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

Yalda Sedaghat
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Cellular and Molecular Medicine)
GRADE / DEGREE

Department of Cellular and Molecular Medicine
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Molecular Genetic Characterization of *Jing* during *Drosophila* Embryonic Central Nervous System and Tracheal System Development

TITRE DE LA THÈSE / TITLE OF THESIS

Margaret Sonnenfeld
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

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

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Antonio Colavita

Marc Ekker

Henry Krause

Alexander Mackenzie

Gary W. Slater
LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES /
DEAN OF THE FACULTY OF GRADUATE AND POSTDOCORAL STUDIES

MOLECULAR GENETIC CHARACTERIZATION OF *JING* DURING *DROSOPHILA* EMBRYONIC CENTRAL NERVOUS SYSTEM AND TRACHEAL SYSTEM DEVELOPMENT

Yalda Sedaghat

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in
Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

Your file Votre référence
ISBN: 0-494-11019-8
Our file Notre référence
ISBN: 0-494-11019-8

Dedication

This thesis is dedicated to my parents.

Acknowledgements

I am indebted to the many people who have influenced and inspired me both scientifically and personally. I would like to thank my supervisor, Dr. Margaret Sonnenfeld for her guidance during the course of my studies. I would also like to thank members of my advisory committee, Dr. Luc Sabourin, Dr. Marie-Andree Akimenko, Dr. David Park and especially Dr. Valerie Wallace for her guidance and support during the course of writing my thesis.

I would like to thank Dr. Ruth Slack and Dr. Jean Marc Renaud for their insightful guidance and support during the course of my studies, as well as other members of the department Dr. Paul Albert, Dr. Stephen Gee, Dr. Stephen Lee, Dr. John Copeland, and Dr. Robert Haché from BMI department for their advise, discussion, time and effort. I would also like to thank members of Dr. Albert's lab especially Neena Kushwaha and Anastasia Rogaeva, who were always available for great scientific discussion, advice and guidance.

Wilson Miranda was an excellent mentor in the lab, who always offered guidance, advice and assistance. Cathy Fan, Nasrin Barazesh, Xuetao Sun and Chris Delvecchio were great colleagues as well as good friends, and were always available for great discussions, as well as other members of the lab, Carla Wood, Nikia Kearns, Helga Agah, Vanessa Scanga, Momena Davood, Paymaan JafarNejad, and Tatiana Morozova. Gillian Lord, Donna Hooper, Sylvie Deblois, Denyse Blais, Claudine Tateham and Blanche Dinelle have always been more than helpful by providing an excellent environment and friendship. I would like to thank student representatives Kelly McClellan and Margaret Czesak for their support. Kim Wong and Rainy Tang have always provided great

would also like to thank George Chouchani from Carleton University for his guidance during the course of writing my thesis.

There have been many people in CMM department and department of BMI, both past and present, who have helped to create a pleasant and fun working environment and offered guidance and advice, Nima Niknejad, Hanan Abramovici and Rosa Guzzo, to name a few.

This work wouldn't have been possible without the love, encouragement and support of my parents, other members of my family and friends.

Abstract

The aim of the thesis was to study the molecular genetic and function of *jing*, a novel gene having important role in central nervous system (CNS) and respiratory (tracheal) system development during *Drosophila* embryogenesis.

We established by sequencing the gene that *jing* has three C_2H_2 zinc finger motifs, and several putative transcriptional regulatory sequences. *jing* plays an essential role in controlling CNS midline and tracheal cell differentiation. Mutation results in the appearance of a premature stop codon in the middle of the second zinc finger decreases survival ratio in those cells. This effect appears to be a result of the functional relationship between *jing* and the *Egfr* (*Epidermal growth factor receptor*) pathway in the developing CNS midline and trachea. *jing* is also a target of Trachealess:: Tango (Trh::Tgo) heterodimers, two members of the bHLH-PAS transcription factors, which play essential role in the development of CNS and trachea. However, *jing* expression is under multiple control mechanisms including auto-inhibition, inhibition by the POU domain transcription factor Drifter and the ETS transcription factor Aop. On the other hand, *jing* is required for the balance between repression and activation that regulates Trh- and Tgo-mediated *btl* transcription and subsequent growth factor levels to control signaling during the primary and secondary branching processes in the respiratory system.

In conclusion, *jing* is a downstream target of Trh::Tgo complex and its function is required for CNS midline and tracheal cell development during *Drosophila* embryogenesis.

Table of Contents

Chapter Two

"The *jing* Zn-finger transcription factor is a mediator of cellular differentiation in the *Drosophila* CNS midline and trachea"

Chapter Three

Chapter Four

List of Abbreviations

AC	Anterior Commissure
ac	*achaete*
armadillo	*arm*
Arnt	Aromatic hydrocarbon Receptor Nuclear Translocator
bnl	*branchless*
BHLH-PAS	basic-helix-loop-helix-PAS
btd	*buttonhead*
btl	*breathless*
cas	*castor*
CNS	Central Nervous System
comm.	*commissureless*
CA (C)	Contralateral Axons
crb	*crumbs*
dpp	*decapentaplegic*
dl	*delta*
DC	Deutocerebral
DE-cadherin	DE-cad
discs lost	*dlt*
esg	*escargot*
EMS	Ethyl Methane Sulphonate
ems	*empty spiracles*

en	*engrailed*
ER	Endoplasmic Reticulum
fas I	*fasciclin I*
fas II	*fasciclin II*
FGF	Fibroblast Growth Factor
FGF-R	Fibroblast Growth Factor Receptor
fra	*frazzled*
GMC	Ganglion Mother Cell
gh	*grainyhead*
glial cells missing	*gcm*
hb	*hunchback*
IA (I)	Ipsilateral Axons
jing	*jing*
kr	*kruppel*
LC	Longitudinal Connectives
l'sc	*lethal of scute*
MGA	midline glia anterior
MGM	midline glia medial
MGP	midline glia posterior
MAPK	Mitogen-activated protein kinase
MA (M)	Motor Axons
NB	Neuroblast
n	*notch*

net	*netrin*
otd	*orthodenticle*
PC	Posterior Commissure
PNS	Peripheral Nervous System
PC	protocerebral
pnt	*pointed*
period	*per*
robo	*roundabout*
sty	*sprouty*
slt	*slit*
sim	*single-minded*
slp	*sloppy paired*
sc	*scute*
SJ	septate junction
SAC	sub-apical complex
stardust	*sdt*
trh	*trachealess*
tgo	*tango*
tll	*tailless*
TC	Tritocerebral
TGF	Transforming Growth Factor
TJ	tight junction
UAS	Upstream Activation Sequence

