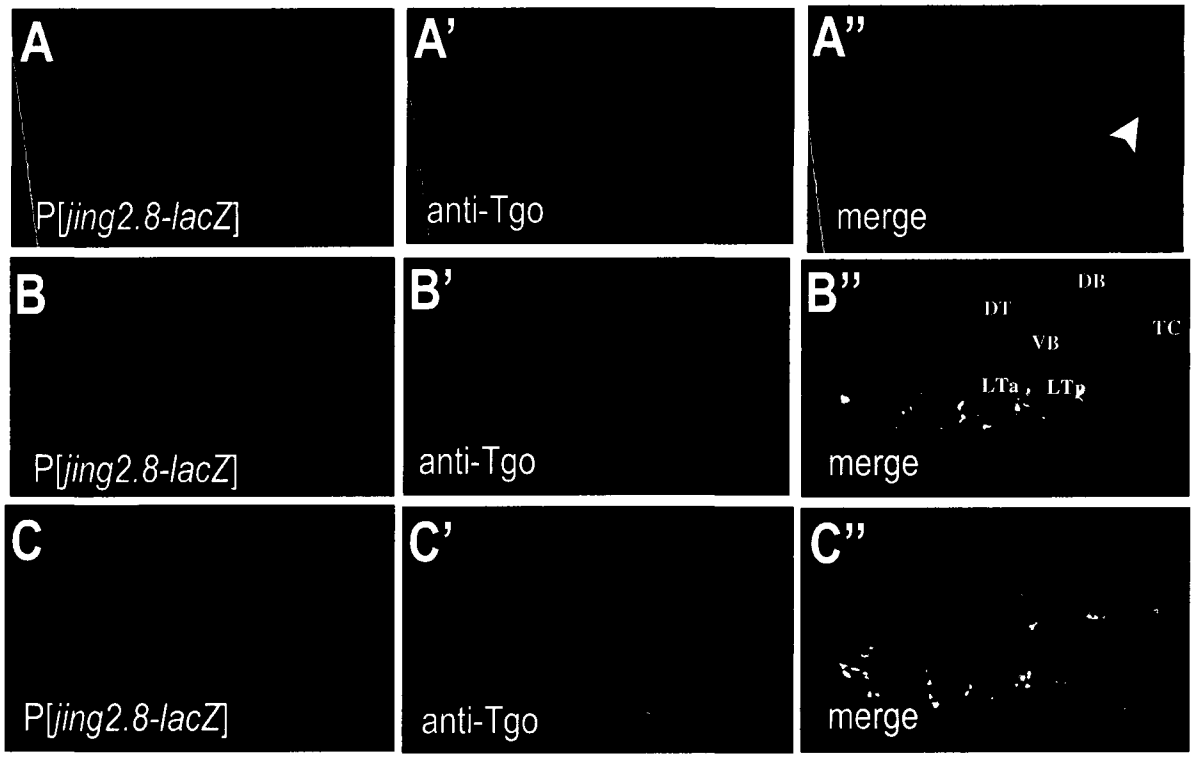


Fig.30. *jing2.8-lacZ* is expressed in all tracheal cells throughout all embryonic stages of tracheal development. Panels show confocal images of embryos carrying *jing2.8-lacZ* double labeled with anti-β-gal (green) and anti-Tgo (red). Embryos are shown with anterior to the left and in sagittal views. *jing2.8-lacZ* is expressed in tracheal pits during stage 12 (A-A″, arrowhead). During stage 15 (B-B″, C-C″), *jing2.8-lacZ* is expressed in all the parts of the trachea (B-B″, C-C″). Dorsal trunk (DT), dorsal branch (DB), visceral branch (VB), lateral trunk anterior (LTa), lateral trunk posterior (LTp), and transverse connective (TC).

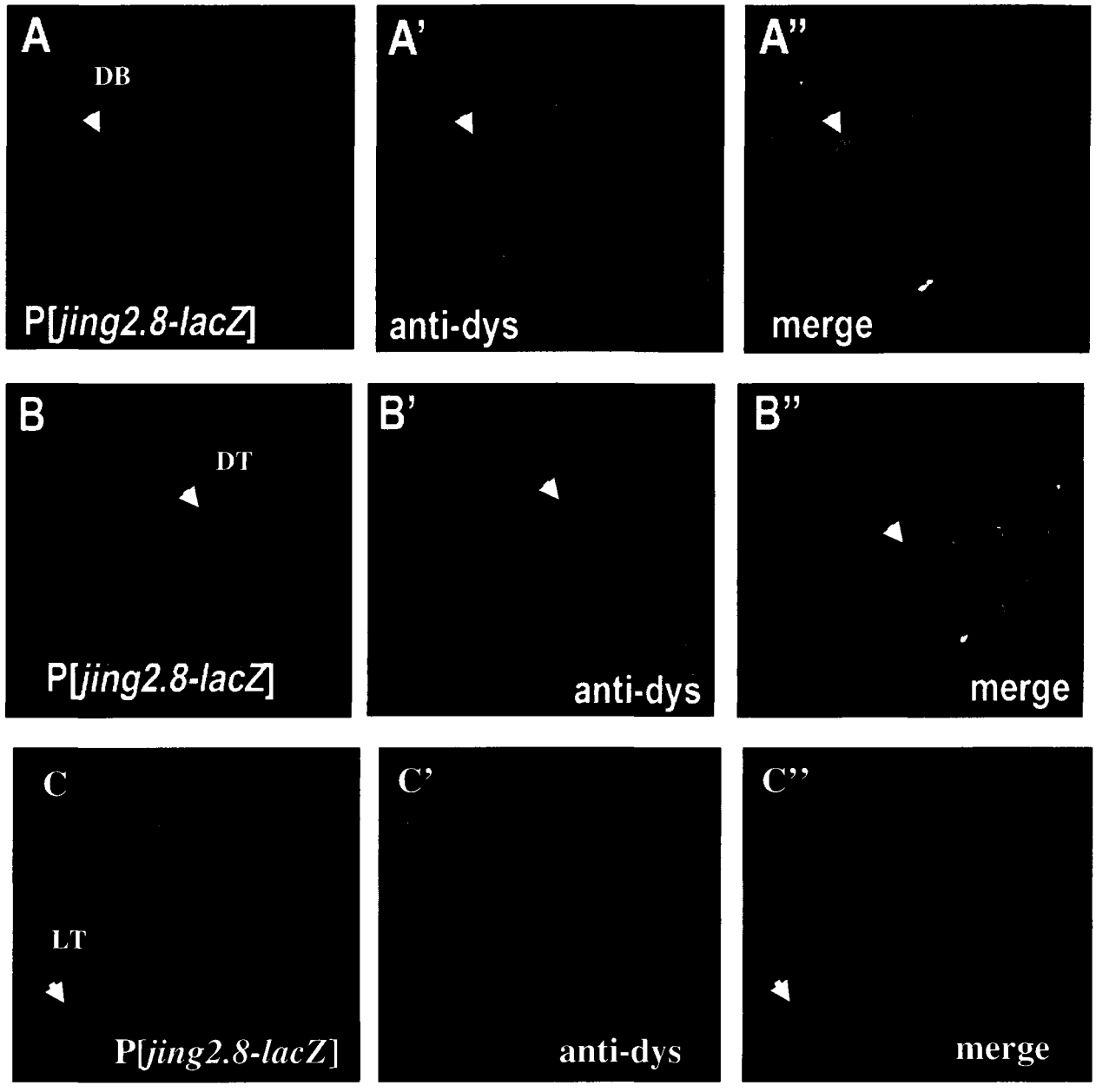


Fig.31. *jing2.8-lacZ* expression in all branch specific fusion cells. Panels show confocal images of embryos carrying *jing2.8-lacZ* double labeled with anti-β-gal (red) and anti-Dys (green) (anti-Dysfusion). (A-A″) A stage 16 embryo is shown in dorsal view with posterior to the right. Arrowhead indicates dorsal branch fusion cells. (B-B″) A stage 14 embryo is shown in sagittal view with anterior down. Arrowheads indicate dorsal trunk fusion cell. (C-C″) A stage 12 embryo is shown in sagittal view anterior left. Arrowhead indicates lateral trunk posterior fusion cell.

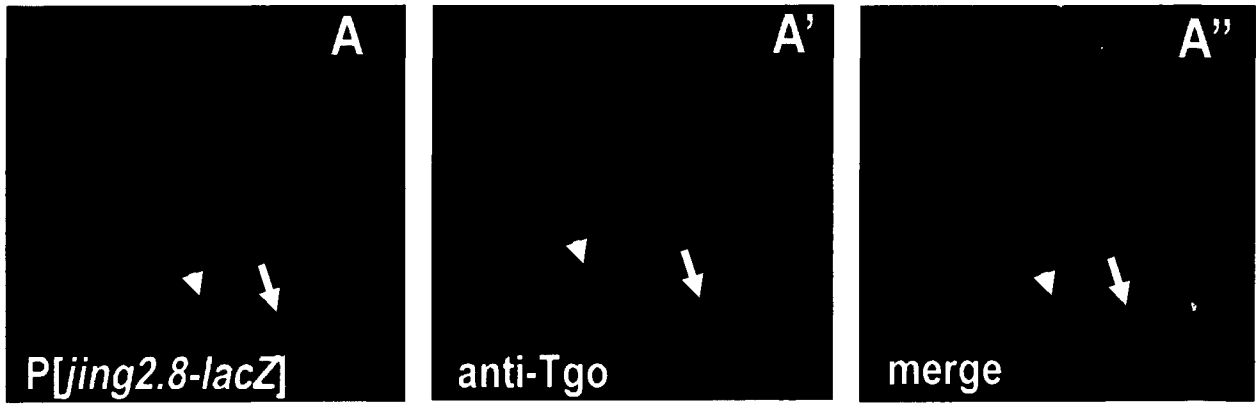


Fig.32. *jing2.8-lacZ* expression in the fusion cells and posterior spiracles. Panels show confocal images of embryos carrying *jing2.8-lacZ* and double labeled with anti-β-gal (green) and anti-Tgo (red). A stage 15 embryo is shown with anterior to the left and in dorsal view. *jing2.8-lacZ* is expressed in the fusion cells (arrowhead) and in posterior spiracles (arrow).

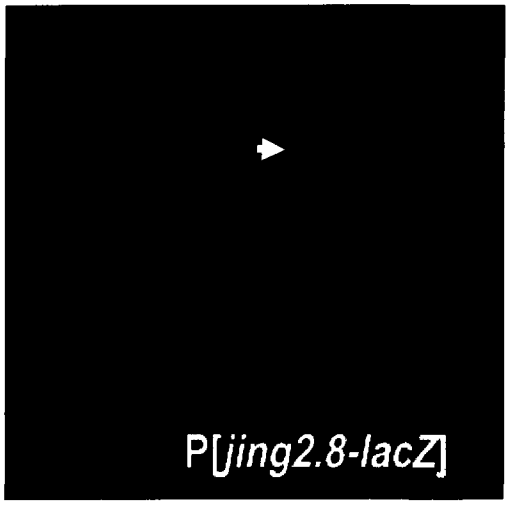


Fig.33. *jing2.8-lacZ* expression in the early ectodermal stripes. A stage 10 embryo stained with anti-β-gal is shown in sagittal view with anterior down. Arrowhead indicates ectodermal stripe.

Expression pattern of the *jing1.3-lacZ* reporter construct

Since the 1.5kb fragment does not recapitulate the *jing* expression pattern, but the 2.8kb fragment does, and to determine if any additional activation sites are present in the 1.3 kb fragment (see Fig.15) of the *jing* 5' regulatory region we used *in vivo* enhancer studies. We hypothesized that these sites might be responsible for the activation of *jing* expression in the trachea and in segmental ectodermal stripes, because the 1.5 kb fragment didn't activate expression in these cells whereas *jing2.8-lacZ* did.

A 1.3 kb sequence from the *jing* 5' regulatory region was amplified by PCR from *Drosophila* w^{1118} genomic DNA and cloned into the pCaSpeRhs43βgal vector. Transgenic lines were generated by injection of recombinant DNA into *Drosophila* embryos using standard procedures (Spradling A.C. and Rubin G.M., 1982). Three independent transgenic lines were tested to minimize experimental error due to random incorporation of the insertion into the fly genome.

All *jing1.3-lacZ* transgenic lines showed *lacZ* expression in segmental ectodermal stripes (Fig.34) as detected by immunostaining with anti-β-gal and anti-Engrailed. Engrailed is a homeodomain-containing protein (Poole et al., 1985) that binds to DNA with sequence specificity similar to that of several other homeodomain transcription factors (Desplan et al., 1988). In *Drosophila*, Engrailed acts as a segment-polarity gene in early embryonic development. *engrailed* is expressed in segmental ectodermal stripes and anti-Engrailed antibody was used as the segmental ectodermal stripes-specific antibody. Since *jing1.5-lacZ* is not expressed in segmental ectodermal stripes (Fig.35) we conclude that the sites required for *jing* expression in segmental ectodermal stripes are located in the 1.3 kb fragment of the *jing* 5' regulatory region.

jing1.3-lacZ is not expressed in the tracheal cells as detected by double staining with anti-β-gal and anti-Tango (n=9) (Fig.36). The results from the *lacZ* expression analyses show that both 1.5 kb and 1.3 kb fragments of the *jing5'* regulatory region are required for *jing* expression in the trachea during early and late stages of embryogenesis. To conclude, tracheal *jing* expression requires combinatorial transcriptional

activation via the sites present in 1.5 kb and 1.3 kb fragments of the *jing* regulatory region. Expression patterns of *jing1.5-lacZ*, *jing1.3-lacZ*, and *jing2.8-lacZ* reporters are summarized in Figure 37.

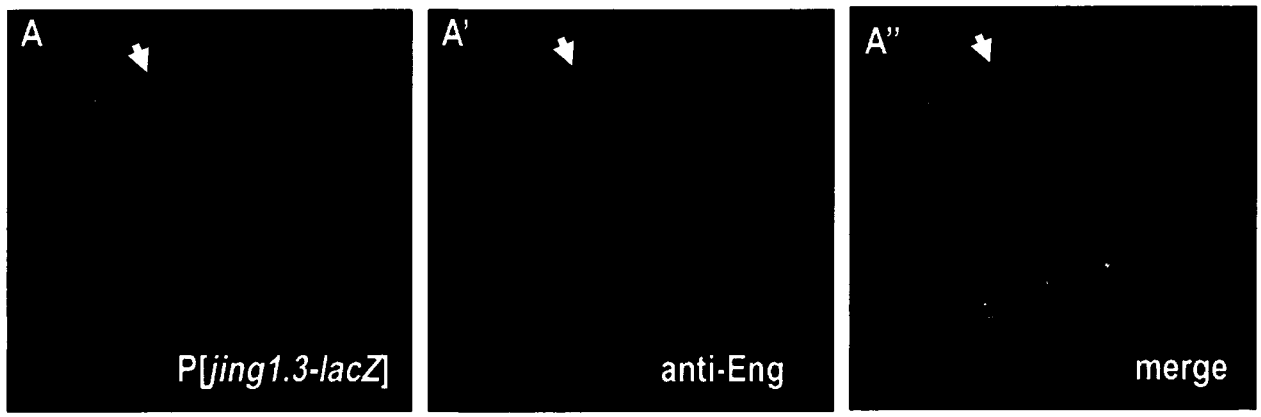


Figure 34. *jing1.3-lacZ* expression in segmental ectodermal stripes. Confocal image of a stage 13 embryo stained with anti-β-gal (green) (A) and anti-Engrailed (Eng) (red) (A') is shown in ventral view with anterior to the left. Arrowhead indicates an ectodermal stripe.

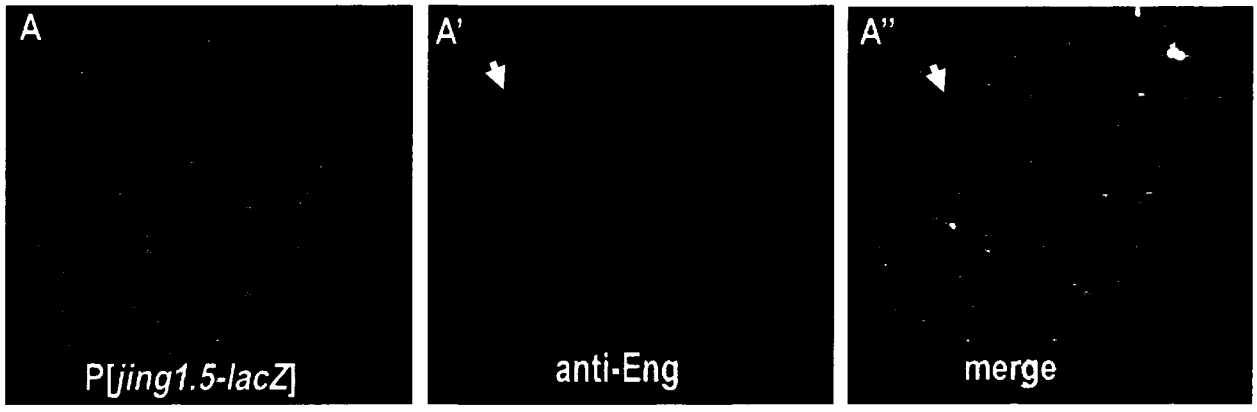


Figure 35. *jing1.5-lacZ* is not expressed in segmental ectodermal stripes. A stage 14 embryo stained with anti-β-gal (green) (A) and anti-Engrailed (Eng) (red) (A') is shown in ventral view with anterior to the left. Arrowhead indicates an ectodermal stripe.