VNC	Ventral Nerve Cord
VUM	Ventral Unpaired Median neurons
wg	*wingless*
ZA	Zonula Adherens

Table of Figures

Chapter One

Chapter Two

Chapter Three

Chapter Four

Chapter Seven

Appendices

Preface

The experimental results are presented in manuscript format, in accordance with the guidelines set forth by the University of Ottawa Faculty of Graduate Studies and Research. Experimental findings are preceded by an overview of *jing* expression and its requirement for central nervous system and trachea during *Drosophila* embryonic development (Chapter Two). Proper function and dosage of *jing* in embryonic brain scaffold patterning and neuronal and glial cell survival are introduced in Chapter Three. In Chapter Four, molecular, biochemical and genetic studies show that *jing* is transcriptionally regulated by two members of bHLH-PAS transcription factors, Trachealess and Tango. The role of Jing in the regulation of *breathless* expression and the consequences of *jing* loss-and gain-of-function in tracheal cell fusion are also introduced in Chapter Four. The relationship between *jing* and the *ras1* pathway is discussed in Chapter Seven (appendices).

Chapter One

General Introduction

General Introduction

A. *Drosophila* as a model system

In order to gain molecular insight into developmental procedures, one should use a model system that is tractable with genetic analysis. Small organisms with short generation times are well-suited for genetic studies. One of the most popular model organisms possessing strong advantages for experimental research is the fruit fly, *Drosophila melanogaster*.

There are a variety of studies currently underway that may validate the fruit fly as an *in vivo* model for analyzing genes involved in different aspects of development. Many mutations in conserved genetic pathways have been found, including those controlling development and physiology. Since homologous genes control early developmental events as well as functional components of *Drosophila* and vertebrate, the fly is the simplest existing model system that can be used to assay genes involved in different pathways.

A. 1. Advantages of using *Drosophila*

The evolutionary conservation of the development and function of the fly system, in addition to the genetic tools for studying fundamental processes governed by gene function and multigenic interactions all provide strong advantages for *Drosophila* as a model system.

Drosophila is an easy-to-handle and a well understood model system. It is small, with a short life cycle of just two weeks, and is affordable and simple to keep in vast numbers. Mutant flies containing defects in any of several thousand genes are available. In addition, the entire genome has recently been sequenced. This broad variety of genetic

tools available to *Drosophila* research offers many technical advantages for rapid screening through large numbers of genes. Thus, it is important to use *Drosophila* as a vehicle for analyzing genetic pathways in humans.

A. 1. i. Life cycle of *Drosophila*

The *Drosophila* egg is half a millimeter long. At 25°C, it takes one day after fertilization for the embryo to develop and hatch into larva. The larva eats and grows continuously for four days after hatching (first, second and third instars). After two days as a third instar larva, it moults one more time to form an immobile pupa. Over the next four days, the body is completely remodeled to give the adult winged form, which then hatches from the pupal case and is fertile within about 12 hours.

A. 2. Available genetic methods in *Drosophila* system

There are many specific genetic approaches available in the *Drosophila* model system. These include chemical mutagenesis, misexpression strategy by using Gal4/UAS system and P-element enhancer trap detection system, which examine gene function.

A. 2. i. Chemical mutagenesis

Chemical mutagenesis is the most advantageous with a high mutation rate and wide target range. The most efficient method to make mutations in *Drosophila* is to feed flies with ethyl methane sulphonate (EMS) (Lewis and Bacher, 1968). EMS is an alkylating agent and the most commonly used mutagen in *Drosophila* since it is easy to apply and causes the highest frequency of mutations. EMS induces point mutations (single-base changes), which interrupt gene function by causing missense or nonsense mutations.

Since most G/C base pairs are potential targets for EMS mutagenesis, the possibility of inducing a mutation in a particular gene can be related to the size of the gene.

A. 2. ii. Misexpression strategy by using GAL4/UAS

Expressing a gene in cells in which it is not normally expressed is a compelling way of determining its function because the identity of the cell is fixed by its gene expression. Normally, *Drosophila* genes are characterized by loss-of-function phenotypes; however, studying ectopic expression phenotypes can be valuable as well. In the case of genetic redundancy, where there is no loss-of-function phenotype available, ectopic expression can provide the functional information.

The first step is to generate flies that express a transcriptional activator in a variety of patterns. Use of a transcriptional activator that has no endogenous targets in *Drosophila* is critical to ensure that the system will allow only the ectopic expression of the gene of interest. The GAL4 system allows the selective expression of any cloned gene in a wide variety of cell- and tissue-specific patterns in *Drosophila* and the expression of yeast transcriptional activator GAL4 can be directed by the promoter of interest. GAL4 in turn directs transcription of the GAL4-responsive (UAS) target gene in an identical pattern. The system's key feature is that the GAL4 gene and UAS–target gene are initially separated into two distinct transgenic lines. In the GAL4 line, the activator protein is present, but has no target gene to activate. In the UAS–target gene line, the target gene is silent because the activator is absent. It is only when the GAL4 line is crossed to the UAS–target gene line that the target gene is turned on in the progeny. Meanwhile, the *Drosophila* system allows the rapid generation of the lines in which the ectopic expression of the gene of interest can be tested in different cell types (fig. 1) (Brand and

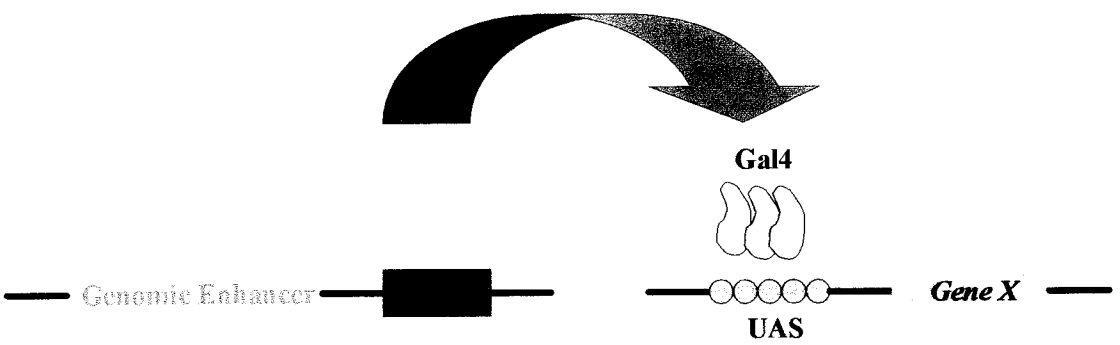


Figure 1. Misexpression strategy by using Gal4/UAS system. The GAL4 system allows the selective expression of any cloned gene in a wide variety of cell- and tissue-specific patterns in *Drosophila* and the expression of yeast transcriptional activator Gal4 can be directed by the promoter of interest. GAL4 in turn directs transcription of the GAL4-responsive (UAS) target gene in an identical pattern. (Adapted and modified from Brand and Perrimon, 1993).