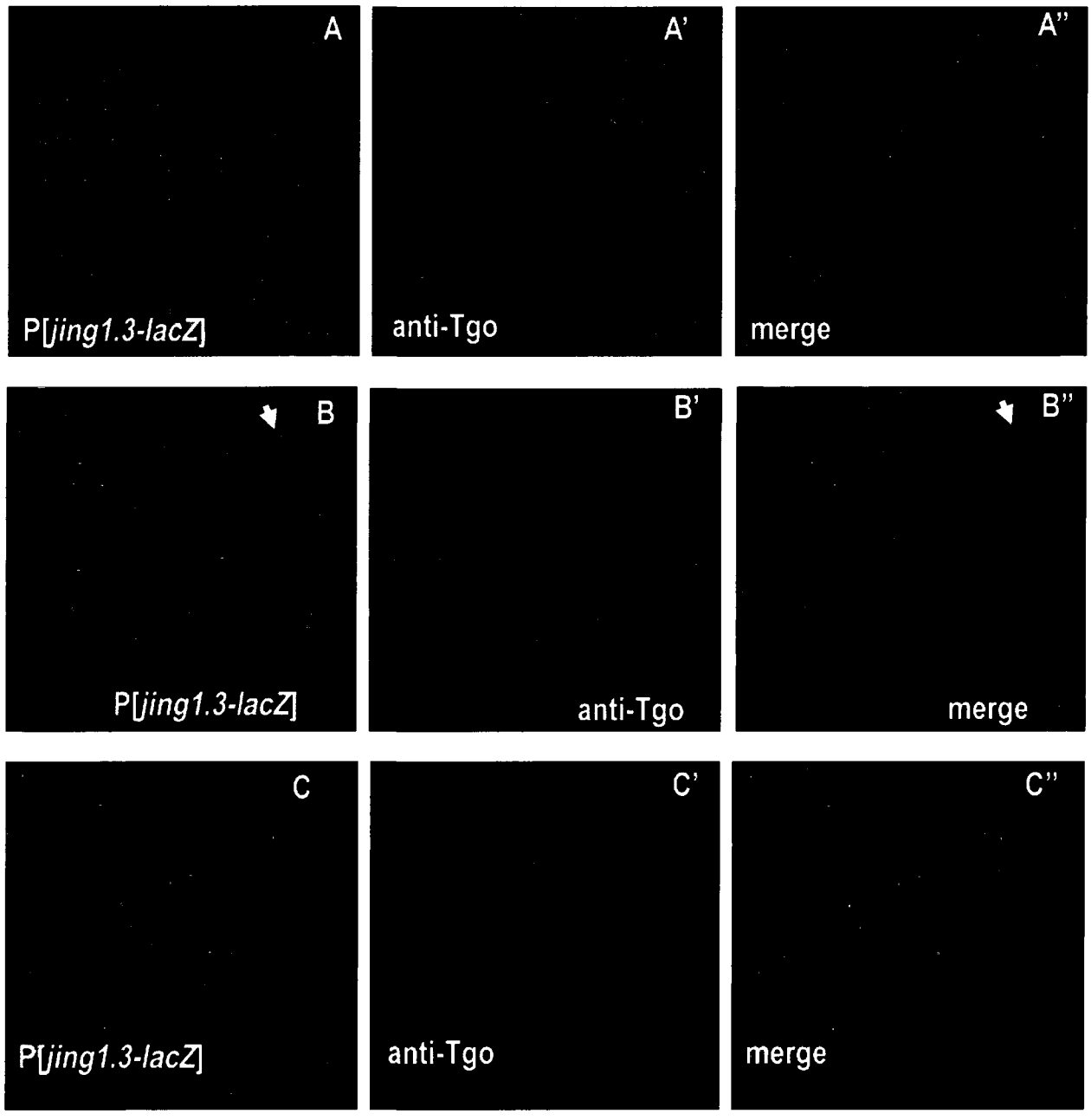


Figure 36. *jing1.3-lacZ* is not expressed in the trachea. (A-A'') A stage 15 embryo stained with anti-β-gal (green) (A) and anti-Tango (red) (A') is shown in sagittal view with anterior to the left. (B-B'') A stage 15 embryo stained with anti-β-gal (B) and anti-Tango (B') is shown in sagittal view with anterior to the left. The arrowheads indicate segmental ectodermal stripe expression of *jing1.3-lacZ*. (C-C'') A stage12 embryo stained with anti-β-gal (C) and anti-Tango (C') is shown in sagittal view.

Expression patterns of *jing1.5-lacZ*, *jing1.3-lacZ*, and *jing2.8-lacZ* reporters

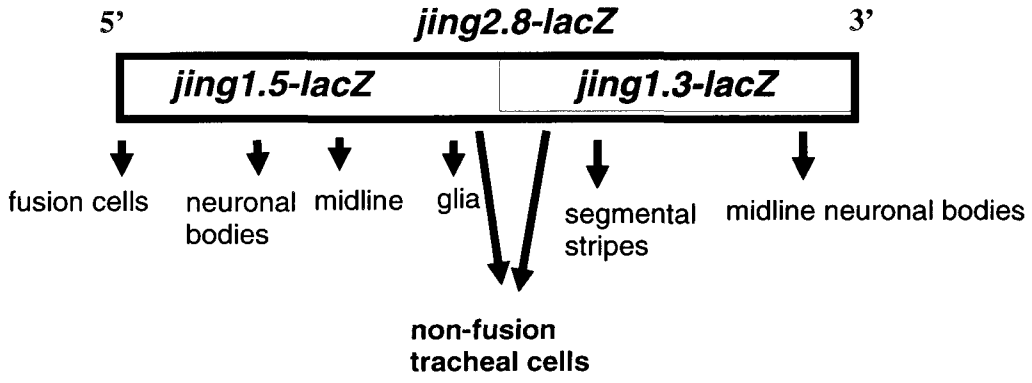


Figure 37. Expression patterns of *jing1.5-lacZ*, *jing1.3-lacZ*, and *jing2.8-lacZ* reporters.

2. Combinatorial transcriptional regulation of *jing* expression. *jing* responsiveness to RTK signaling

Previous studies showed that Jing is required for both EGFR- and FGFR-dependent tyrosine kinase signaling during tracheal development (Sonnenfeld M. et. al., 2004). *jing*'s zinc fingers are similar to those in the mammalian AEBP2 transcription factor, and Jing protein is present in the nuclei of most tracheal cells throughout development, which suggests a regulatory role for *jing* in tyrosine kinase signaling. We therefore studied its relationship with bHLH-PAS, POU and ETS-domain transcription factors which control FGFR and EGFR signaling in the *Drosophila* embryonic trachea.

To determine whether *sim, trh, dfr, pnt* are required for *jing* expression, the *jing1.5-lacZ* reporter was stably incorporated into homozygous dfr^{E082}, pnt^{E039}, sim^{H9}, trh^{2} mutant backgrounds. To investigate whether *jing* is responsive to RTK signaling the *jing1.5-lacZ* reporter was stably incorporated into homozygous bnl^{1} and rho^{7M} mutants. bnl^{1} is a hypomorphic allele of FGF like chemoattractant Branchless. Branchless is a key component in the FGFR signaling (see Introduction section 6). rho^{7M} is a null allele of the transmembrane protein Rhomboid, receptor of Spitz. Rhomboid is essential for EGFR signaling (see Introduction section 6). To determine whether the tracheal activation of the *jing1.5-lacZ* reporter by ETS-domain transcription factor Pnt is dependent on the integrity of consensus ETS DNA binding sites we used deletion site analysis.

2.1 Regulation of *jing1.5-lacZ* expression by the Single-minded bHLH-PAS transcription factor

First, *jing1.5-lacZ* expression in wild type embryos was compared with *jing1.5-lacZ* expression in homozygous sim^{H9} null mutant embryos (Fig. 38). The basis of this experiment is that the *jing*1.5 kilobase enhancer element contains three consensus CME binding sites for Sim::Tgo heterodimers (Crews S., 1998). Single-minded is a master regulator whose function is required for all CNS midline transcription and development (Crews S, 1998). As expected, sim^{H9} mutant embryos lost *jing1.5-lacZ* expression in the CNS midline. sim^{H9} is a null allele (Sedaghat Y. et.al., 2002). Our data are consistent with previous results

showing an absence of *jing-lacZ* enhancer trap expression in the CNS midline in homozygous sim^{H9} mutant embryos (Sedaghat Y. et.al., 2002). We conclude that Single-minded regulates *jing* expression in the midline. This result also confirms the CNS midline expression of *jing1.5-lacZ.*

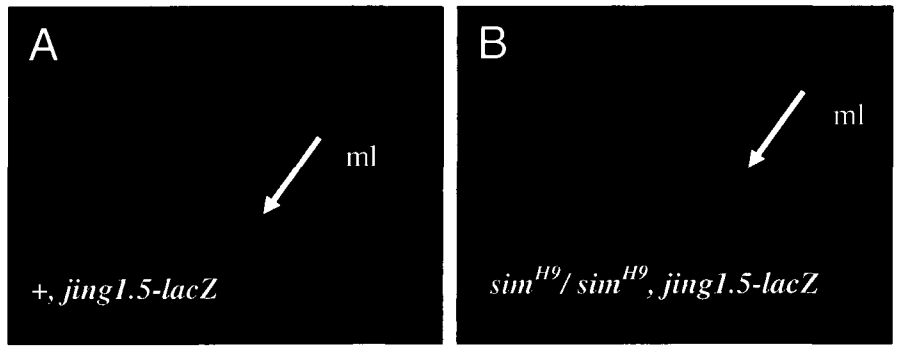


Fig.38. *jing1.5-lacZ* CNS midline expression requires sim function. The *jing5'* regulatory region contains three Sim::Tgo DNA binding sites with consensus sequence ACGTG (Central midline elements, CMEs). Embryos containing the *jing1.5-lacZ* enhancer were stained with mouse monoclonal anti-β-gal and Alexa Fluor488 (green). Confocal images of stage 13 embryos are shown with anterior to the left in ventral view. (A) Midline (ml) expression of *jing1.5-lacZ* is indicated by the arrow. (B) A homozygous null sim^{H9} mutant carrying *jing1.5-lacZ. jing1.5-lacZ* is not expressed in the CNS midline (ml).

2.2 *trh*, *dfr*, and *pnt* are required for endogenous *jing* mRNA expression

2.2.1 *trachealess*

Receptor tyrosine kinase (RTK) signaling determines development of the *Drosophila* embryonic trachea and it is regulated by Trh::Tgo heterodimers, the Pou-domain transcription factor Drifter, and the ETS-domain transcription factor Pointed (Ohshiro T. et al., 2002). *jing* is required for both EGFR and FGFR signaling in the developing trachea (Sonnenfeld M. et al., 2004). Therefore investigation was carried out to determine the relationship of *jing* with bHLH-PAS, Pou and ETS transcription factors known to regulate EGFR and FGFR signaling during embryonic tracheal development (Klambt, 1993; Gabay L. et. al., 1996; Wappner P. et al, 1997; Anderson M.G. et. al., 1996; Boube M. et al, 2000; Zelzer E. and Shilo B-Z., 2000).

Trachealess (Trh) is a master transcription factor in the *Drosophila* trachea controlling expression of many tracheal genes (Isaac D. and Andrew D., 1996; Llimargas M. and Casanova J., 1997; Boube M. et al., 2000). It forms heterodimers with Tgo and binds CME consensus DNA binding sites to initiate gene transcription (Ohshiro and Saigo, 1997). We therefore determined if *trh* was required for endogenous *jing* expression during tracheal development. To analyze *jing* transcription during embryogenesis, digoxigenin-labeled *jing* RNA probes were prepared as previously described (Sonnenfeld M. et. al., 2004).

At the beginning of tracheal development, cells which form tracheal pits are distinguished from surrounding cells by nuclear localization of the Trh and Tgo (Isaac D. and Andrew D., 1996; Wilk R. et al., 1996; Sonnenfeld M. et al., 1997; Ohshiro T. and Saigo K., 1997; Zelzer E. et al., 1997; Ward M. et al., 1998). Early developing tracheal cells are competent to respond to EGF and FGF signaling because they express *rhomboid* (*rho*) and *breathless* (*btl*) genes, respectively, which are controlled by Trh and Tgo transcriptional activities (Ohshiro T. and Saigo K., 1997; Llimargas M. and Casanova J., 1999). In the absence of Trh, tracheal cells do not invaginate and tubules do not form. Therefore severe trh^{8} mutant embryos do not have tracheal tubes (Sonnenfeld M. et al., 1997). trh^{2} hypomorphic mutant embryos show

defects in tracheal branch formation, tracheal cell differentiation, and weak Tgo immunoreactivity (Fig.42A'). We detected reductions in *jing* mRNA expression in the trachea of homozygous trh^2 mutant embryos (Fig.39B) hybridized with a digoxigenin-labeled *jing* RNA probe as compared with wild type *jing* mRNA expression (Fig.39A). Strong mRNA expression in the brain confirms that tracheal reductions are not an artifact of immunohistochemistry. We conclude that Trh is required for endogenous *jing* mRNA expression.

Fig.39. *trh* is required for *jing* mRNA expression. Bright field images of embryos hybridized with Digoxigenin-labelled *jing* anti-sense riboprobes and stained with anti-Dig. Embryos are oriented with anterior left and ventral down in sagittal views. (A) *jing* mRNA is present in a segmentally repeated pattern (arrow) in stage 12-3 wild-type embryos. (B) A stage 12-3 homozygous trh^2 embryo with reduced tracheal *jing* expression (arrow) while expression in the brain is strong (arrowhead).

2.2.2 *pointed*

Pnt may regulate *btl* expression combinatorially with Trh::Tgo (Ohshiro T. et al., 2002; see Introduction section 4B3). To determine whether Pnt is required for *jing* mRNA expression, we analyzed the effects of loss of *pnt* function on endogenous *jing* mRNA expression. Homozygous *pnt* mutant embryos were hybridized with digoxigenin-labeled *jing* RNA probes. Significant reductions were detected in *jing* mRNA expression in the trachea, but not in the brain of the homozygous pnt^{E039} mutant embryos (Fig.40B) compared with wild-type embryos (Fig.40A). We conclude that Pnt is required for endogenous *jing* mRNA expression.

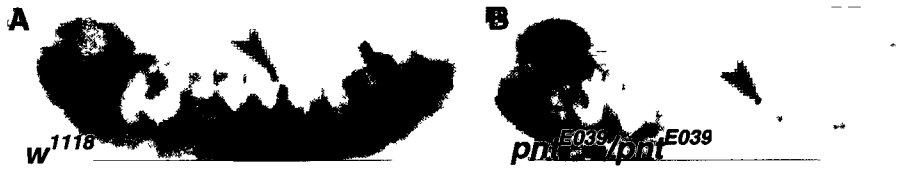


Fig.40. *pnt* is required for *jing* mRNA expression. Bright field images of embryos hybridized with Digoxigenin-labelled *jing* anti-sense riboprobes and stained with anti-Dig. Embryos are oriented with anterior left and ventral down in sagittal views. *in situ* hybridization of whole mount embryos with *jing* anti-sense riboprobes. (A) A wild-type stage 13 embryo showing segmental *jing* mRNA expression (arrowhead). (B) *jing* transcripts are reduced in the trachea of a stage 13 homozygous pnt^{E039} embryo (arrowhead) but remain comparatively strong in the brain (white arrow).

2.2.3 *dfr*

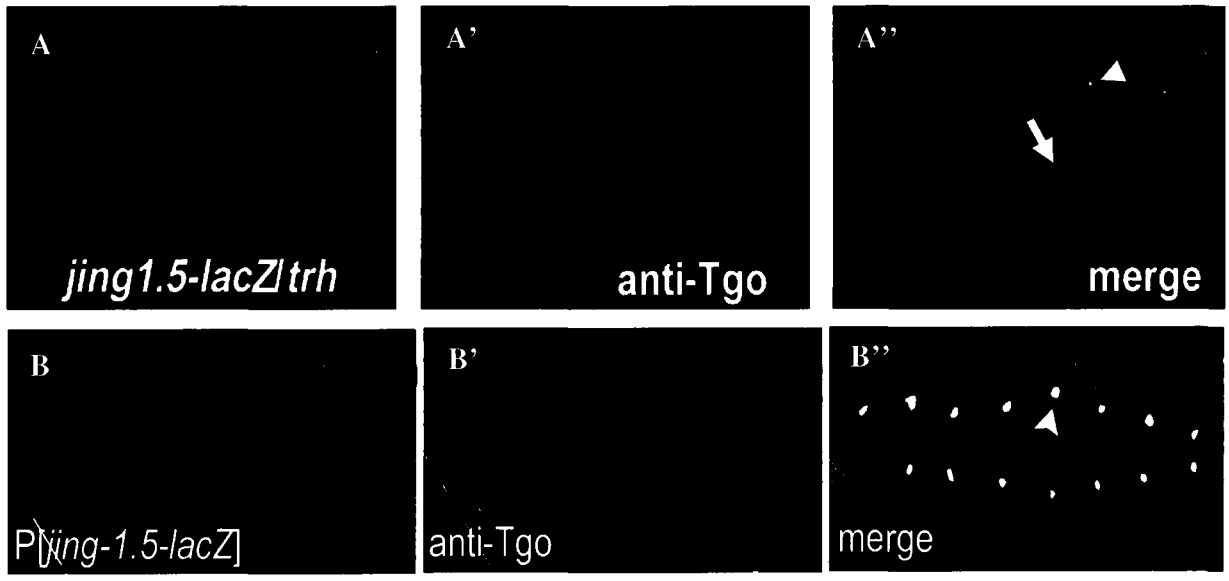
During primary branching, *btl* expression is regulated by the Pou-domain transcription factor Drifter/Ventral Veinless (Dfr/Vvl) (Anderson et.al., 1995). To determine whether Dfr is required for *jing* expression, we analyzed endogenous *jing* mRNA expression in homozygous dfr^{E082} mutant embryos by *in situ* hybridization with digoxigenin-labeled *jing* RNA probes. In homozygous dfr^{E082} mutant embryos, *jing* mRNA expression was significantly reduced in the trachea but not in the brain (Fig.41B). We conclude that Dfr is required for endogenous *jing* mRNA expression.

Fig.41. *in situ* hybridization with *jing* anti-sense riboprobe. (A) *jing* mRNA expression (arrow) in a wild-type stage 12 embryo. (B) A stage 13 homozygous dfr^{E082} mutant embryo with strong *jing* mRNA expression in the brain (arrowhead) and weak expression in dorsal and ventral regions of the trachea (arrow). Embryos are shown with anterior to the left and ventral down.

2.3 Analysis of *jing1.5-lacZ* reporter expression in *trh*, *dfr*, and *pnt* mutant embryos

2.3.1 Trachealess and Tango are required for *jing1.5-lacZ* expression *in vivo*

We next determined whether *jing* tracheal expression was regulated by *trh* since the *jing*1.5kb enhancer element contains three Trh::Tgo DNA binding sites (CME). The *jing1.5-lacZ* reporter was stably incorporated into flies carrying the trh^2 mutation balanced over TM3, *ultrabithorax* (*ubx*)-*lacZ*. Embryos were stained with rabbit polyclonal anti-β-gal to identify the pattern of *jing1.5-lacZ* activation in homozygous trh^2 embryos lacking a *ubx* staining pattern and double labeled with anti-Tgo. To determine if *jing1.5-lacZ* expression was affected in trh^2 mutant embryos we quantified β-gal expression using confocal images of the embryos. In the dorsal branch of the homozygous trh^2 mutant many tracheal fusion cells were able to migrate to the dorsal midline but they did not always strongly express *jing1.5-lacZ* as compared with strongly expressing adjacent cells (Fig. 42A). Expression of *jing1.5-lacZ* was also down regulated in the dorsal trunk fusion cells in the trh^2 mutant background (Fig.42A''). The results on the graph (Fig.42C) show the increase in the number of segments with weak ß-gal expression in the homozygous trh^2 mutant embryos compared with wild-type. We conclude that *trh* is required for *jing1.5-lacZ* expression in tracheal



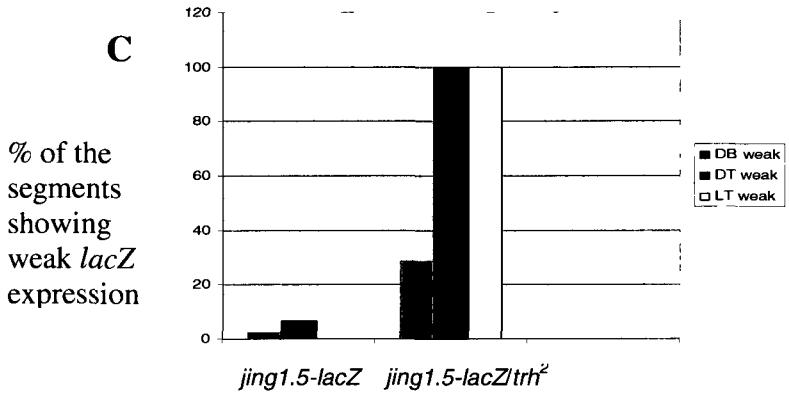


Fig.42. *jing1.5-lacZ* expression is reduced in the fusion cells of trh^2 homozygous mutant embryos. (A-B'') Confocal images of embryos double labeled with anti-β-gal (green) and anti-Tgo (red). (A-A'') A stage 16 homozygous trh^2 mutant embryo shown in sagittal view. The arrow indicates a dorsal trunk fusion cell and the arrowhead indicates a dorsal branch fusion cell. (B-B'') Control *jing1.5-lacZ* transgene expression is shown in dorsal view with posterior to the right. The arrowhead in B'' indicates a dorsal branch fusion cell. (C) Quantification of the percentage of segments with weak *lacZ* expression in w^{1118} embryos and in homozygous trh^2 mutant backgrounds. DB-dorsal branch fusion cells, DT-dorsal trunk fusion cells, LT- lateral trunk fusion cells. Db fusion cells *jing1.5-lacZ* transgene n=123 metameres counted, Dt fusion cells *jing1.5-lacZ* transgene n=115 were counted; Db fusion cells *jing1.5-lacZ/trh*^{2} transgene n=78 metameres counted, Dt fusion cells *jing1.5-lacZ/trh*^{2} transgene n=87 metameres counted, LT fusion cells *jing1.5-lacZ/trh*^{2} transgene n=51 metameres counted.

fusion cells. In addition, some dorsal branch fusion cells showed weak Tgo immunoreactivity which was not observed in control *jing1.5-lacZ* embryos.

2.3.2 Ectopic activation of the *jing1.5-lacZ* reporter by *trh* and *dfr/vvl*

To test whether *jing1.5-lacZ* expression can be activated by Trh in cells other than fusion cells, we used the UAS/Gal4 over-expression system (Material and Methods, section 1; Brand and Perrimon, 1993). Ectopic expression of *trh*, in combination with *dfr/vvl*, activates the *btl* target gene (Fig.43E; Boube et.al, 2000; Zelzer and Shilo, 2000). To determine if *jing1.5-lacZ* could be ectopically activated, expression of *trh* and *vvl* was induced by the pair-rule gene *paired* (*prd*). In wild-type stage 13 embryos, *jing1.5-lacZ* is expressed in the CNS midline and lateral ectoderm (Fig.43A) (Sun X. et al., 2006). However, ectopic activation was observed in an alternating pair-rule pattern in stage 14 and 13 embryos containing the *prd*-Gal4 driver, UAS-*trh*, *vvl* and *jing1.5-lacZ* (Fig. 43B, C). Stage 14 embryos containing the *prd*-Gal4 driver, UAS-*trh* alone, and *jing1.5-lacZ* did not show ectopic activation of *jing1.5-lacZ* (Fig. 42D). In addition, deletion of CME2 and CME3 from the *jing1.5-lacZ* reporter reduced tracheal but not CNS expression (Fig.44B), when compared with wild-type (44A). Together, these results provide evidence that Trh::Tgo in combination with Drifter/Vvl may directly regulate *jing* transcription *in vivo*.

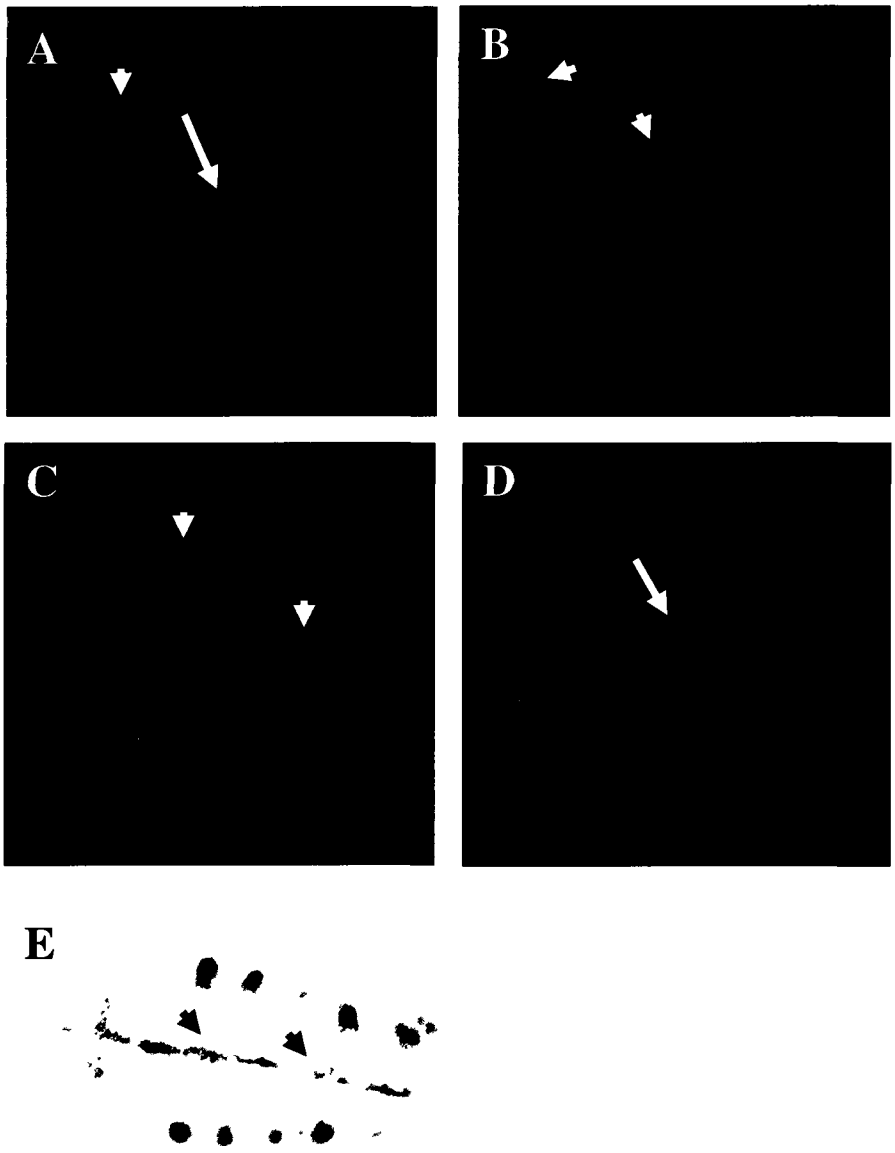


Fig.43. Ectopic induction of *jing1.5-lacZ* expression by Trh and Dfr (A-D). Confocal images of whole-mount embryos stained with anti-ß-Galactosidase. (A, D) *jing1.5-lacZ* expression in a wild-type stage 13 embryo in the CNS midline (arrow) and in the lateral ectoderm (arrowhead); (B, C) *jing1.5-lacZ* is ectopically activated in cells ventral and lateral to the CNS midline in every other segment (arrowheads) in stage 14 (C) and 13 (B) embryos expressing *UAS-trh* and *UAS-vvl* under control of the pair-rule gene *paired* (*prd*) (*prd-Gal4*). (D) *jing1.5-lacZ* is not activated ectopically in stage 14 embryos expressing *UAS-trh* alone under control of the pair-rule gene *paired* (*prd*) (*prd-Gal4*) (CNS midline, arrow). (E) Activation of *btl* expression in a *prdGal4/UASvvltrh* embryo, *in situ* hybridization with a *btl* Dig-labeled RNA probe. *btl* expression is ectopically activated in cells ventral and lateral to the CNS midline in every other segment (arrowheads). Embryo is shown in ventral view with anterior left.

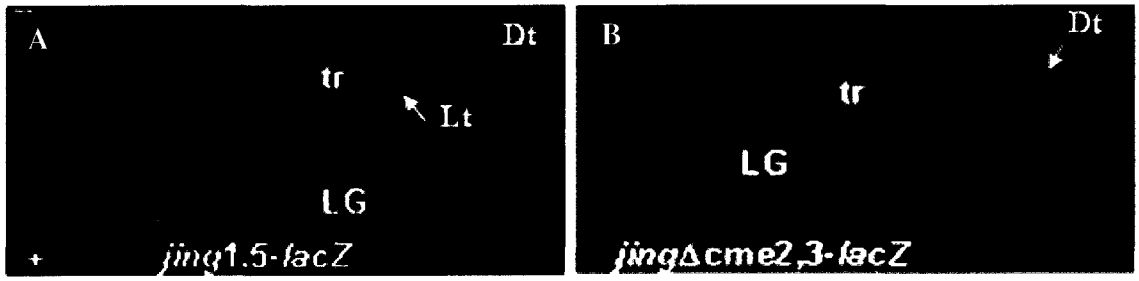
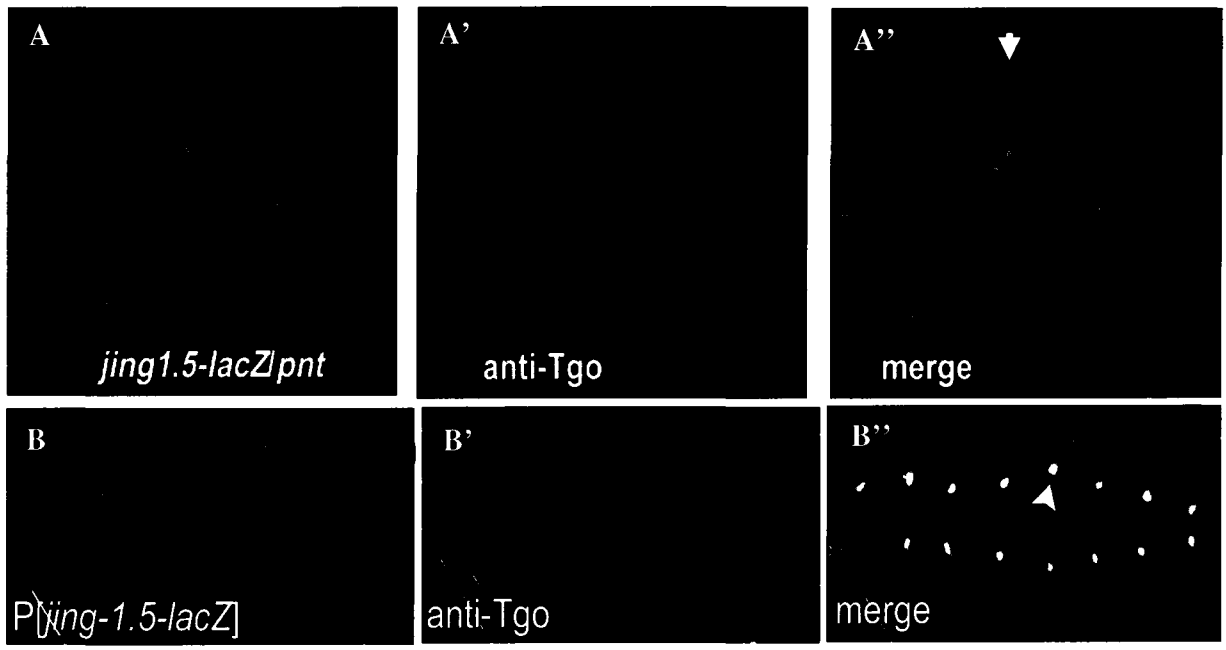


Fig.44. *jing1.5-lacZ* expression after deletion of 2 CMEs. (A) Wild-type *jing1.5-lacZ* is strongly expressed in the trachea (tr), longitudinal glia (LG), and dorsal trunk (DT) during stage 15. (B) Expression is barely visible in the stage 15 trachea after deletion of Trh::Tgo binding sites CME 2 and 3, but it is still present in the longitudinal glia as these cells do not express Trh.

2.3.3 *jing1.5-lacZ* activation in tracheal fusion cells requires the Pointed ETS transcription factor

To determine whether *pnt* regulates *jing*, the *jing-1.5-lacZ* reporter was stably incorporated into homozygous *pnt* mutants balanced with TM3, *ubx-lacZ*. Embryos were double labeled with anti-Tgo and anti-β-gal antibodies (Fig.45). Homozygous pnt^{E039} embryos show severe down-regulation and losses in *jing1.5-lacZ* expression in Tgo-positive fusion cells of the dorsal branch (Fig.45 A-A'') compared with control *jing1.5-lacZ* embryos (Fig.45 B-B''). Weak *jing1.5-lacZ* expression was observed in 53% of the pnt^{E039} mutant embryos compared with control wild-type *jing1.5-lacZ* expression (Fig.45 C). We conclude that *jing1.5-lacZ* expression is regulated by Pnt.