Perrimon, 1993). This UAS line, in the absence of GAL4, will be viable even if the ectopic expression of the target gene is lethal.

A. 2. ii. a. Vectors for directed gene expression

Two Gal4 vectors were designed to target GAL4 expression to specific cell types, pGaTB and pGawB. To target the expression to a particular tissue, promoters can be cloned upstream of Gal4 in pGaTB vector. To direct the expression of GAL4 in a specific location on the genome, a pGawB enhancer detection vector can be used. The GAL4 binding sites are available in pUAST vector. This vector is designed to direct GAL4-dependent transcription of the gene of interest. There are five tandemly arrayed and optimized GAL4 binding sites located in pUAST, followed by the sequence of the gene of choice (Brand and Perrimon, 1993).

This system can be used not only to misexpress a fly gene in a particular tissue, but moreover, to obtain loss-of-function information by targeting a disrupted form of a gene. Knockdown transgenic flies can be generated by using RNA interference (RNAi) strategy in a GAL4/UAS binary system. On the other hand, some human diseases can be modeled in *Drosophila* by using a targeted mutation of homologous genes in *Drosophila* or by expressing a human transgene in *Drosophila* (Wittmann et al., 2001, Muqit and Feany, 2002, Chen and Feany, 2005). The fact that the fly is capable of recapitulating similar phenotypes as that of humans, the use of Drosophila allows us to provide more solid data by taking advantage of genetic analysis in order to understand the underlying pathways. This can be possible by applying the GAL4/UAS system and generating transgenic flies carrying human genes.

A. 2. iii. P-element enhancer trap detection system

The P-element has been one of the main tools of *Drosophila* genetics since it was developed for transgenesis in 1982 (Rubin and Spradling, 1982). The subsequent development of a variety of systems based on the transposon have provided a range of powerful and flexible tools for genetics. Transposable elements, which are sequences of DNA and move within their host genome, were discovered in 1940s by Barbara McClintock. *Drosophila* has one of the best characterized transposons known as P-element. P-element transposition occurs by the transposase enzyme through a cut and paste mechanism (Kaufman and Rio, 1992).

Molecular genetic studies of embryogenesis in *Drosophila* have played an important role in our understanding of similar developmental processes in higher organisms. To have a better understanding of a gene function and its effects on the development of a specific tissue, suitable markers should be made to recognize the defects caused by the malfunction of the gene of interest. In some cases, antibodies generated against particular gene products serve as good markers to analyze gene function. Another method to generate cell specific markers in *Drosophila* is a system that allows us to use *β-galactosidase (lacZ)* gene of *E. coli* as a reporter gene (O'Kane and Gehring, 1987; Wilson et al., 1989). In this system the *lacZ* gene is fused to the *P-transposase* gene and the *β-galactosidase* expression is under the control of the *transposase* promoter. This fusion is part of a P-element construct.

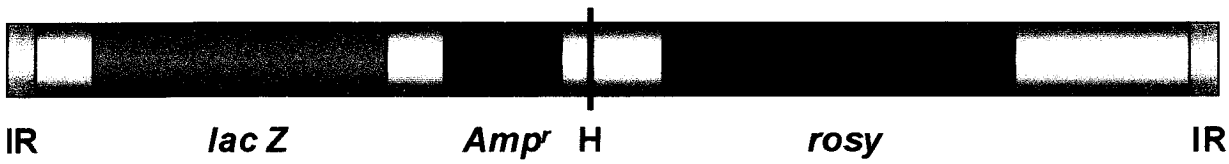


Figure 2. Structure of the P-element enhancer detector. The key features of the P-element are shown. It contains the *lacZ* fusion gene from an in-frame translational fusion of the *E. coli lacZ* reporter gene, an ampicillin resistance gene, the *rosy* gene as an eye color marker, *Hind*III (H) restriction site (the enzyme used for plasmid rescue) and inverted repeat (IR) motifs. The P-elelment is usually 10-11 Kb in size (O'Kane and Gehring, 1987; Wilson et al., 1989).

The construct carries an eye color marker (e.g. *rosy* depending on the type of the enhancer-trap construct) and an antibiotic resistance gene (fig. 2). The P-element construct can then be integrated to different locations in the genome. The P-transposase promoter can be affected by genomic regulatory elements, which can regulate the expression of the fusion gene. It seems that a large variety of the regulatory elements are enhancers and therefore this is known as the P-element-mediated enhancer trap detection system (Bellen et al., 1989). The protein expression pattern of the endogenous target gene, and *lacZ* reporter gene, are overlapping in early embryos indicating that the cloned target gene regulatory region is fully functional and the *β-galactosidase* expression can reflect or mimic the expression pattern of a nearby gene (Wilson et al., 1989).

On the other hand, a fundamental goal of genetics is to identify and mutate every gene in model organisms such as *Drosophila.* P-elements are very useful in genetic dissection of fly development and gene function. The Berkeley Drosophila Genome Project (BDGP) has generated single P-element insertion strains where each mutates unique genomic open reading frames. These strains facilitate further genetic and molecular studies of the disrupted genes since the insertion of the P-element nearby a gene can interrupt its function.

The presence of the eye color marker on the P-element helps to isolate the transgenic lines possessing the P-element. Plasmids possessing the P-element carry sequences which are necessary for propagation as circular plasmids in E. coli, which allows the DNA that flanks the transgenic construct to be cloned by plasmid rescue. In this strategy, the construct, which carries a plasmid backbone and antibiotic resistance gene will be cut and

circularized again followed by transformation into bacteria, which can be recovered following antibiotic selection.

A. 3. *Drosophila* Eye

In 1910, the first *Drosophila melanogaster* mutant was isolated by Morgan when he discovered a white eyed fly among his collection of wild-type red eyed flies. Since that time, the compound eye of *Drosophila* has been an invaluable model system for studying fundamental biological questions in development and physiology. This is not only because, as Morgan demonstrated, adult eye phenotypes are easy to detect, but also because unlike most organs in the fly, the eye is tolerant to genetic disruption of basic biological processes.

A. 3. i. The eye as a useful tissue to study genetic interactions

The adult *Drosophila* eye is the product of a series of precise developmental events that begin in the embryo and proceed through the larval, pupal, and adult stages. Studying the role of genes *in vivo* is important but difficult and it seems that over two thirds of all *Drosophila* genes show no obvious loss-of-function phenotype. Therefore, misexpression analysis in the *Drosophila* eye has proven to be a practical first step towards clarifying the *in vivo* function of a gene of interest. The goal of the mis-expression approach is to produce an eye phenotype that reveals a role for the misexpressed gene within a biological process or a phenotype that can be used in a genetic screen to find new components of a biological process. Rough or small eye phenotypes are the most common results observed following mis-expression of genes. These phenotypes are easily detected using a light microscope (Simone, 1994).

Screens for genes which dominantly modify a phenotype caused by mis-expression or mutation of a gene in the developing eye, have led to the identification of new components of biological pathways and to the determination of their order of action. With a line that produces an intermediate phenotype, both suppressors and enhancers of the phenotype that encode positive and negative regulators of the process, respectively, can be identified in a single-generation (F1) screen. Furthermore, the simplicity of these F1 screens allows large numbers of flies to be examined.

Examples of using the eye to identify new genes in a particular pathway include the elucidation of both the *sevenless* signaling pathway (Simone, 1994 , Zipursky and Rubin, 1994) and the phototransduction cascade (Zuker, 1996). The weakness of these assays is that they typically identify only the genes that are limiting in a pathway (*i.e.*, where elimination of one copy of the gene product results in a recognizable change in the sensitized phenotype).

A. 3. ii. The adult eye structure

The adult compound eye shows a regular hexagonal array of approximately 750 facets, the lenses of the eyes or ommatidia. An adult ommatidium is a combination of 8 photoreceptors and 11 accessory cells, and pigment cells. The small mechanosensory parts of the eye are bristles that are composed of neurons, glia, and two support cells. Eye bristles direct their sensory axons into the brain (fig. 3) (Basler and Hafen, 1991; Wolff and Ready, 1991a,b).

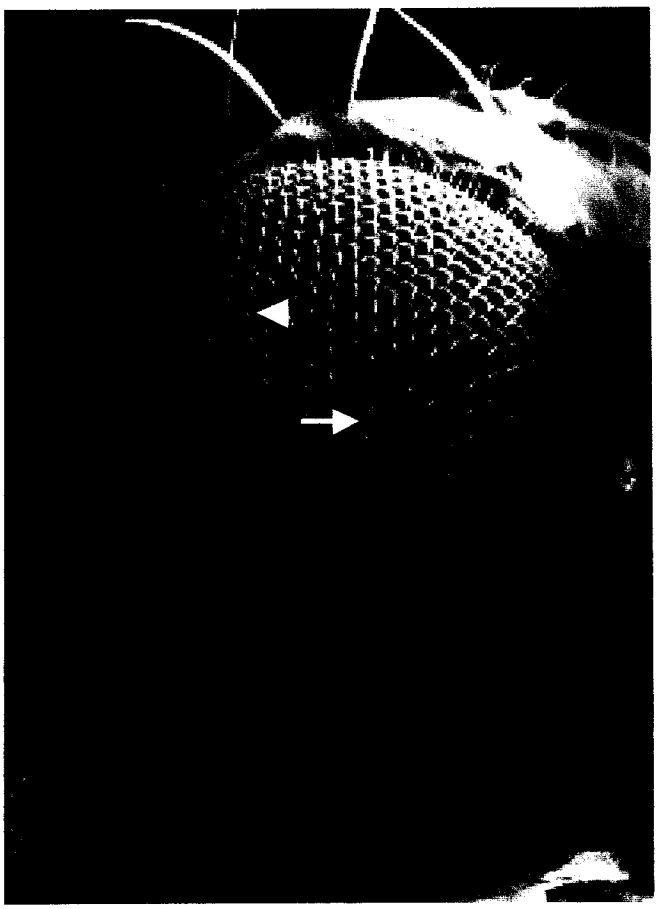


Figure 3. *Drosophila* compound eye. Scanning electron micrograph of an adult wild-type eye. The adult compound eye shows a regular hexagonal array of approximately 750 ommatidia and bristles, which are the small mechanosensory parts of the eye; ommatidia (arrow) and bristle (arrowhead) (Sedaghat et al., 2005).

In this study we use *Drosophila* as a model system to study fundamental processes governed by two groups of transcription factors: C_2H_2 zinc finger and bHLH-PAS proteins. bHLH-PAS transcription factors control a variety of developmental and physiological events in both vertebrates and invertebrates. It is essential to identify additional *cis* and *trans*-acting factors that dictate transcriptional specificity and cross-regulation of these bHLH–PAS proteins.

B. Transcription factors

Transcription factors are a group of regulatory proteins that bind to DNA transcription- control elements and stimulate or repress transcription. There are variety of structural motifs that interact with specific DNA sequences and transcription factors can be classified based on the type of these DNA binding domains.

In this section, some of the well-known classes of transcription factors are briefly described.

B. 1. Zinc finger proteins

In this family of proteins, there are regions that fold around a central Zn^{2+} ion. This structure was first found in DNA binding domains but there are proteins with this structural motif that do not bind to DNA (Park et al., 1999). There are different classes of zinc finger proteins such as: C_2H_2 zinc finger, which is one of the most common DNA binding motifs in eukaryotic transcription factors, C_4 zinc finger and C_6 zinc finger (Miller et al., 1985; Pavletich and Pabo, 1991 and 1993).

C_2H_2 zinc finger was initially found in transcription factor IIIA, which is required for 5S rRNA genes transcription by RNA polymerase III (Miller et al., 1985). Each repeating unit, which has two cysteine and two histidine residues, binds one zinc ion through the cysteine and histidine side chains. This compact domain can insert its α helix into the major groove of DNA.

In this project we studied a gene called *jing*. Jing has C_2H_2-type zinc finger motifs. The closest mammalian homologue of Jing is mouse AEBP2 that has three tandemly repeated sequence motifs related to the Gli-Kruppel C_2H_2-type zinc finger (He et al., 1999). This protein regulates *aP2* gene expression through binding to AE1 site (He et al., 1995 and 1999). The *aP2* or *adipose P2* gene encodes the adipose fatty acid-binding protein, which is important for triglyceride metabolism during adipocyte differentiation. CEB/Pα binds to the AE1 site and activates the *aP2* transcription (Cornelius et al., 1994; MacDougald and Lane, 1995; Spiegelman and Flier, 1996). AEBP2 represses the transcription of *aP2* and this repression role requires the proper function of the middle zinc finger (He et al., 1999).