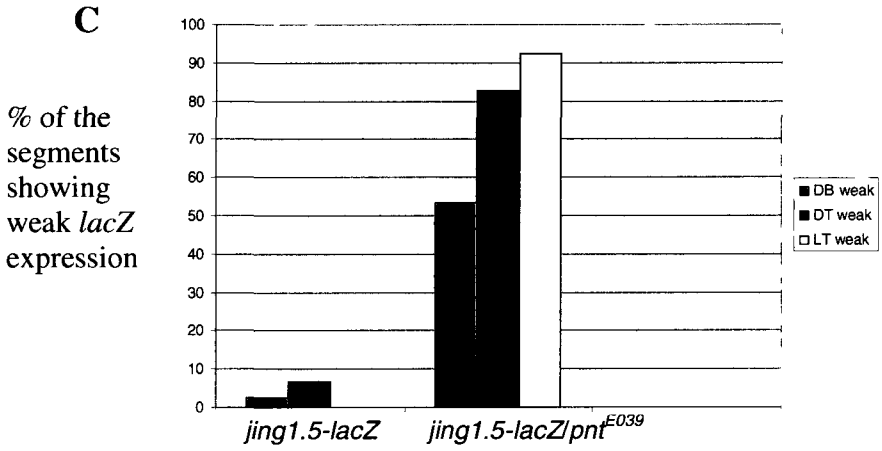


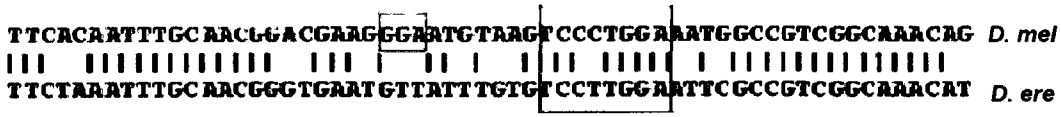
Fig.45. *jing1.5-lacZ* expression is regulated by *pnt*. Panels A-B'' show embryos double labeled with anti-Tgo (red) and anti-β-gal (green). (A-A'') *jing1.5-lacZ* expression is reduced in the dorsal branch fusion cells in a stage 14 *pnt* homozygous mutant embryo. (A-A'') Embryo is shown in dorsal view with anterior down; arrowhead indicates dorsal branch fusion cell. (B-B'') Control embryo expressing *jing1.5-lacZ* transgene is shown in dorsal view with posterior to the right. Arrowhead in B'' indicates a dorsal branch fusion cell. (C) Graph represents the % of segments showing weak *lacZ* expression in w^{1118} embryos and in a homozygous pnt^{E039} mutant background. DB-dorsal branch fusion cells, DT-dorsal trunk fusion cells, LT- lateral trunk fusion cells. Db fusion cells *1.5jing-lacZ* transgene n=123 metameres were counted, Dt fusion cells *1.5jing-lacZ* transgene n=115 metameres were counted; Db fusion cells *jing1.5-lacZ/pnt*^{E039} transgene n=163 metameres were counted, Dt fusion cells *jing1.5-lacZ/pnt*^{E039} transgene n=120 metameres were counted, LT fusion cells *jing1.5-lacZ/pnt*^{E039} transgene n=103 metameres were counted.

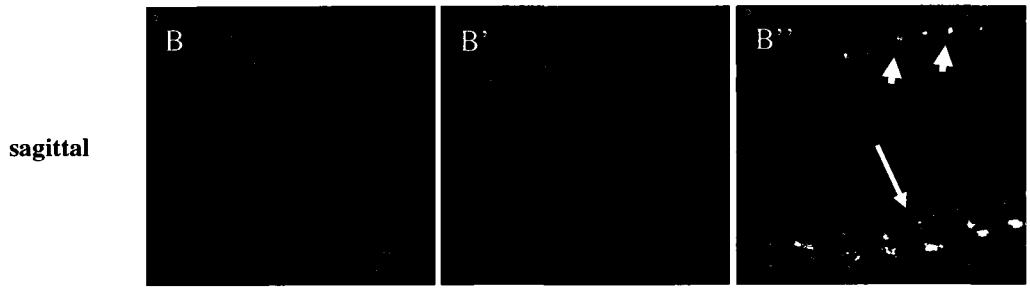
2.3.4 Activation of *jing1.5-lacZ* reporter by ETS-domain transcription factor Pnt is dependent on the integrity of consensus ETS DNA binding sites

Since there is evidence that *jing1.5-lacZ* expression is regulated by the ETS-domain transcription factor Pointed (Fig.45), and to further investigate whether *jing* is responsive to FGFR signaling in the trachea, potential Pnt binding sites were deleted from the *jing* regulatory region to analyze the consequences to *jing1.5-lacZ* expression in the tracheal fusion cells. Pointed is phosphorylated by activated MAPK (mitogen-activated protein kinase) and is one of the downstream targets in the *btl* positive feedback loop (Fig.1, Ohshiro et.al., 2002). Pnt regulates *btl* expression by binding a consensus TCC..GGA DNA binding site in the *btl* 5' regulatory region (Nye J.A. et. al., 1992; Ohshiro T. et. al., 2002). There is a similar consensus site, TCCCTGGA, in the 5′ *cis*-regulatory region of the *jing*1.5 enhancer that is conserved in the *Drosophila* sibling species *D.ere* (Fig.46A). We chose two adjacent sites, GGA and TCCCTGGA, to be deleted from the *jing1.5-lacZ* reporter in a construct called *jing1.5Δpnt-lacZ.* Transgenic lines were generated by injection of recombinant DNA into *Drosophila* embryos using standard procedures (Spradling A.C. and Rubin G.M., 1982). Five independent transgenic lines were tested for *lacZ* expression.

Deleting the sites from the *jing1.5-lacZ* reporter significantly reduced tracheal fusion cell expression in Tgo-positive dorsal branch fusion cells (Fig.46C-C″) while CNS expression remained comparable to wild-type (Fig.46B-B″). During stage 15 *jing1.5-lacZ* expression in dorsal branch fusion cells was reduced by 53% (n=84) after Pnt binding site deletion (Fig.46E). Quantification results (Fig.46G) illustrate the percent of Tgo-positive fusion cells with reduced *jing1.5-lacZ* expression in w^{1118} embryos and those carrying the *jing1.5ΔPnt-lacZ* transgene stained with anti-ß-Galactosidase. Our data suggest that *jing* expression in dorsal branch fusion cells may be under control of tyrosine kinase signaling.

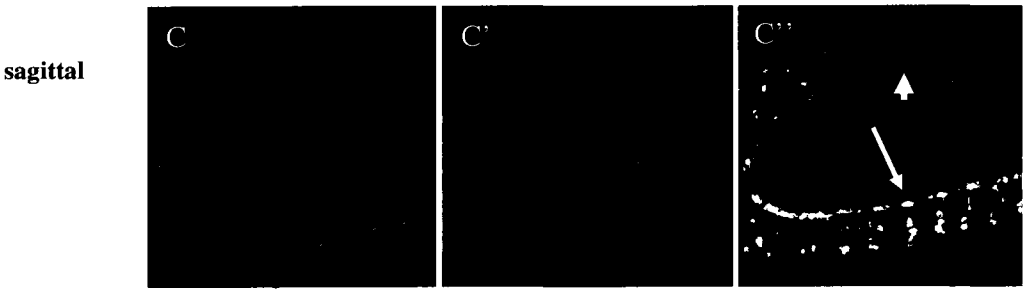
A



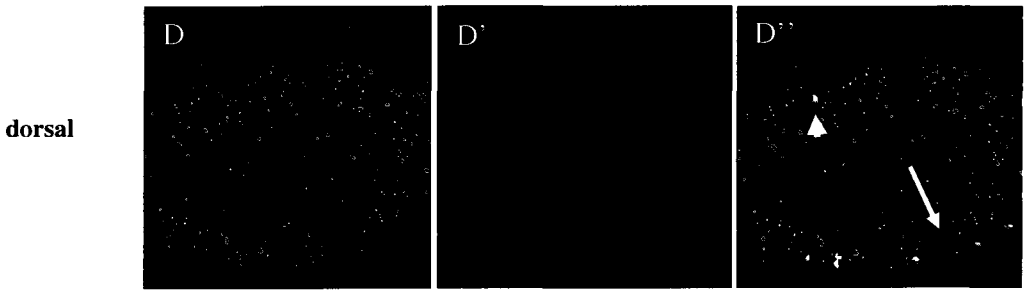


sagittal

jing1.5-lacZ anti-Tgo merge

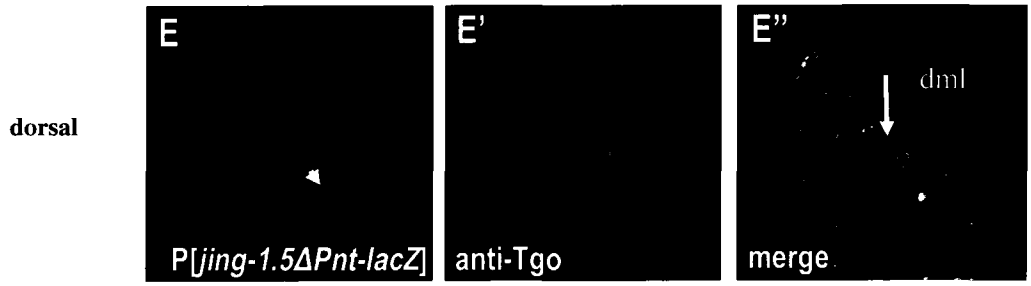


sagittal



dorsal

jing1.5ΔPnt-lacZ anti-Tgo merge



dorsal

dorsal

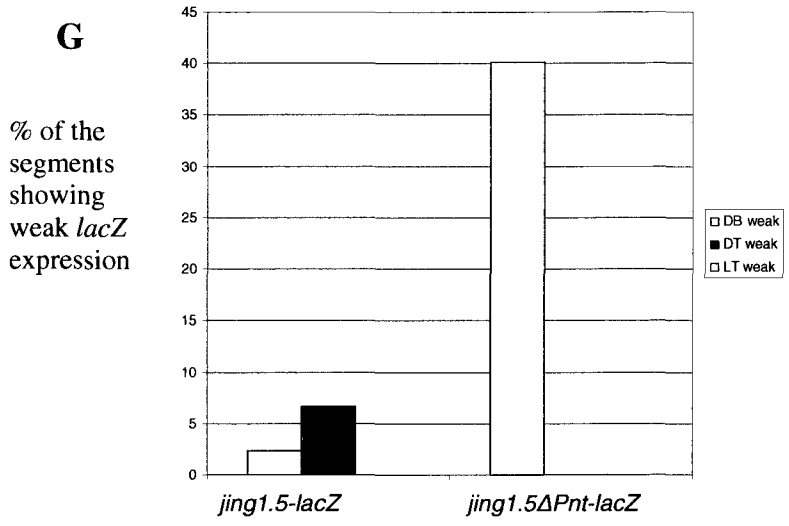


Fig.46. Deletion of the putative *pnt* DNA binding sites from *jing1.5-lacZ* (*jing1.5ΔPnt-lacZ*) is associated with significant reductions in tracheal expression. (A) A putative Pnt/Aop binding site (TCC.....GGA) in the *jing1.5* regulatory sequence is conserved in the *Drosophila* sibling species, *D. erecta* (*ere*). Red boxes indicate putative Pnt/Aop binding sites. Single ETS-domain^{AOP} binding site 5'GGA3' (small left red box) and double inverted repeat ETS-domain^{AOP} binding site (right big red box). Binding is stronger to the double inverted repeat binding site. (B-F) Confocal images of embryos double-labeled with anti-β-gal (green) and anti-Tgo (red) with anterior left in sagittal (B-C'') and dorsal (D-F'') view. (B-B'') Wild-type expression of *jing1.5-lacZ* (B), Tango (B'), and a merge of the two (B'') in the dorsal branch fusion cells (arrowhead) and the CNS (arrow). (C-C'') *lacZ* expression of the *jing1.5ΔPnt-lacZ* transgene in stage 16 embryos remains strong in the CNS (arrow) but is absent from dorsal branch fusion cells (arrowhead) even though they are Tgo-positive. (D-D'') Dorsal view of a stage 13 embryo showing remaining *lacZ* expression in the dorsal branch fusion cells (arrowhead) and strong Tgo immunoreactivity with no corresponding *lacZ* expression (arrow). (E-E'') An *jing1.5Δpnt-lacZ* embryo stage 15 in a dorsal view showing Tgo-positive fusion cells in the dorsal midline (arrow, dml) with reduced *lacZ* expression (arrowhead). (F) A control embryo carrying the *jing1.5-lacZ* transgene is shown in dorsal view with posterior to the right. Arrowhead indicates a dorsal branch fusion cell. (G) The graph illustrates the percent of Tgo-positive fusion cells with reduced *jing1.5-lacZ* expression in w^{1118} embryos and those carrying the *jing1.5Δpnt-lacZ* transgene (stages 13-15). DB-dorsal branch fusion cells, DT-dorsal trunk fusion cells, LT- lateral trunk fusion cells, Db fusion cells. Dt fusion cells *1.5jing-lacZ* transgene n=115 metameres were counted, Db *jing1.5-lacZ* n=123 metameres; *jing1.5ΔPnt-lacZ* transgene in the dorsal branch n=84 metameres were counted.

2.3.5 Drifter (Dfr) is required for activation of the *jing1.5-lacZ* reporter

To determine whether *jing* tracheal fusion cell expression is regulated by *dfr* the *jing1.5-lacZ* reporter was stably incorporated into flies carrying the dfr^{E082} mutation. In homozygous Drifter dfr^{E082} mutant embryos, *jing1.5-lacZ* expression was significantly reduced in Tgo-positive fusion cells. Tgo-positive cells that had migrated to the dorsal midline had weak *jing1.5-lacZ* expression as compared with control *jing1.5-lacZ* embryos (Fig.47B-B''). We conclude that *jing1.5-lacZ* expression is regulated by Dfr. The percent of weak *jing1.5-lacZ* expression was significantly higher in dfr^{E082} mutant background than in the wild-type (Fig.47C).

2.4 RTK signaling is required for *jing1.5-lacZ* expression

To further investigate whether *jing* expression is responsive to FGFR and EGFR signaling, the *jing1.5-lacZ* reporter was stably incorporated into homozygous bnl^{1} and rho^{7M} mutants, respectively, balanced with TM3, *ubx-lacZ*. Branchless is a chemoattractant (Ohshiro T et al., 2002). The receptor of Branchless is Breathless (FGFR; see Introduction, section 1, 6A2). Rhomboid (Rho) is a transmembrane protein which regulates processing of the activating ligand Spitz. The receptor of Spitz is EGFR (Gabay L. et al., 1997) (see Introduction, section 6A1).

2.4.1 FGFR signaling is required for *jing1.5-lacZ* expression

Tracheal cells at branch tips are exposed to high levels of the Bnl ligand (Ohshiro T. et al., 2002). Furthermore, *btl* is required for *pnt* expression in tip and fusion cells in the dorsal branch (Sutherland D. et al., 1996; Ohshiro T. et al., 2002) and we have shown that *pnt* regulates *jing1.5-lacZ* fusion cell reporter expression (Fig.45). We therefore hypothesized that *bnl* would be required to regulate *jing1.5-lacZ* expression possibly through Pointed transcription factor.

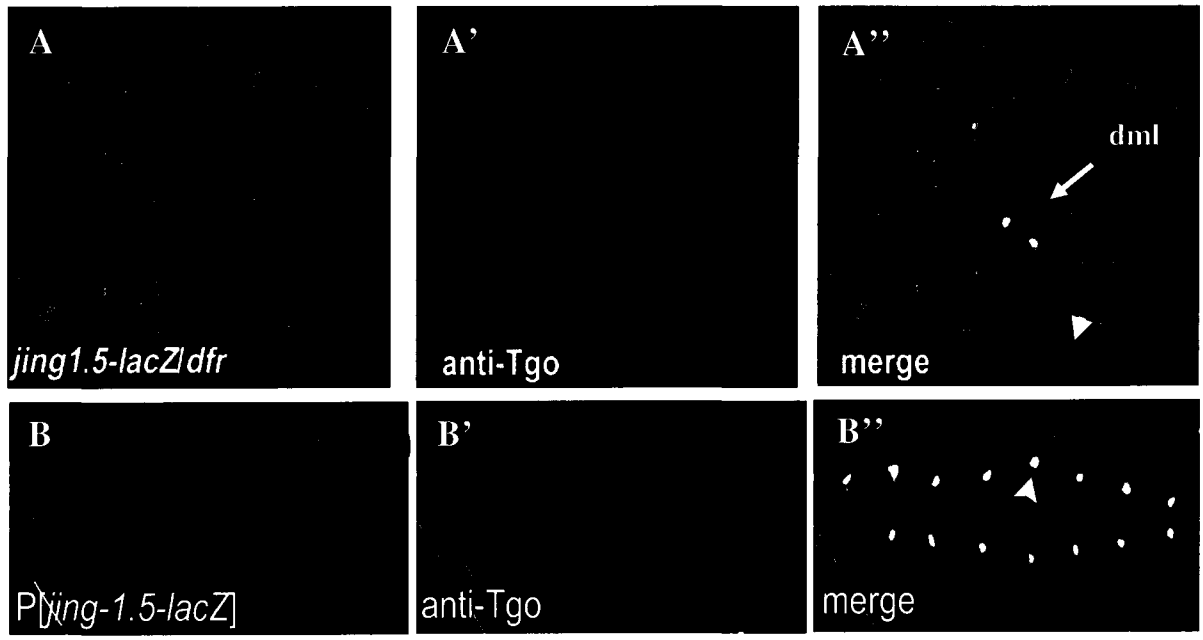
In embryos mutant for *bnl* and its receptor *btl,* tracheal cells invaginate but do not branch or have filopodial extension (Ohshiro et al., 2002). Localized misexpression of Bnl can direct branch formation and outgrowth to new positions. Generalized misexpression activates later programs of tracheal gene expression and branching, resulting in a massive network of branches (Sutherland D. et al., 1996). To determine whether FGFR signaling is required for *jing* expression we examined *jing1.5-lacZ* expression in bnl^{1} homozygous mutant embryos (Fig.48). In embryos homozygous for bnl^{1}, primary branch formation did not occur and *jing1.5-lacZ* expression levels were drastically reduced in Tgo-positive fusion cells (Fig.48 A-A'') compared with control *jing1.5-lacZ* embryos (Fig.48B-B"). For example, only 4.8% of Tgo-positive dorsal branch fusion cells expressed *jing1.5-lacZ* (stages 13-15, n=45). In comparison, in the wild-type embryos 95% of Tgo-positive dorsal branch fusion cells express *jing1.5-lacZ* (Fig.47B-B'', n=123). We conclude that *jing* expression is regulated through the Bnl/Btl (FGFR) pathway in tracheal fusion cells.