B. 2. BHLH-PAS proteins

The DNA sequence analysis of several proteins such as the Myc family, Myo D in mammalians, Daughterless, Achaete-Scute, and Twist in *Drosophila,* identified two amphipathic helices in which the hydrophobic residues are highly conserved between these proteins (Peyrefitte et al., 2001; Spicer et al., 1996; Oellers et al., 1994). This region has approximately 60 amino acids and is involved in DNA binding and dimerization. The region forms a helix-loop-helix structure adjacent to a highly charged region. Considering the structure and function, these proteins are considered as transcription factors that can

bind to their tissue-specific sequence and regulate the transcription of target genes (Murre et al., 1989 a,b).

The basic-helix-loop-helix-PAS (bHLH-PAS) transcription factors control a variety of developmental and physiological events including neurogenesis, tracheal, salivary duct formation, toxin metabolism, circadian rhythms, response to hypoxia, and hormone receptor function. bHLH-PAS proteins usually function as dimeric DNA-binding protein complexes. Therefore a functional unit is usually composed of heterodimer complexes. These heterodimers are composed of one protein that is broadly expressed and, another whose expression or function is restricted (Crews, 1998).

Some of the members of bHLH transcription factors have an extended region that is highly conserved. This conserved domain was originally found in the *Drosophila* Period (Per), vertebrate Arnt and Sim proteins, and it was called the PAS domain. There are two 51 amino acid direct repeats present in the PAS domain.

The bHLH domain in these transcription factors is located near the amino terminus. The basic region binds DNA and the HLH domain contributes to dimerization. These residues are followed by the PAS domain. The carboxy-terminal residues contain transcriptional activation domains or repression domains. The PAS domain can mediate a number of biochemical functions. It is used for dimerization between PAS proteins, small molecule binding, and interactions with non-PAS proteins (Crews, 1998).

bHLH-PAS proteins could act as lineage-specific developmental regulatory proteins. Three members of this family are *single-minded* (*sim), tango (tgo),* and *tracheales (trh). sim* is a master regulator whose function is required for all CNS midline transcription and development. The proper function of *trh* is required for tracheal development. In both

cases the presence of *tgo* (the other partner in the heterodimer complexes) is necessary (Crews, 1998).

B. 2. i. *single-minded (sim)*

The *Drosophila single-minded* (*sim*) gene controls transcription and development of the CNS midline cell lineage, which is required for the formation of the CNS (Nambu, 1996; Crews, 1998). In *sim* homozygous mutant embryos, midline cells are absent, longitudinal axons are affected, and the ladder-shaped axon scaffold is collapsed. This phenotype is embryonically lethal. Conversely, ectopic expression of Sim gives rise to the induction of midline cell fates in the entire ventral ectoderm (Nambu et al., 1991).

Sim forms heterodimers with Tango and the complex binds midline enhancer elements (ACGTG core sequence) that are present in the promoters of target genes (Crews, 1998). Mammals have two *sim* genes, *sim1* and *sim2* (Michaud and Fan, 1997). Both *sim* genes are expressed in the developing CNS. *sim2* is a candidate gene for Down's syndrome based on its expression pattern and localization to chromosome 21. Mutant analyses have demonstrated that *sim1*, like its fly counterpart, is required to control the development of specific cell types within the central nervous system (Michaud et al., 1998; Michaud et al., 2000).

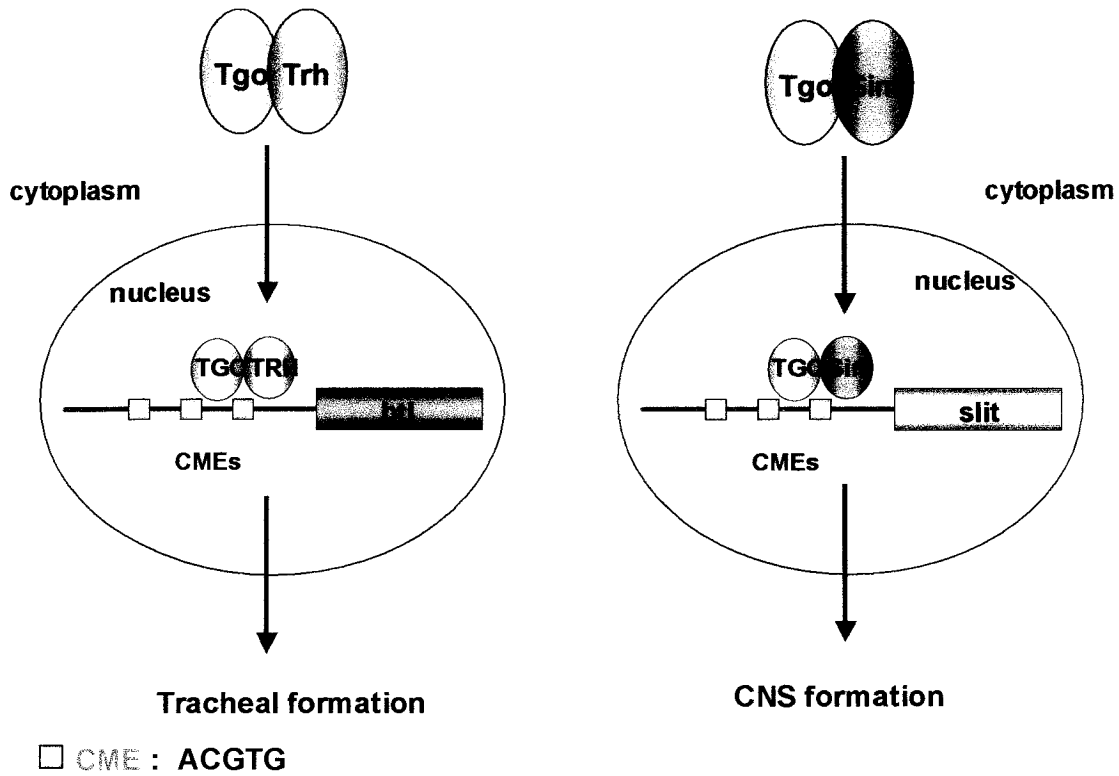


Figure 4. bHLH-PAS proteins and their transcriptional regulatory systems during the trachea and CNS formation. Trh and Sim form heterodimers with Tango and transfer this protein into the nucleus. The complex binds midline enhancer elements (ACGTG core sequence) that are present in the promoters of target genes in order to regulate the transcription (Adapted and modified from Crews, 1998).

B. 2. ii. *trachealess (trh)*

Trachealess (Trh) is another protein belonging to the bHLH-PAS family, which is expressed in the tracheal pits and developing tracheal tubule. It is also expressed in two other tubular structures: the posterior tracheal spiracles, and the salivary duct (Isaac and Andrew 1996; Wilk et al. 1996). All three structures do not form in *trh* mutant embryos, confirming the crucial role of *trh* in the formation of Tracheal system, tracheal spiracles and salivary duct. *trh* ectopic expression results in both the formation of extra tracheal pits in segments that normally do not form pits, and to the expression of tracheal markers on the ectoderm (Wilk et al. 1996).

B. 2. iii. *Tango (tgo)*

The *Drosophila tgo* gene is orthologous to the Aromatic hydrocarbon receptor nuclear translocator (Arnt). Tgo functions as a dimerization partner for a number of bHLH-PAS proteins (Sonnenfeld et al., 1997; Crews, 1998). Tgo protein is expressed in all cells in the embryo and thus overlaps with Sim expression in the CNS midline cells and Trh expression in the tracheal cells. In the absence of a bHLH-PAS partner protein, Tgo is found in the cytoplasm. However, in the presence of a partner protein, they dimerize, translocate into the nucleus, bind DNA, and activate transcription (Fig. 4) (Ward et al., 1998). *Drosophila* contains five bHLH-PAS proteins: Sim, Sima, Ss, Trh and Dys, which dimerize with Tgo (Emmons et al., 1999; Lavista-Llanos et al. 2002; Jiang and Crews, 2003).

B. 2. iv. Central Midline Element (CME)

Drosophila FGF receptor is encoded by the *breathless (btl)* gene. *btl* is required for proper branching of the tracheal system as well as midline glial migration (Klambt et al.,

1992). *btl* expression in the developing trachea is regulated by direct interaction between Trh::Tgo heterodimers with three identical central midline elements (CMEs; ACGTGs) located in the minimum enhancer region of the *btl* gene (Ohshiro and Saigo, 1997). It has also been shown that Sim::Tgo heterodimers that are essential for *btl* expression in midline precursor cells and *slit* in the glial cells, share DNA targets that are in common with Trh::Tgo. This indicates that two different basic helix-loop-helix-PAS protein complexes act through the same target sites *in vivo* (Wharton and Crews, 1993). It is also thought that additional nucleotide sequences flanking CMEs may serve as additional cis-regulatory elements for tracheal expression. *In vivo* studies on the transgenic flies which lacked the CME motifs in the *btl* promoter demonstrated the necessity for Trh::Tgo transcriptional activity on *btl* (Fig. 4) (Ohshiro and Saigo, 1997; Crews, 1998).

Since *jing* is expressed in the central nervous system (CNS) and respiratory (tracheal) system during *Drosophila* embryogenesis and three bHLH-PAS proteins that were introduced above have key roles in the development of both systems, CNS and tracheal structure will be described in the following section.

C. Central nervous system

The *Drosophila* central nervous system (CNS) is a model system for studying the molecular genetic mechanisms that control CNS midline cells differentiation and axon scaffold patterning. The CNS in *Drosophila* is segmented into units called neuromeres that are composed of neuron, glia, and projected axons. Each neuromere arises from the ventral neuroepithelium and is composed of three regions (two neurogenic regions

separated by a midline region). The neurogenic regions generate the neurons, a small number of midline neurons, and glial cells are generated by the midline region (Fig. 5).

C. 1. Neurogenic region

The neurogenic region consists of a sheet of ectodermal cells that will later give rise to neuronal precursor cells called neuroblasts (NB). Two classes of genes are required for neuronal precursor cell formation: (1) Proneuronal genes, which are necessary for the identification of a certain neuroectodermal cell, at a particular time, to become competent to form a neuroblast (Ghysen and Dombly-Chaudiere, 1989), and (2) neurogenic genes. In each group of equivalent neuroectodermal cells, only one cell becomes a NB and neurogenic genes prevent other cells in the group to become neuroblasts.

C. 2. Midline region

At the cellular blastoderm stage, there are two transcription factors: *twist* and *snail*, which are activated. These genes activate the transcription of *single-minded (sim),* which results in the molecular specification of midline precursors (Nambu et al., 1990; Crews et al., 1988). At this stage, the midline precursors are located in two parallel stripes along the anterior/posterior axis. As gastrulation proceeds, these two stripes of midline precursors join together at the ventral midline to form a single row. Divisions and differentiation of the cells lead to the formation of neuron and glia. Midline function will be discussed later in more details.

The Drosophila CNS consists of a bilaterally symmetric group of neurons separated by a group of CNS midline cells. Genetic and molecular studies have shown that multiple