2.4.2 *jing* is responsive to EGFR signaling in trachea and in midline glia

Our data shown in Fig.49 confirmed *jing*'s responsiveness to EGFR signaling in the trachea as *rho* was required for *jing1.5-lacZ* expression in the tracheal fusion cells. In rho^{7M} homozygous mutant embryos, *jing1.5-lacZ* expression was down regulated in the Tgo-positive fusion cells as compared with control *jing1.5-lacZ* embryos (Fig.49). To further examine if EGFR signaling was required for *jing* expression, homozygous *rho* mutant embryos were hybridized with digoxigenin-labeled *jing* RNA probes (Fig.50). *jing* mRNA expression was significantly reduced in homozygous *rho* mutant embryos compared with wild-type embryos.

To further address if *jing* expression is controlled by tyrosine kinase signaling in the midline glia (Sonnenfeld M. et al., 2004), we analyzed whether *jing1.5-lacZ* expression was affected by EGFR signaling. *jing1.5-lacZ* activation was assessed in response to over-expression of the EGFR-activating proteins, Rhomboid (rho) and secreted spitz (sSpi) in CNS midline cells using the *single-minded* (*sim*)-Gal4

driver. Previous studies have shown that tyrosine kinase signaling activated by *Drosophila* EGFR in CNS midline glia is required for their survival (Sonnenfeld and Jacobs, 1994; Stemerdink and Jacobs, 1997). Persistent tyrosine kinase signaling in these cells allows for the survival of extra glia that would normally undergo apoptosis (Sonnenfeld and Jacobs, 1995). Ectopic *jing1.5-lacZ* expression was detected in surviving midline glia in response to *rho* and *sspi* CNS midline over-expression (Fig.51 B, C, arrows). Our results show that *jing* expression is responsive to EGFR signaling in midline glia.



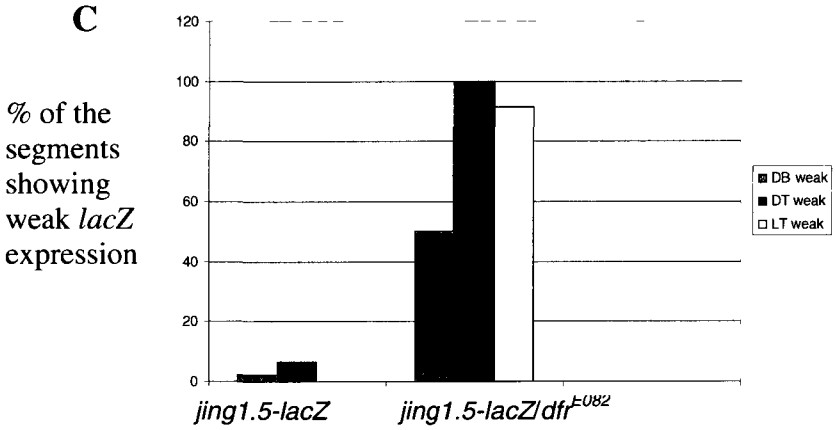


Fig.47. Down regulation of *jing1.5-lacZ* expression in the fusion cells in a dfr^{E082} homozygous mutant. Embryos were double labeled with anti-β-gal (green) and anti-Tgo (red). (A-A″) A stage 15 embryo is shown in dorsal view. The arrowhead indicates a dorsal branch fusion cell, arrow indicates the dorsal midline (dml). (B-B″) Control *jing1.5-lacZ* transgene expression in a stage 13 embryo is shown in dorsal view with posterior to the right. Arrowhead indicates a dorsal branch fusion cell. (C) Graph represents the % of the segments showing weak *lacZ* expression in w^{1118} embryo and in homozygous dfr^{E082} mutant background. DB-dorsal branch fusion cells, DT-dorsal trunk fusion cells, LT- lateral trunk fusion cells. Db fusion cells *1.5jing-lacZ* transgene n=123 metameres counted, Dt fusion cells *1.5jing-lacZ* transgene n=115 metameres were counted; Db fusion cells *jing1.5-lacZ/*dfr^{E082} transgene n=90 metameres counted, Dt fusion cells *jing1.5-lacZ/*dfr^{E082} transgene n=56 metameres counted, Lt *jing1.5-lacZ/*dfr^{E082} transgene n=51 metameres counted.

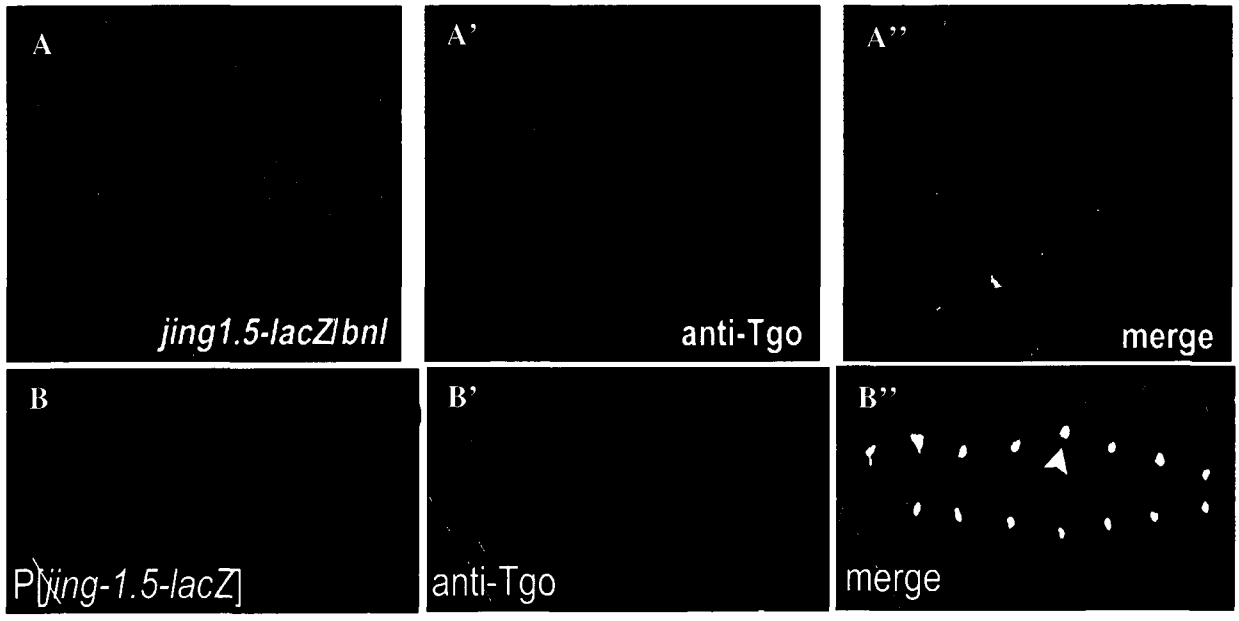


Fig.48. *jing1.5-lacZ* expression is regulated by FGFR signaling. (A-A'') *jing1.5-lacZ* expression in a homozygous *bnl* mutant embryo. Confocal images of embryos double labeled with anti-β-gal (green) and anti-Tgo (red). A stage 14 embryo is shown in sagittal view. (B-B'') Control embryo expressing the *jing1.5-lacZ* transgene is shown in dorsal view with posterior to the right. Arrowhead indicates a dorsal branch fusion cell.

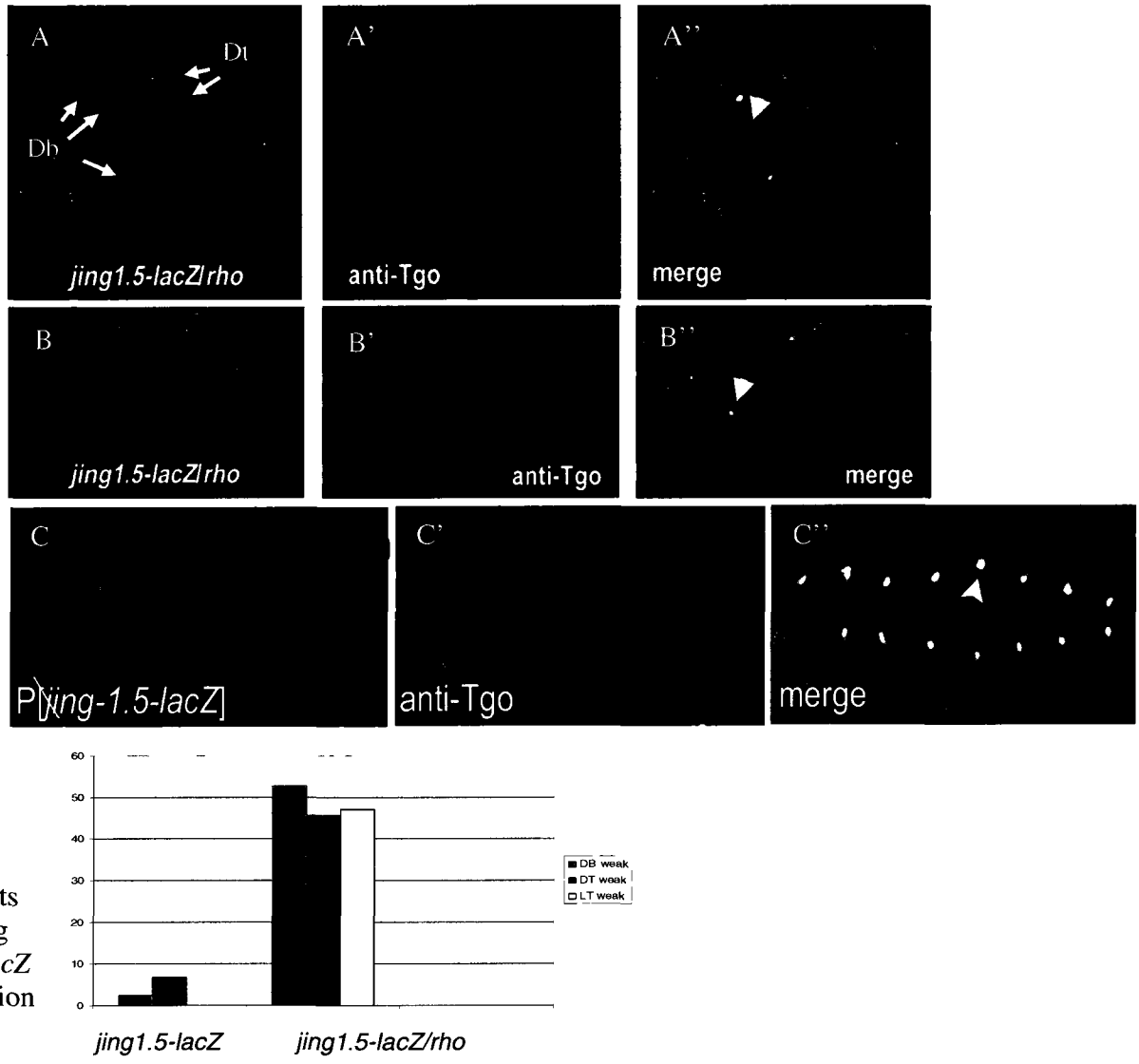


Fig.49. *jing1.5-lacZ* expression is regulated by EGFR signaling in the trachea. Panels A and B show reductions of *jing1.5-lacZ* expression in the fusion cells in *rhomboid* (*rho*) homozygous mutant. Embryos carrying *jing1.5-lacZ* were double labeled with anti-β-gal (green) and anti-Tgo (red). (A-A'') A stage 13 embryo is shown in sagittal view with anterior up. Arrows in A indicate dorsal branch (Db) and dorsal trunk (Dt) fusion cells expressing *lacZ*. The arrowhead in A'' indicates a Tgo-positive cell not expressing *lacZ*. (B-B'') A stage 14 embryo is shown in dorsal view with anterior left. The arrowhead indicates a fusion cell. (C-C'') Control embryo showing *jing1.5-lacZ* transgene expression in dorsal view with posterior to the right. Arrowhead indicates a dorsal branch fusion cell. (D) The graph represents the percentage of segments showing weak *lacZ* expression in w^{1118} embryos and in homozygous rho^{7M} mutant embryos (stage 13-15). DB-dorsal branch fusion cells, DT-dorsal trunk fusion cells, LT- lateral trunk fusion cells. *jing1.5-lacZ/rho* Db n=87, Dt n=45, Lt n=38 metameres counted; *jing1.5-lacZ* Db n=123, Dt n=115 metameres counted.

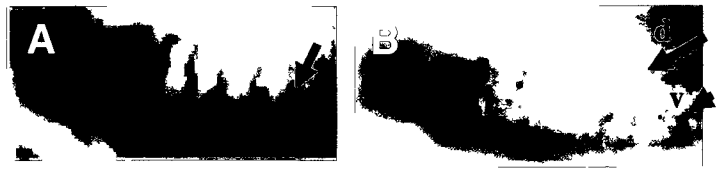


Fig.50. *in situ* hybridization with *jing* anti-sense riboprobe. (A) *jing* mRNA expression (arrow) in a wild-type stage 12 embryo. (B) *jing* mRNA expression (arrow) is reduced in the dorsal (d) and ventral (v) regions of the trachea in homozygous rho^{7M43} mutant embryos.

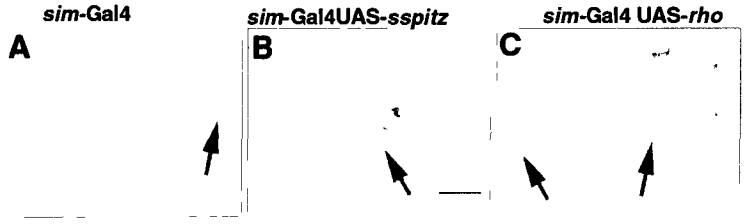


Fig.51. *jing1.5-lacZ* expression is regulated by tyrosine kinase activity. Expression of *jing-1.5-lacZ* was assessed in response to overexpression of the EGFR-activating proteins, Rhomboid (Rho) and secreted Spitz (sSpitz). Panels A, B and C show the CNS midline in embryos stained with anti-βgal monoclonal antibody. Midline expression of UAS-*rho* (C) and UAS-*sspitz* (B) transgenes induces ectopic *jing-1.5-lacZ* expression (B and C, arrows) in surviving midline glia in both genetic backgrounds, thus establishing responsiveness to tyrosine kinase signaling.

3. *jing* is required autonomously for maximal *btl* expression in the tracheal placodes and primary branches

Our studies of *jing* regulatory sequences and *jing* mRNA levels establish that *jing* is a target of Trh/Tgo and of Btl-mediated Pnt regulation (see Results, section 2). Our *in vivo* data are strongly supported by *in vitro* data showing that Trh::Tgo can activate *jing1.5-lacZ* expression in Luciferase reporter assays and bind to the *jing*1.5 element in chromatin immunoprecipitation assays (Sonnenfeld M. et al., submitted). Furthermore, it was previously shown that *jing* function is required for Btl-dependent MAPK activity in the trachea (Sonnenfeld M., et. al., 2004). Therefore it was hypothesized that Jing may be an integral component of the tyrosine kinase positive feedback loop by regulating *btl* expression. To test this hypothesis we used RNA interference and dominant negative interactions to reduce *jing* function specifically in tracheal cells. The consequences to *btl* expression were determined by *in situ* hybridization using a digoxigenin-labeled *btl* RNA probe (Janody et al., 2000).