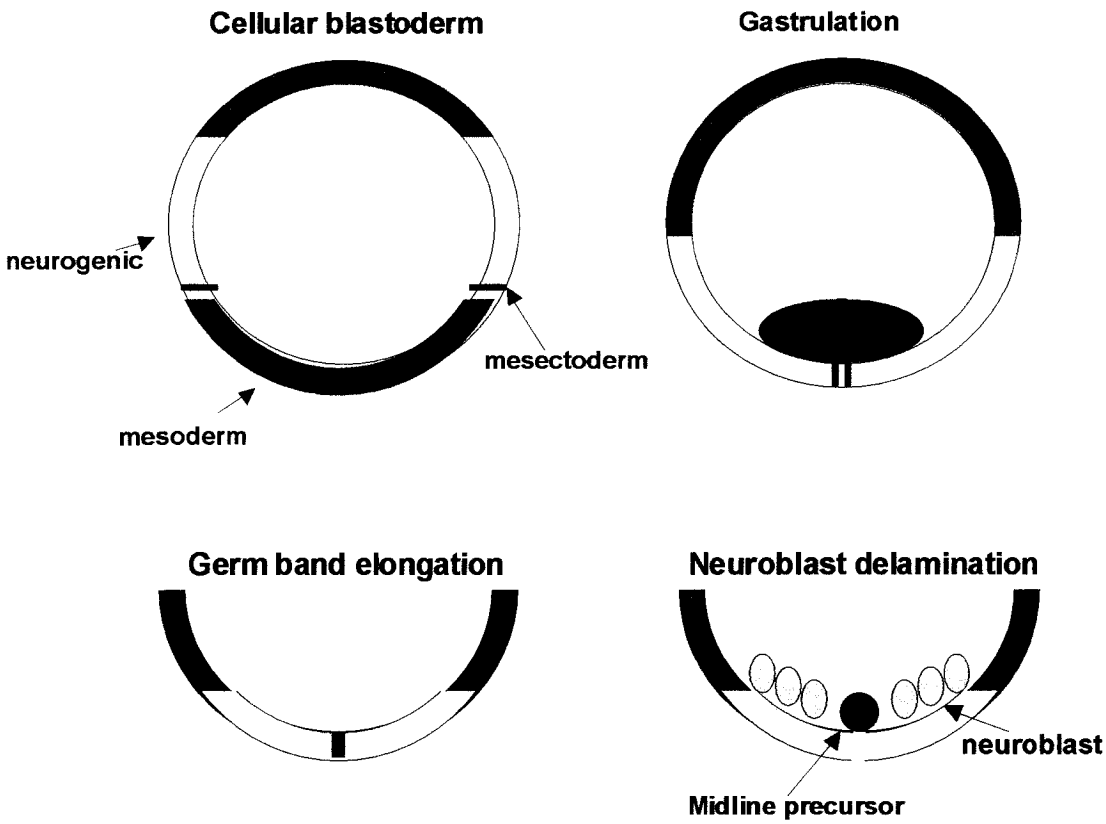


Figure 5. Development of the neurogenic region and ventral midline. Midline development occurs along with four stages of embryonic development. During the blastoderm stage, midline precursor cells (indicated in dark blue stripes) are placed on both sides of the mesodermal anlage and separate the presumptive mesoderm from the presumptive neurogenic ectoderm. During gastrulation, mesodermal cells invaginate and locate side by side at the ventral midline. During germ-band elongation, these cells form a row of eight cells per segment. Finally, these precursor cells delaminate from the ventral ectoderm into the same layer where neuroblasts are located; divide and give rise to the neurons and glia of the midline cells (Adapted and modified from Goodman and Doe, 1993).

transcriptional activators function in a cooperative concentration-dependent mode in combination with a transcriptional repressor to form the axon scaffold. The *Drosophila* embryonic nervous system can be subdivided into the ventral nerve cord (VNC) and the brain (Goodman and Doe, 1993).

C. 3. Ventral Nerve Cord

The *Drosophila* embryonic ventral nerve cord (VNC) has a ladder-like structure consisting of longitudinal connectives (LC) and the anterior and posterior commissures (AC and PC). Different neuronal and glial cell types are found along these axonal pathways. Midline glia are adjacent to the commissures; longitudinal glia are associated with the connectives. Peripheral glial cells set the boundaries between the CNS and PNS and enwrap the peripheral nerves (Fig. 6) (Goodman and Doe, 1993).

C. 3. i. Proneuronal genes

The VNC arises from a bilaterally symmetrical two-dimensional sheet of cells, representing the ventral neurogenic region of the ectoderm. Through the expression of proneural genes of the Achaete–Scute Complex [*achaete (AC), scute (sc)* and *lethal of scute (l'sc)*], and the gene *ventral nervous system defective (vnd)* at precise locations, groups of neuroectodermal cells called proneural clusters acquire the potential to become neural precursor cells termed neuroblasts (NBs, Skeath 1992). Loss-of-function of either AS-C or *vnd* results in the loss of roughly 25% of all segmental NBs (Jimenez and Campos-Ortega, 1990). Since embryos with double mutation for the AS-C and *vnd* lack roughly

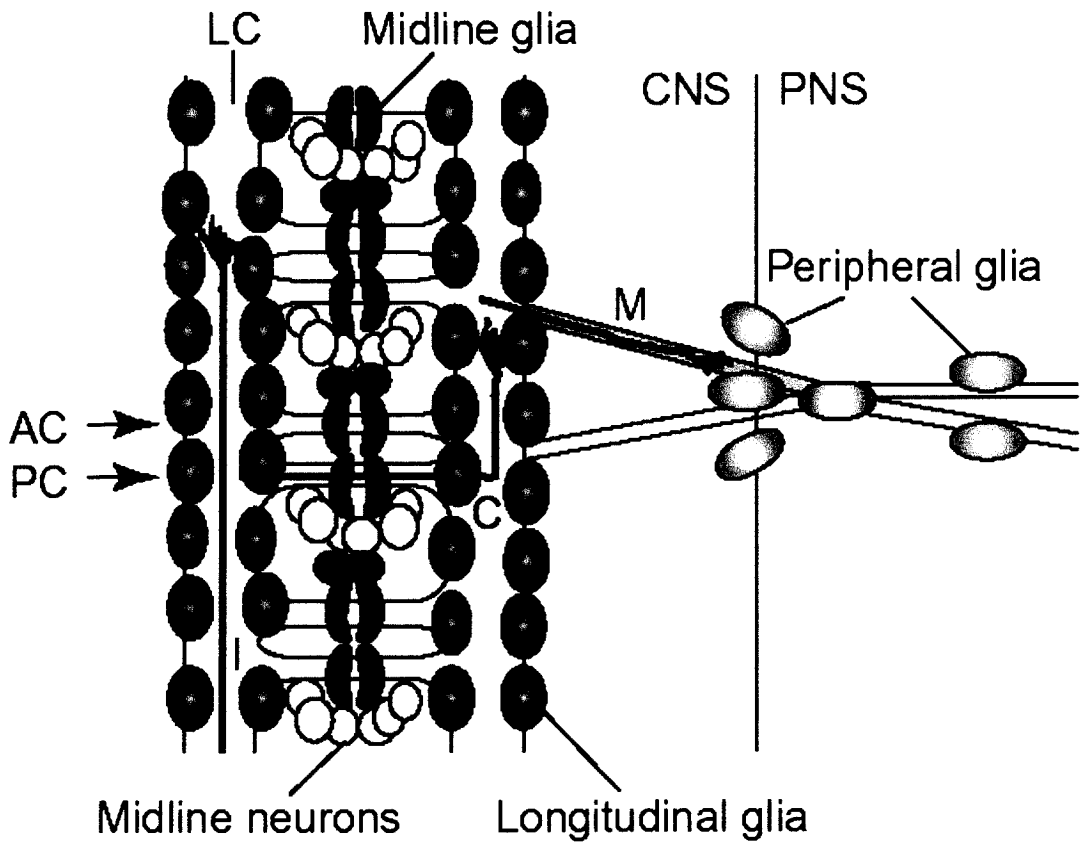


Figure 6. *Drosophila* CNS. The *Drosophila* embryonic nervous system has a ladder-like structure, consisting of longitudinal connectives (LC) and the anterior and posterior commissures (AC and PC). Different glial cell types are found along these axonal pathways. Midline glia are adjacent to the commissures; longitudinal glia are associated with the connectives. Peripheral glial cells set the boundaries between the CNS and PNS and enwrap the peripheral nerves. Abbreviations: C, contralateral axons; I, ipsilateral axons; M, motor axons. (Adapted from Goodman and Doe, 1993).