First, *jing* expression was reduced in tracheal cells by RNA interference (RNAi) using the Gal4/UAS system (Brand and Perrimon, 1993). *UAS-jingRNAi* transgene (Sun et al., 2006) expression was directed into the trachea either by *btl-Gal4* (Fig.52B) or ubiquitously by the *armadillo* (*arm-Gal4*) driver (Fig.52D). In w^{1118} embryos, *btl* is expressed in every tracheal cell during all the stages of tracheal development (Ohshiro T. et al., 1997; Fig.52A, Introduction Fig.2). However, *btl* mRNA expression levels were reduced during stage 12 after expression of *UAS-jingRNAi* transgenes in tracheal cells (*btl-Gal4* driver, Fig.52B) and ubiquitously (*arm-Gal4* driver, Fig.52C) as compared with that in control stage 12 w^{1118} embryos (Fig.47A). Similar results were observed by tracheal expression of a *UAS-jing* fusion protein missing the five putative transactivation domains in the amino terminus (*jing-Δ5N*), ubiquitously expressed under control of *arm-Gal4* (Fig.52D). In the dominant-negative background, down-regulation of endogenous *jing* levels causes downregulation of *btl* levels.

In wild type stage 15 embryos, *btl* transcripts are highest in the dorsal, visceral and ganglionic branches and are reduced in the dorsal trunk (Fig.52E). At the same stage, only remnants of *btl* expression are visible in homozygous $jing^3$ mutants (Fig52F). Targeted knock down of *jing* expression was also associated with reductions in *btl* expression in the dorsal branch (Fig52H) and ganglionic branches (Fig52J,L) and *btl* mRNA does not enter the ventral nerve cord (VNC) (52J, arrow) as it does in control embryos (Fig.52I, arrow). In the ganglionic branches, some cells with reduced *btl* mRNA were observed close to but not at the final destinations in the VNC (Fig.52L, arrows). This reveals that the migration machinery was intact but not functioning properly. These results establish that proper *jing* function is required autonomously for maximal *btl* expression during primary branching.

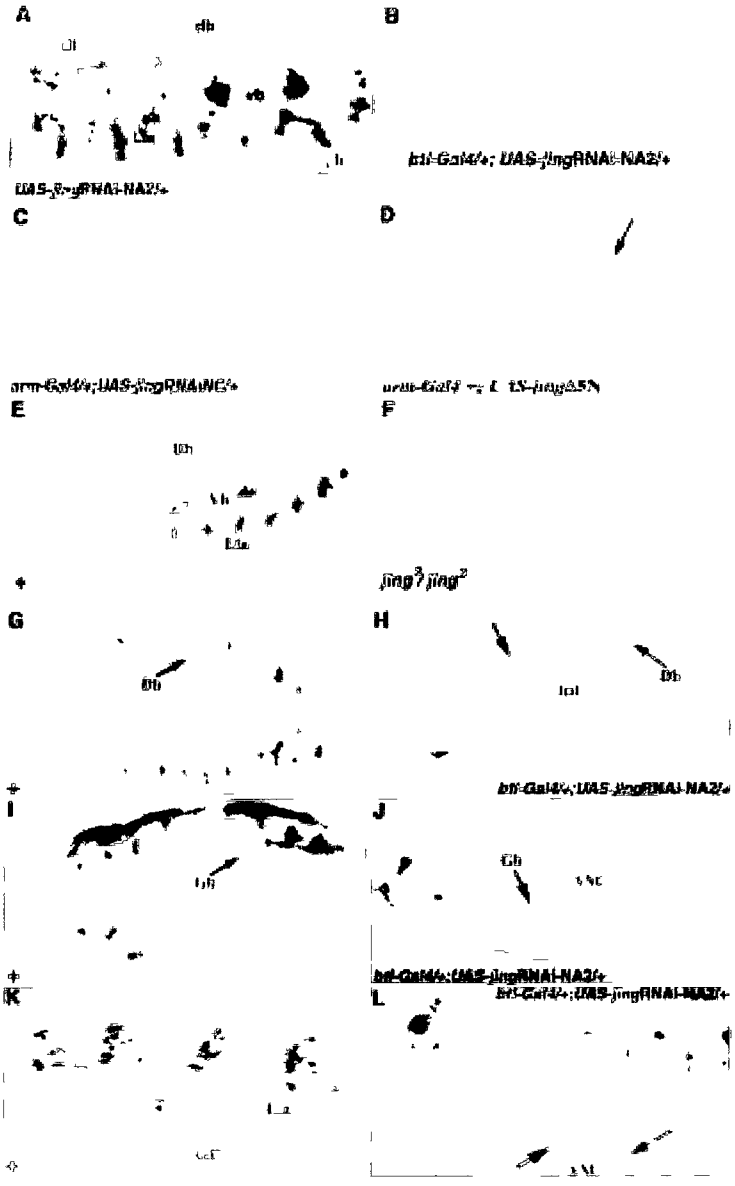

A
B
C
D
E
F
G
H
I
J
K
L

Fig.52. *jing* is required for embryonic *btl* mRNA expression. All whole-mount embryos are labeled with digoxigenin-labeled *btl* RNA probes (A-L). Shown are control embryos (A, E, G, I, K), a homozygous *jing*^{3} mutant embryo (F), embryos expressing *jing* RNAi transgenes in the trachea (driven by *btl-Gal4 or arm-Gal4* as denoted) (B, C, H, J, L), and over-expressing *UAS-jingΔ5N* under control of the *arm-Gal4* driver (D). Whole-mount embryos are shown with anterior left and in sagittal (A-F, K and L), ventral (I and J) and dorsal (G and H) views. (A) A wild-type stage 12 embryo showing each tracheal branch. (B-D) *btl* transcript levels are reduced in the stage 12 embryos expressing *jing* RNAi transgenes in all tracheal cells (B) and ubiquitously (C) as well as dominant negative transgenes ubiquitously (*UAS-jingΔ5N*) (D). (F) Significantly reduced *btl* transcript levels in a stage 14 homozygous *jing* mutant embryo. (E) Comparatively, in a similarly staged control embryo, *btl* is expressed in the Lta, Ltp, Db, and Vb. (G and H) Dorsal branches approaching the dorsal midline (dml) have reduced *btl* expression (arrows) in *btl-Gal4/jingRNAi* embryos as compared with the dorsal branches (Db) of a wild-type embryo (G). (I) *btl* expression in the ganglionic branch (Gb) enters the VNS of a stage 14 wild-type embryo. (J) Reduced *btl* expression (arrow) in the ganglionic branches (Gb) of a stage 14 embryo expressing *jing* RNAi in tracheal cells. (K and L) Enlarged views of *btl* expression in the anterior lateral trunk (Lta) and ganglionic branch (Gb) of a stage 14 embryo. (L) *jing*RNAi-NA2 transgene expression in tracheal cells results in significant reductions in *btl* expression in the Gb. Arrows denote Gb cells that have migrated to but not within the ventral nerve cord (VNC). Scale bars: 50 μm

4. *jing* mutant embryos show defects in tracheal cell migration

jing1.5-lacZ and *jing2.8-lacZ* reporters are strongly expressed in tracheal fusion cells (Fig.22, 23, 26D-D'', 31, 32). We therefore analyzed fusion cell development and tubulogenesis in *jing* loss-of-function backgrounds. Embryos homozygous for the hypomorphic $jing^3$ allele (Sedaghat Y. et.al., 2002) or expressing *jing* RNAi transgenes in the trachea show gaps in the dorsal trunk during stage 13 (Fig.53B,C) compared with a continuous tubule in control w^{1118} embryos (Fig.53A). We next analyzed development of dorsal branches in $jing^3$ mutant embryos using anti-Tgo as a tracheal marker (Sonnenfeld M. et al., 1997) and *jing1.5-lacZ* as a fusion cell marker. In stage 13 homozygous $jing^3$ mutant embryos, the number of cells in the dorsal branch of each tracheal metamere (6/branch; n=102) was similar to that in the w^{1118} control embryos, but the rate of cell migration lagged in some branches causing a double row of cells to form (Fig.53D, arrows) instead of a single row as in w^{1118} embryos (Fig.53J, Db) or in unaffected tracheal metameres (Fig.53D", Fc).

Reductions and losses in *jing1.5-lacZ* fusion cell expression were observed in the dorsal branch of stage 13 homozygous $jing^3$ mutants possibly due to *jing* autoregulation. In migrating dorsal branches, *jing1.5-lacZ* expression was strong in the fusion cells (Fig.53D' and D'', fc) situated properly adjacent to the lead cell (Fig.53D'', Lc). However, in other dorsal branches, fusion cells migrated properly but their *jing1.5-lacZ* expression levels were reduced (Fig.53D'' unlabeled arrows). This suggests that the fusion cells were not differentiating properly although their migration machinery was intact. In tracheal metameres where dorsal branch migration lagged, *jing1.5-lacZ* expression was further reduced again suggesting that these cells were not differentiating properly.

In embryos expressing *jing* RNAi transgenes in the trachea under control of the *btl*-Gal4 driver we also observed defects in dorsal branch cell migration. In stage 15 w^{1118} embryos, almost all dorsal branch fusion cells migrated to the dorsal midline (Fig.53E, fc). However, in *jing* knockdown embryos there were defects in the localization of cells at the tips of the dorsal branches. For example, dorsal branches invaded

adjacent metameres (Fig.53F, arrowhead). In more severe cases, tip cells and Db cells were absent (Fig.53G, large arrowhead) or misplaced (Fig 53G, small arrowhead).

Defects in the migration of cells in the lateral trunk and dorsal trunk were also associated with reduced *jing* function. In the dorsal trunk of homozygous $jing^3$ mutant embryos, there were frequent breaks (Fig.53I, arrowhead) or the two cell-wide Tgo-positive rows were reduced to one (Fig.53D, arrowhead). Also observed were lags in the migration of cells in the ganglionic branch (Fig.53I, arrow, Gb) and reductions in the number of cells in the visceral branch (Fig.53I, arrow, Vb). In w^{1118} embryos (Fig.53H, J) every tracheal branch is clearly visible and 9 dorsal trunk cells are clustered around each growing dorsal branch. This number increase to 13 in homozygous $jing^3$ mutant embryos suggesting defects in cell migration. These results show that *jing* function is required for cellular migration in most tracheal branches.

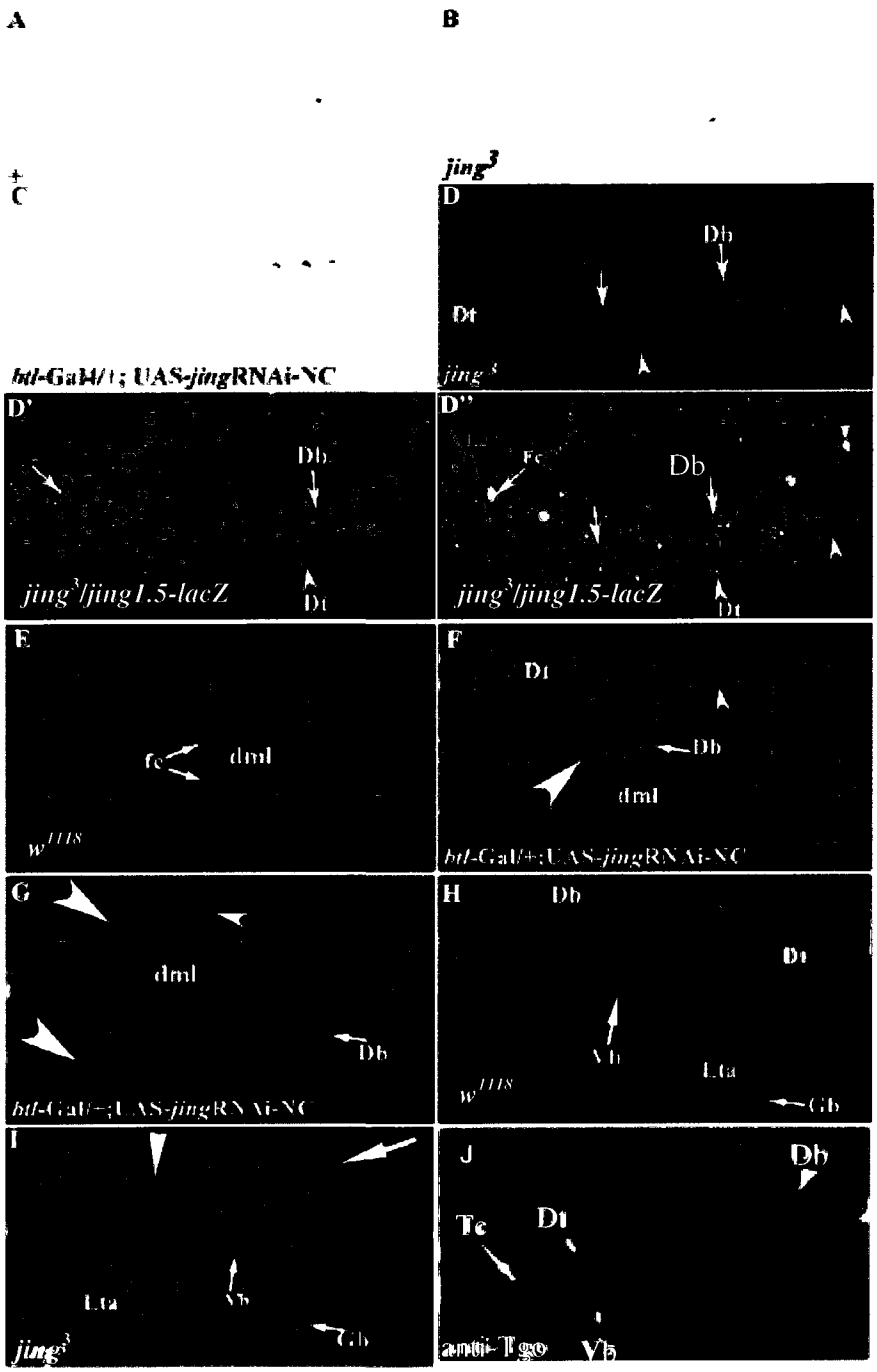

A
B
C
btl-Gal4/+; UAS-jingRNAi-NC
jing3
D
Db
Dt
jing3
D'
Db
jing3/jing1.5-lacZ
Dt
D''
Fe
Db
jing3/jing1.5-lacZ
Dt
E
fe
dml
w1118
F
Dt
Db
dml
btl-Gal4/+; UAS-jingRNAi-NC
G
dml
Db
btl-Gal4/+; UAS-jingRNAi-NC
H
Db
Dt
Vb
Lta
w1118
Gb
I
Lta
Vb
Gb
jing3
J
Db
Te
Dt
anti-Tgo
Vb

Fig.53. *jing* is autonomously required for proper cell migration during primary branching. (A-C) Anti-lumen 2A12 staining of early stage 13 whole-mount embryos. (A) A w^{1118} embryo with an intact dorsal trunk (arrowhead). (B) A $jing^{3}$ homozygous mutant embryo shows breaks in the dorsal trunk (arrowhead). (C) An embryo expressing *jing* RNAi transgenes under control of *btl*-Gal4 driver shows breaks in the dorsal trunk (arrowheads). (D-J) Confocal images of whole-mount embryos stained with anti-Tgo (red) and anti-β-gal (green) are shown with anterior left in sagittal (D, H-J) and dorsal (E-G) views. (D-D″) Aberrant development of the dorsal branch (Db) and dorsal trunk (Dt) in a $jing^{3}$ homozygous mutant embryo. Dorsal trunk cells are lost or reduced in width (arrowheads) and *jing1.5-lacZ* expression is drastically reduced (D′, arrowhead). In some dorsal branches, cells have not migrated as far as in others and the branch is two cells wide (D, arrows). *jing1.5-lacZ* expression in these branches is absent or drastically reduced (D′, Db arrow). In adjacent metameres, strong (D″, arrow Fc) and weak (D″, small arrowhead) *jing1.5-lacZ* expressing fusion cells are properly positioned next to lead cells (Lc) (D'', blue arrowhead). (E) In a stage 15 wild-type embryo, dorsal branch cells have reached the dorsal midline (dml). Fusion cells (fc) remain closest to the dml. (F and G) Stage 15 embryos with *jing* RNAi-NC transgene expression in tracheal cells under control of the *btl*-Gal4 driver. (F) The tip cells migrate into adjacent segments (large arrowhead) after reaching the dml suggesting fusion of neighboring branches. Small arrowhead indicates a dorsal trunk (Dt) break. (G) In more severe cases, dorsal branch cells have not migrated to the dml (large arrowheads) or are slower (small arrowhead). (H) In w^{1118} stage 14 embryos, all branches are clearly visible. (I) In stage 14 $jing^{3}$ mutant embryos, dorsal trunk cells have not migrated toward adjacent segments leaving gaps (arrowhead). Dorsal branches do not form in some metameres (arrow), ganglionic branch (Gb) cell migration is slow (small arrow) and there are fewer cells in the visceral branch (Vb).

Discussion

The trachea of the *Drosophila melanogaster* is a popular model for studies of branching morphogenesis and in particular cell differentiation, cell motility and cell guidance (Hogan and Yingling, 1998; Metzger and Krasnow, 1999). The formation of *Drosophila* trachea follows well-conserved processes of tubule growth including cell migration, cell guidance, cytoskeletal rearrangement characteristic to cell shape changes, cellular fusion and intracellular lumen formation (Manning and Krasnow, 1993; Metzger and Krasnow, 1999). Cell migration and cell fusion mechanisms are similar between vertebrates and invertebrates. For example, vertebrate Fgf10 is homologous to *Drosophila* FGF (Min H. et al., 1998). Elucidating the mechanisms regulating these processes is essential to understand what controls the behavior of highly migratory cells during lung development and invasive processes such as tumor formation.

Tubulogenesis is dependent on which tyrosine kinase is activated and also on the degree, duration and localization of the activation. For example, during hepatocyte growth factor-induced tubulogenesis, both transient high level activation and low level activation of ERK1/2 and PI3-K pathways are required (Gaul et al., 2000; Karihaloo et al., 2004; Ueland et al., 2004; Karihaloo et al., 2005). Low level ERK activation induces the ETS-1 transcription factor during branching morphogenesis (Hu et al., 2004). In contrast, high levels of ERK activation at the membrane are required for focal adhesion turnover and cell migration (Ishibe et al., 2003). In addition, transient MAPK stimulation induces cell proliferation while sustained and strong MAPK activity promotes neuronal differentiation of the same cell (Marshall, 1995). Lastly, the development of human carcinomas and malignant glioblastomas has been associated with overexpression of EGFR or a block in EGFR endocytosis (Sawano et al., 2002; Verveer et al., 2000; Blume-Jensen and Hunter, 2001). In *Drosophila* trachea, the level of dp-ERK was shown to be crucial not only for cell invagination and cell migration, but also for determining distinct cell fates within the tracheal branches (Gabay L. et al., 1997). Therefore, it is clear that the signal threshold of tyrosine kinases controls

cell fates and therefore understanding the inhibitory and stimulatory components regulating this process is critical.

Tyrosine kinase signaling is a key force driving tracheal cell differentiation. The last mitotic cell division in trachea occurs at stage 10 which is regulated by EGFR which also controls invagination. The following stages involve directed cell migration regulated through the action of the FGFR (*btl*) (Llimargas M. and Casanova J., 1999; Wappner P. et al, 1997; Glazer L. and Shilo B.Z., 2001; Gabay L. et al, 1997; Ohshiro T. et al, 2002).

In the developing *Drosophila* trachea, transcriptional regulation is precisely coordinated with growth factor signaling to induce the appropriate cellular response such as cell migration and cell fate determination (Ikeya and Hayashi, 1999; Llimargas and Casanova, 1997; Wappner et al, 1997; Llimargas and Casanova, 1999). Expression of *btl* is regulated by several transcription factors. All of them form an interconnected mechanism of combinatorial transcriptional regulation in the *Drosophila* trachea. Furthermore, previous data indicate a strong feedback loop in the regulation of *btl* expression (Ohshiro T. et al, 2002).

I examined the role of the Jing zinc finger protein in embryonic tracheal development and in the regulation of *btl* expression. First of all, *jing* is expressed in most tracheal cells. Moreover, it shows genetic interactions with *trh*, *dfr, pnt,* and other genes involved in FGFR and EGFR signaling. Furthermore, *jing* is involved in the regulation of EGFR-dependent and FGFR-dependent MAPK activity in tracheal placodes and pits (Sonnenfeld M. et al., 2004). Finally, *jing* can also induce the EGFR pathway and is required for EGFR signaling and expression of EGFR in midline glia (Sonnenfeld M., et al, 2004). I show that the *Drosophila* Jing zinc finger protein plays a critical role in *btl* regulation and migration of tracheal cells. Furthermore, I show that *jing* expression levels are controlled by bHLH-PAS, ETS, and POU domain transcription factors which are implicated in combinatorial regulation of *btl* expression during primary

branching (Boube et al., 2000; Ohshiro et al., 2002; Zelzer and Shilo, 2000), and that *jing* is responsive to tyrosine kinase signaling through EGFR and requires EGFR and FGFR signaling for expression.

Regulation of jing expression by bHLH-PAS transcription factors

jing is required for both EGFR and FGFR-dependent tyrosine kinase signaling during early tracheal development (Sonnenfeld M., et al, 2004) which makes it critical to understand how its expression is controlled. The *jing5' cis*-regulatory region contains three CME consensus DNA binding sites previously shown to bind Trh::Tgo (Ohshiro T. and Saigo K., 1997). For this reason, we expected that Trh::Tgo may directly activate *jing* expression (Sedaghat Y. et. al., 2002). To test this hypothesis, 1.5kb of sequence containing the CMEs from the *jing5'* cis-regulatory region was fused with *lacZ* and its enhancer activity was studied in embryos collected from transgenic flies. Since Trachealess is expressed in every tracheal cell (Wilk et al., 1996), I expected that a 1.5kb sequence containing three CMEs would be activated in all developing tracheal cells. However, *jing1.5-lacZ* is expressed mostly in one type of tracheal cell lineage known as the fusion cells from stage 12. This was determined by anti-Dys/anti-β-gal double labeling using a fusion cell-specific antibody recognizing the Dysfusion protein (Jiang and Crews, 2003). I show that *trh* function is required for *jing1.5-lacZ* expression in the fusion cells. Nevertheless, this regulation can be indirect because of the inter-regulation of *dys* and *trh*. Prior to stage 12, *trh* is required for *dys* expression and then *dys* down-regulates expression of *trh* in the fusion cells (Jiang and Crews, 2003). However, it is possible that the reporter is activated by Dys::Tgo heterodimer which is expressed in the fusion cells from stage 12 onward (Jiang and Crews, 2003). To avoid restriction of *jing1.5-lacZ* activation to the fusion cells, I tested whether Trh::Tgo could ectopically activate the *jing1.5-lacZ* reporter in Dys-negative cells under control of the *prd-Gal4* driver. When over-expressed ectopically under control of the *prd-Gal4* driver, UAS-Trh, in combination with UAS-Dfr, was able to activate *jing1.5-lacZ* expression but not UAS-Trh

alone. Together, these results show that *jing* is a downstream target (direct or indirect) in the Trh::Tgo pathway in the developing trachea.

jing mRNA is significantly down-regulated in hypomorphic trh^2 mutant embryos by stage 12. Given that in a wild-type embryo, Jing protein co-localizes with Trh::Tgo prior to stage 12 when Dys first appears in tracheal fusion cells (Sedaghat Y. et. al., 2002; Jiang and Crews, 2003) it is possible that *jing* expression is regulated combinatorially by Trh::Tgo and unidentified factors in all tracheal cells excluding the fusion cells. My *in vivo* data indicating regulation of *jing* expression by Trh::Tgo are strongly supported by *in vitro* data showing that Trh::Tgo can activate *jing1.5-lacZ* expression in Luciferase reporter assays and bind to the *jing1.5* element in chromatin immunoprecipitation assays (Sonnenfeld et.al., submitted). In addition, deletion site analysis showed that integrity of the CME consensus binding sites is essential for the activation of the 1.5kb reporter (Sedaghat Y., unpublished data) suggesting that 1.5kb reporter enhancer activity is controlled by bHLH-PAS transcription factor.

There is 1.3kb of 5′ regulatory sequence between the *jing1.5* enhancer element and the *jing* open reading frame. This suggested that additional regulatory sites required for *jing* tracheal expression may be present in this region. We chose 2.8kb fragment from *5′ jing* regulatory sequence including 3 CMEs since bHLH-PAS transcriptional activation can also be of combinatorial nature when the presence of CMEs is required (Ohshiro T et al., 2002). I was able to recapitulate tracheal expression pattern more similar to endogenous *jing* (Sedaghat Y. et. al., 2002) with a second larger 2.8kb *lacZ* reporter from the *jing* 5′ *cis*-regulatory region. *jing2.8-lacZ* was activated in all tracheal branches and in early tracheal pits as confirmed by anti-Tgo/anti-β-gal double labeling. The absence of the additional CMEs binding sites in this sequence implies that factors other than Trh::Tgo are required for *jing* expression in a non-fusion type tracheal cells. Moreover, *jing1.3-lacZ* reporter lacking CMEs is not expressed in the trachea supporting our conclusion that CMEs are required for the activation of *jing* expression in the trachea. We conclude that Trh::Tgo heterodimers regulate *jing* in combination with unidentified transcription factors whose DNA binding sites

are in the additional 1.3kb regulatory sequences in the *jing2.8* element. The combinatorial nature of transcriptional regulation involving CME DNA binding sites has been shown in the *Drosophila* CNS midline and trachea in *in vivo* reporter studies of the *btl* regulatory region (Ohshiro and Saigo, 1997; Ma Y. et al., 2000). The *jing2.8-lacZ* and *jing1.3-lacZ* reporters activation pattern also revealed the presence in the 1.3kb fragment of a binding site(s) necessary for the expression in segmental ectodermal stripes, an expression pattern previously reported for a *jing* enhancer trap construct (Sedaghat Y. et. al., 2002).

I also show that bHLH-PAS transcription factor Single-minded can activate *jing1.5-lacZ* expression in the CNS midline consistent with previous results for *jing* enhancer trap construct (Sedaghat Y. et. al., 2002). To conclude, Sim likely regulates expression of *jing* in the CNS midline.

Jing regulatory function is required in the positive-feedback loop that controls btl expression

During the primary branching process, tracheal cells migrate from the pits towards the surrounding chemoattractant Branchless which activates FGFR on the membrane of the tracheal tip cells (Samakovlis et al., 1996). Tyrosine kinase signaling in the trachea follows the universal cascade involving activation of the Ras/MAPK pathway. In general, a dorsal branch typically consists of five or six cells near the branch tip, one that becomes a terminal cell (tip cell) and another that becomes a fusion cell, and three or four cells that form the branch stalk (Ghabrial A.S. and Krasnow M., 2006).

Among the cells of the dorsal branch Btl activation is only required in the leading tip cells (Gabay et al., 1997; Ghabrial and Krasnow, 2006). During migration, Btl-dependent di-phosphorylated ERK is an indication of tyrosine kinase activity and is highest in the tip cells (Gabay et al., 1999). Although *btl* is expressed in the fusion and stalk cells, its activation in these cells is not required for successful migration (Ghabrial and Krasnow, 2006). It is the highest level of *btl* activity that selects one cell to become a tip cell and then Pnt is required for further differentiation of the tip cell (Samakovlis et al., 1996; Ghabrial and

Krasnow; 2006). Our studies of *jing2.8-lacZ* expression pattern clearly show the highest level of enhancer activation at the tips of the tracheal branches, suggesting that *jing* is expressed most strongly in the highly migratory regions with the highest level of *btl* expression.

FGFR signaling regulates the transcription of many genes (Ohshiro et al., 2002). In the developing trachea, *btl* expression is amplified through a positive feedback loop involving activation of Pnt and regulation of its own expression in the dorsal, ventral, and visceral branches (Ohshiro et al., 2002). In particular, *btl* is required for *pnt* expression in the tip cells and the fusion cells of the dorsal branch. The expression pattern of *jing2.8-lacZ* reporter at the tips of primary tracheal branches suggested that Jing is present where Btl function is most needed. Furthermore, regulation of the *jing1.5-lacZ* reporter suggested that *jing* may be regulated by *btl* since *btl* is a target of Trh::Tgo (Ohshiro and Saigo, 1997).

The ligand of *btl* is the chemoattractant *bnl*, which activates the expression of many genes at later developmental stages such as *btl, pnt,* and *pruned DSRF*, and controls gene expression in tracheal tips (Ohshiro et al., 2002). Thus, regulation of *jing1.5-lacZ* reporter expression by *bnl* in tracheal fusion cells is consistent with *jing* functioning in the Btl signaling pathway. Supporting evidence includes the requirement of Pnt for *jing* expression. Significant reductions were observed in endogenous *jing* mRNA in the trachea of homozygous *pnt* mutants indicating that *jing* requires *pnt* transcriptional regulation. Reduction in the widespread tracheal expression of *jing* mRNA in *pnt* mutants suggests a regulation of *jing* not only in fusion cells, but possibly also in the tip cells. In addition, the integrity of putative Pnt DNA binding sites was required for maximal *jing1.5-lacZ* enhancer activity (Ohshiro et al., 2002). Pnt and Aop/Yan compete for the common ETS binding target sites, GGA, in order to regulate gene expression (Gabay et al., 1996). Seventeen (17) GGA sites are present in the 1.5kb sequence but only one region is associated with an inverted repeat (TCC..GGA) similar to the site bound by Aop/Yan (Ohshiro et al., 2002). One or more of the additional ETS DNA binding sites in the 1.5kb *jing* regulatory region may be functional given the persistence of weak *jing1.5ΔPnt-lacZ* expression in the tracheal fusion cells. *jing1.5-lacZ* expression was

also significantly reduced in mutants homozygous for multiple *btl* pathway genes as well as dfr^{B129} and rho^{7M}.

Previous studies have shown that *pnt* is required for maximal *btl* expression during primary branching (Ohshiro et al., 2002; Zelzer and Shilo, 2000). We show that a *pnt* mutant reduces *jing* expression specifically in fusion cells. We therefore proposed that *jing* may regulate *btl* expression given the putative trans-activation domains in its amino terminus and its three potential DNA binding zinc fingers. We showed that *jing* is required for *btl* expression *in vivo* using in situ hybridization. Reducing *jing* expression by using *jing* RNA interference construct targeted to tracheal cells or $jing^3$ mutant background indicates an autonomous role for *jing* in *btl* activation in most tracheal branches. In the ganglionic branches, some cells with reduced *btl* mRNA expression were observed close to but not at their final destination in the ventral nerve cord. This reveals that the migration machinery was intact but not functioning properly. The truncated ganglionic branches and corresponding reductions in *btl* expression in $jing^3$ homozygotes are consistent with this proposal. In *jing* mutants, dorsal branches were present but their rates of cell migration were reduced and there were changes in their migratory directionality. Reducing *jing* expression by RNAi resulted in the crossing of tracheal metamere boundaries by dorsal branches once they have reached the dorsal midline. The basis for this phenotype is not known but it may be due to the lack of response to guidance cues at the dorsal midline or defects in additional signaling pathway working together with Btl to control tracheal branch migration.

Jing regulatory function is responsive to EGFR signaling in trachea and CNS midline

Dfr/vvl is required for the expression of *rho* and the Dpp receptor, *thick veins*, in the tracheal pits (Llimargas and Casanova, 1997; Zelzer and Shilo, 2000). Jing is present in the tracheal pits and we show that EGFR-mediated tyrosine kinase signaling regulates *jing* expression and vice versa (Sonnenfeld et al.,

2004). *jing1.5-lacZ* expression was significantly reduced in mutants homozygous for EGFR pathway genes dfr^{B129} and rho^{7M}. In the CNS midline glia, *jing1.5-lacZ* expression was activated in the additional glial cells occurring due to the stimulation of the EGFR signaling through over-expression of the secreted *spitz* (*sspitz*) or *rhomboid* (*rho*). To conclude, *jing* is responsive to EGFR signaling in both trachea and CNS midline.

Conclusions

1.5 kb fragment of the *jing* 5' regulatory region drives reporter expression into the tracheal fusion cells, midline, and CNS

2.8 kb fragment of the *jing* 5'regulatory region contains additional binding sites involved in the activation of *jing* expression in the trachea. It drives expression into all tracheal lineages, midline, CNS, and segmental ectodermal stripes

1.3 kb fragment of *jing* 5'regulatory region contains sites required for *jing* expression in the segmental ectodermal stripes, and for midline and tracheal expression of *jing*

Trachealess and Tango are required for *jing1.5-lacZ* expression in vivo

Drifter/Vvl is required for *jing1.5-lacZ* expression in vivo

Pointed is required for *jing1.5-lacZ* expression in vivo

Integrity of the Pointed binding sites is required for *jing1.5-lacZ* expression in vivo

jing is responsive to tyrosine kinase signaling in the trachea and in the midline glia

jing function is required for proper cell migration and tubulogenesis

jing is required autonomously for maximal *btl* expression during primary branching

Future goals

The sites required for the endogenous *jing* expression in the segmental ectodermal stripes can be identified using the data from *jing1.3-lacZ* reporter studies. The 1.3 kb *jing* 5' regulatory region can be further dissected to localize the sequences required for the induction of the expression in the segmental stripes pattern.

The midline expression patterns of the *jing1.5-lacZ* and *jing1.3-lacZ* reporters can be compared. The CME-independent midline expression of the *jing1.3-lacZ* reporter raises the question which transcription factors regulate late neuronal midline expression of *jing* independently from Sim::Tgo heterodimers. Among other transcription factors activating midline expression we propose to investigate Pointed, Aop, and Dfr/Vvl. The *jing1.3-lacZ* reporter will be stably incorporated into the respective mutant backgrounds and the resulting midline phenotypes determined.

Involvement of other transcription factors such as Spalt into the regulation of *jing* expression in the trachea can be verified using deletion site analysis. Spalt consensus binding sites can be removed from 1.5 kb and 2.8 kb fragments of *jing 5'* regulatory region and the resulting tracheal phenotypes can be analyzed. *jing1.5-lacZ* and *jing2.8-lacZ* reporters also can be stably incorporated into the *spalt* mutant backgrounds.

References

Anderson M.G., Certel S.J., Certel K., Lee T., Montell D.J., and Johnson W.A. Function of the *Drosophila* POU domain transcription factor Drifter as an upstream regulator of Breathless receptor tyrosine kinase in developing trachea. Development, 122, 4169-4178, 1996.

Beitel G.J. and Krasnow M.A. Genetic control of epithelial tube size in the *Drosophila* tracheal system. Development 127, 3271-3282, 2000.

Bellusci S., Grindley J., Emoto H., Itoh N. And Hogan B.L.M. Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development. 1997. 124: 4867-4878.

Blume-Jensen P., Hunter T. 2001. Oncogenic kinase signaling. Nature 17, 355-365.

Boube M., Llimargas M., Casanova J. Cross-regulatory interactions among tracheal genes support a co-operative model for the induction of tracheal fates in the *Drosophila* embryo. Mechanisms of Development, 91, 271-278, 2000.

Brand A.H. and Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development, 118, 401-415, 1993.

Brodu V. and Casanove J. The RhoGAP *crossveinless-c* links *trachealess* and EGFR signaling to cell shape remodeling in *Drosophila* tracheal invagination. 2006. Genes Dev. 20: 1817-1828.

Bellusci S., Grindley J., Emoto H., Itoh N. And Hogan B.L.M. Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development. 1997. 124: 4867-4878.

Celli G., LaRochelle W.J., Mackem S., Sharp R., and Merlino G. Soluble dominant-negative receptor uncovers essential roles for fibroblast growth factors in multi-organ induction and patterning. EMBO J. 17, 1642-1655, 1998.

Crews S.T. Control of cell lineage-specific development and transcription by bHLH-PAS proteins. Genes and Development. 1998. 12: 607-620.

Culi J, Aroca P, Modolell J, Mann RS. Jing is required for wing development and to establish the proximodistal axis of the leg in *Drosophila melanogaster. Genetics.* 2006; 173:255-266.

Desplan C., Theis J., O'Farrell P.H. The sequence specificity of homeodomain-DNA interaction. Cell. 1988 Sep 23; 54 (7): 1081-1090.

Duchek P., Rorth P. Guidance of cell migration by EGF receptor signaling during *Drosophila* oogenesis. Science, 2001, vol. 291, no. 5501, pp. 131-133.

Emmons R.B., Duncan D., Estes P.A., Kiefel P., Mosher J.T., Sonnenfeld M., Ward M.P., Duncan I., Crews S.T. The Spineless-Aristapedia and Tango bHLH-PAS proteins interact to control antennal and tarsal development in *Drosophila.* Development 126, 3937-3945, 1999.

Fuse N., Hirose S., and Hayashi S. Diploidy of *Drosophila* imaginal cells is maintained by transcriptional repressor encoded by escargot. 1994. Genes Dev. 8, 2270-2281.

Gabay L., Scholz H., Golembo M., Klaes A., Shilo B-Z., and Klambt C. EGF receptor signaling induces *pointed P1* transcription and inactivates Yan protein in the *Drosophila* embryonic ventral ectoderm. Development. 122. 3355-3362. 1996.

Gabay L., Seger R., Shilo B-Z. In situ activation pattern of Drosophila EGF receptor pathway during development. Science, vol.277, 1103-1106, 22 Aug 1997.

Gabay L., Seger R., Shilo B-Z. MAP kinase in situ activation atlas during *Drosophila* embryogenesis. Development, 124, 3535-3541, 1997.

Gaul P., Giordano S., Williams T.A., Rocchi S., Van Obberghen E., Comoglio P.M. 2000. Sustained recruitment of phospholipase C-gamma to Gab1 is required for HGF-induced branching tubulogenesis. Oncogene 19, 1509-1518.

Ghabrial A.S. and Krasnow M.A. Social interactions among epithelial cells during tracheal branching morphogenesis., Nature, vol.441, 8 June 2006.

Glazer L. and Shilo B.Z. The Drosophila FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. 2001. Genes Dev 5, 697-705.

Goodman, C.S., Doe, C.Q. (1993). Embryonic development of the Drosophila central nervous system. Bate, Martinez Arias, 1993 : 1131--1206.

He J.P., Kim S., and Ro H.S. Cloning and characterization of a novel zinc finger transcriptional repressor. A direct role of the zinc finger motif in repression. J. Biol. Chem., 274, 14678-14684, 1999.

Hogan B.L.M., and Yingling J.M. Epithelial/mesenchymal interactions and branching morphogenesis of the lung. Curr. Opin. Genet. Dev. 1998, 8, 481-486.

Ikeya T. and Hayashi S. Interplay of Notch and FGF signaling restricts cell fate and MAPK activation in *Drosophila* trachea. Development. 1999, 126, 4455-4463.

Isaac D.D., Andrew D.J. Tubulogenesis in *Drosophila*: a requirement for the trachealess gene product. Genes Dev. 1996 Jan 1; 10(1): 103-17.

Ishibe S., Joly D., Zhu X., Cantley L.G. 2003. Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis. Mol Cell 12, 1275-1285.

Jacobs JR. The midline glia of Drosophila: a model genetic model for the developmental functions of glia. Progress in Neurobiology, 2000, December, vol. 62, issue 5, pp.475-508.

Jiang L. and Crews S.T. The *Drosophila dysfusion* Basic Helix-Loop-Helix (bHLH)-PAS gene controls tracheal fusion and levels of the Trachealess bHLH-PAS protein. Molecular and Cellular Biology. 2003 Aug; p. 5625-5637.

Karihaloo A., Kale S., Rosenblum N.D., Cantley L.G. 2004. Hepatocyte growth factor-mediated renal epithelial branching morphogenesis is regulated by glypican-4 expression. Mol. Cell Biol 24, 8745-8752.

Karihaloo A., Nickel C., Cantley L.G. 2005. Signals which build a tubule. Expt Neph 100, e40-e45.

Kidd T, Bland KS, Goodman CS. Slit is the midline repellent for the robo receptor in Drosophila. 1999, Cell, Mar.19; 96(6): 785-94

Kim H., Kang K., and Kim J. AEBP2 as a potential targeting protein for Polycomb repression complex PRC2. Nucleic Acids Res., 2009, 37(9): 2940-2950.

Klambt C. The Drosophila gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. Development. 1993. 117. 163-176.

Lee T., Feig L., Montell D.J. Two distinct roles for Ras in a developmentally regulated cell migration. Development, 1996. 122(2):409-18.

Lehninger A.L., Nelson D.L., and Cox M.M. Principles of Biochemistry. Second edition. Worth Publishers. New York. 1998.

Liu Y. and Montell D.J. *jing*: a downstream target of *slbo* required for developmental control of border cell migration. Development. 2001. 128. 321-330.

Llimargas M. and Casanova J. Ventral veinless, a POU domain transcription factor, regulates different transduction pathways required for tracheal branching in *Drosophila*. Development. 1997. 124. 3273-3281.

Llimargas M. and Casanova J. EGF signaling regulates cell invagination as well as cell migration during formation of tracheal system in *Drosophila*. Dev. Genes Evol. 1999. 209. 174-179.

Ma Y., Certel K., Gao Y., Niemitz E., Mosher J., Mukherjee A., Mutsuddi M., Huseinovic N., Crews S.T., Johnson W.A., Nambu J.R. 2000. Functional interactions between Drosophila bHLH/PAS, Sox and Pou transcription factors regulate CNS midline expression of the *slit* gene. J. Neuroscience 20, 4596-4605.

Manning G. and Krasnow M.A. Development of the *Drosophila* tracheal system. In the *Development of Drosophila* (ed. A. Martinez-Arias and M. Bate), Vol. 1, pp. 609-685. Cold Spring Harbor, New York. Cold Spring Harbor Laboratory Press.

Marshall C.J. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80, 179-185.

Metzger R.J. and Krasnow M.A. (1999). Genetic control of branching morphogenesis. Science 284, 1635-1639.

Miettinen P.J., Berger J.E., Meneses J., Phung Y., Pedersen R.A., Werb Z., Derynck R. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. 1995. Nature. 376, 337-341.

Miettinen P.J., Warburton D., Bu D., Zhao J.S., Berger J.E., Minoo P., Koivisto T., Allen L., Dobbs L., Werb Z. and Derynck R. Impaired lung branching morphogenesis in the absence of functional EGF receptor. 1997. Dev. Biol. 186, 224-236.

Min H., Danilenko D.M., Scully S.A., Bolon B., Ring B.D., Tarpley J.E., DeRose M. and Simonet W.S., Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. Genes Dev. 12, 3156-3161. 1998.

Mitchell P.J. and Tjian R. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science, vol. 245, 371-378, 1989.

Mortimer N.T. and Moberg K.H. The drosophila F-box protein Archipelago controls levels of the Trachealess transcription factor in the embryonic tracheal system. Developmental Biology, 312, 560-571, 2007.

Murphy A.M., Lee T., Andrews C.M., Shilo B.Z. and Montell D.J. The breathless FGF receptor homolog, a downstream target of Drosophila C/EBP in the developmental control of cell migration. Development, 1995, 121(8): 2255-63.

Naar A.M., Lemon B.D., and Tjian R. Transcriptional coactivator complexes. Annu. Rev. Biochem. 2001. 70: 475501.

Nambu, J. R., Franks, R. G., Hu, S., and Crews, S. T. (1990). The *single-minded* gene of Drosophila is required for the expression of genes important for the development of CNS midline cells. *Cell* 63, 63-75.

Nambu, J. R., Lewis, J. L., and Crews, S. T. (1993). The development and function of the *Drosophila* CNS midline cells. Comparative Biochemistry and Physiology 104A, 399-409.

Nambu, J. R., Lewis, J. L., Wharton K.A., Jr and Crews, S. T. (1991). The *Drosophila* single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of the CNS midline development. Cell 67, 1-20.

Niewiadomska H., Godt D., Tepass U. DE-Cadherin is required for intercellular motility during *Drosophila* oogenesis. J.Cell Biol. 1999. Feb. 8; 144(3):533-47.

Nye J.A., Petersen J.M., Gunther C.V., Jonsen M.D., Graves B.J. Interaction of murine Ets- 1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. Genes Dev., 6, 975-990, 1992.

Ohshiro T., Emori Y., Saigo K. Ligand-dependent activation of *breathless* FGF receptor gene in *Drosophila* developing trachea. Mechanisms of Development, 114, 3-11, 2002.

Ohshiro T., Saigo K. Transcriptional regulation of breathless FGF receptor gene by binding of TRACHEALESS/dARNT heterodimers to three central midline elements in *Drosophila* developing trachea. Development. 1997 Oct; 124(20):3975-86.

Peters K., Werner S., Liao X., Wert S., Whitsett J., Williams L. Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of mouse lung. EMBO J, 13, 3296-3301, 1994.

Poole S.J., Kauvar L.M., Drees B., Kornberg T. The engrailed locus of *Drosophila*: structural analysis of an embryonic transcript. Cell. 1985 Jan; 40(1): 37-43.

Ribeiro C, Ebner A, Affolter M. *in vivo* imaging reveals different cellular functions for FGF and Dpp signaling in tracheal branching morphogenesis. Dev. Cell 2, 677-683, 2002.

Risau W. Mechanism of angiogenesis. 1997. Nature. 386: 671-674.

Ro H-S. and Roncari D.A.K. The C/EBP-binding region and adjacent sites regulate expression of the adipose P2 gene in human preadipocytes. Mol and Cell Biology, Apr.1991, p.2303-2306.

Rubin G.M. and Spradling A.C. Genetic transformation of Drosophila with transposable element vectors. Science. Vol.218, 1983, pp.348-353.

Samakovlis C., Manning G., Steneberg P., Hacohen N., Cantera R. And Krasnow M.A. 1996. Genetic control of epithelial tube fusion during *Drosophila* tracheal development. Development. 122. 1395-1407.

Sawano A., Takayama S., Matsuda M., miyawaki A. 2002. Lateral propagation of EGF signaling after local stimulation is dependent on receptor density. Dev. Cell 3, 245-257.

Sedaghat Y., Sonnenfeld M. The jing gene is required for embryonic brain development in *Drosophila*. Dev Genes Evol. 2002 Jul; 212(6): 277-287.

Sedaghat Y., Miranda W.F., Sonnenfeld M.J. The jing Zn-finger transcription factor is a mediator of cellular differentiation in the *Drosophila* CNS midline and trachea. Development. 2002 Jun; 129(11): 2591-606.

Schlessinger J. Common and distinct elements in cellular signaling via EGF and FGF receptors. 2004. Science. V.306: 1506-1507.

Schwartz Y.B., Pirrotta V. Polycomb silencing mechanisms and the management of genomic programmes. Nat Rev Genet. 2007 Jan, 8(1):9-22. Review.

Spilde T.L., Bhatia A.M., Mehta S.S., Hembree M.J., Preuett B.L., Ostlie D.J., Prasadan K., Li Z., Snyder C.L., Gittes G.K. Aberrant fibroblast growth factor receptor 2 signaling in esophageal atresia with tracheoesophageal fistula. J. Pediatr. Surg. Jun; 38(6):953-6, 2004.

Spilde T.L., Bhatia A.M., Mehta S., Ostile D.J., Hembree M.J., Preuett B.L., Prasadan K., Li Z., Snyder C.L., Gittes G.K. Defective sonic hedgehog signaling in esophageal atresia with tracheoesophageal fistula. Surgery, Aug;134(2):345-50, 2003.

Spradling A.C. and Rubin G.M. Transposition of cloned P elements into *Drosophila* germ line chromosomes. Science 218, 341-347.

Stemerdink C., Jacobs J.R. Argos and Spitz group genes function to regulate midline glial cell number in *Drosophila* embryos. Development, 124, 3787-3796, 1997.

Sonnenfeld M.J., Jacobs J.R. Mesectodermal cell fate analysis in *Drosophila* midline mutants. Mech. Dev., 46, 3-13, 1994.

Sonnenfeld M.J. and Jacobs J.R. Apoptosis of the midline glia during *Drosophila* embryogenesis: a correlation with axon contact. Development, 121, 569-578, 1995.

Sonnenfeld M., Ward M., Nystrom G., Mosher J., Stahl S., Crews S. The *Drosophila* tango gene encodes a bHLH-PAS protein that is orthologous to mammalian Arnt and controls CNS midline and tracheal development. Development. 1997 Nov; 124(22): 4571-82.

Sonnenfeld M.J., Barazesh N., Sedaghat Y., Fan C. The jing and ras 1 pathways are functionally related during CNS midline and tracheal development. Mech Dev. 2004 Dec; 121(12): 1531-47.

Sun X., Morozova T. and Sonnenfeld M. Glial and neuronal functions of the *Drosophila* homolog of the human SWI/SNF gene *ATR-X* (*DATR-X*) and the *jing* zinc-finger gene specify the lateral positioning of longitudinal glia and axons. Genetics. 2006. 173: 1-17.

Sutherland D., Samakovlis C., and Krasnow M.A. branchless encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. Cell 87, 1091-1101, 1996.

Tanaka-Matakatsu M., Uemura T., Oda H., Takeichi M. and Hayashi S. 1996. Cadherin-mediated cell adhesion and cell motility in *Drosophila* trachea regulated by the transcription factor Escargot. Development. 122. 3697-3705.

Ueland J.M., Gwira J., Liu Z.X., Cantley L.G. 2004. The chemokine KC regulates HGF-stimulated epithelial cell morphogenesis. Am. J.Physiol. renal. Physiol. 286. F581-F589.

Verveer P.J., Wouters F.S., Reynolds A.R., Bastiaens P.I.H. 2000. Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane. Science 290, 567-570.

Wappner P., Gabay L., and Shilo B.Z. Interactions between the EGF receptor and DPP pathways establish distinct cell fates in the tracheal placodes. Development. 1997. 124. 4707-4716.

Warburton D., Schwarz M., Tefft D., Flores-Delgado G., Anderson K.D. and Cardoso W.V. The molecular basis of lung morphogenesis. 2000. Mech Dev. 92, 55-81.

Ward M.P., Mosher J.T., Crews S.T. Regulation of *Drosophila* bHLH-PAS protein cellular localization during embryogenesis. Development 125, 1599-1608, 1998.

Wilk R., Wezman I., Glazer L., and Shilo B-Z. *trachealess* encodes a bHLH-PAS protein and is a master regulator gene in the Drosophila tracheal system. Genes Dev. 10, 93-102, 1996.

Zelzer E., Shilo B-Z. Interaction between the bHLH-PAS protein Trachealess and the POU-domain protein Drifter, specifies tracheal cell fates. Mechanisms of Development, 91, 163-173, 2000.

Zelzer E., Wappner P., Shilo B-Z. The PAS domain confers target gene specificity of *Drosophila* bHLH-PAS proteins. Genes Dev. 11, 2079-2089, 1997.