50% of all segmental NBs, it has been suggested that the ASC and *vnd* control the formation of non-overlapping sets of NBs (Jimenez and Campos-Ortega, 1990). The generation of the initial clustered pattern of proneural gene expression is governed by the combined action of the gene products of the pair-rule and dorsal-ventral patterning genes. These proteins presumably act through a vast array of region-specific enhancers found within the AS-C to carve out clusters of *ac*, *sc,* or *l'sc* gene expression at precise and reproducible coordinates within the *Drosophila* embryo (Skeath et al., 1992). Within each cluster, the cell that comes to express the proneural gene(s) to the highest level becomes the NB or neural stem cell (Cubas et al., 1991). Soon after, the NB, which retains proneural gene expression, initiates a cell-communication pathway (lateral inhibition) mediated by the gene products of the neurogenic genes. The process of lateral inhibition removes both proneural gene expression and neural competency from the remaining cells of the cluster (Skeath and Carroll, 1992; Campos-Ortega, 1993). The others remain in the periphery to develop as epidermoblasts (Campos-Ortega, 1995). Several sequential events of NB segregation result in the formation of 30 NBs per hemisegment (the developmental unit of the VNC) (Campos-Ortega, 1993)

Right after segregation, each NB divides asymmetrically to make smaller secondary precursor cells known as `ganglion mother cells´ (GMCs) at the interior of the embryo. Each GMC then divides once asymmetrically to produce two postmitotic neurons and/or glia. Thus, in each *Drosophila* VNC hemisegment, a group of 30 NBs generates a large number of GMCs which is followed by the production of about 400 postmitotic neurons and glia (Goodman and Doe, 1993).

C. 3. ii. Neuronal cells

Each GMC gains a unique fate. GMC formation involves the sequential expression of a set of transcription factors in almost all NB lineages, which enable GMCs born at different times in a lineage to have different fates. This temporal complex consists of the sequential expression of five genes: *Hunchback (Hb), Kruppel (Kr), Pdm, Castor (Cas),* and *Grainyhead (Gh)* (Fig. 7, Skeath and Thor, 2003). *Hb,* a zinc finger transcription factor, was detected in deep layer neurons; Pdm1/Pdm2 (Pdm), both POU domain transcription factors, were detected in middle layer neurons, and Castor (Cas), previously called Ming (Cui and Doe, 1992), a zinc finger transcription factor, was expressed in the neurons located on the surface (Kambadur et al., 1998). It has been proposed that *hb, pdm,* and *cas* expression may correlate with neuronal birth-order (Pearson, 2004). Kr is another zinc finger transcription factor (Isshiki et al., 2001), which is expressed at low levels in the Hb layer and in a distinct layer between Hb and Pdm. These factors are expressed in the order started with *hb, kr, pdm, cas* and *gh* (with low level of *kr* present during the *hb* expression period). Meanwhile, GMCs are born as the neuroblast transits through this sequential expression and the GMC and its neuronal progeny will maintain the gene expression profile present in the neuroblast at the time the GMC was born (Pearson, 2004).

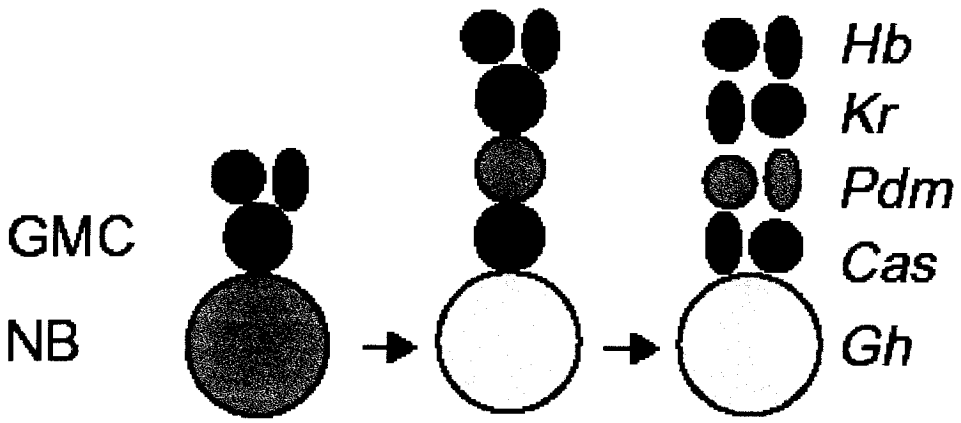


Figure 7. The temporal gene cascade during neuronal cell development. The temporal expression of *Hb* (red), *Kr* (blue), *Pdm* (green), *Cas* (purple), and *Gh* (light blue) leads to diversification of the GMCs generated by each NB. Most GMC divisions are, in turn, asymmetric (oval versus circle shape) and generate postmitotic sibling cells of different types. (Adapted from Skeath and Thor, 2003).

First-born GMCs that express *hb* can produce interneurons, motor neurons, or glia depending on the spatial identity of the neuroblast. GMCs expressing *kr, pdm,* and *cas* can generate motor neurons or interneurons and, in some cases, glia depending on the neuroblast lineage (Isshiki et al., 2001). *hb* loss-of-function in the CNS resulted in death or cell fate transformation of the early-born GMCs in both lineages without affecting later-born cells in the lineages. Over-expression of *hb* in the CNS resulted in continuous production of first-born GMCs even more than the normal number of GMCs made by that lineage. Over-expression of *kr* also resulted in continuous production of GMCs. *kr* loss-of-function caused cell death or cell fate transformation of first-born GMCs. Therefore, *hb* and *kr* are the potential regulators of early cell identity (Pearson, 2004).

C. 3. iii. Glial cells

C. 3. iii. a. Glial cell type and nervous system formation

Cell–cell interactions have a key role in establishing the complex pattern of neuronal connections in the developing nervous system. Both Glia and neuron have a supportive function in guiding the axons.

Glial cells in *Drosophila* are categorized according to their positions within the nervous system, CNS, or peripheral nervous system (PNS). Glia in the *Drosophila* CNS are divided into four groups: (1) surface-associated glia, which are associated with CNS surface, consisting of perineurial and subperineurial glia, (2) Cell body-associated glia, which wrap neuronal cell body, and (3) Neuropil-associated glia consisting of interface glia (including longitudinal glia), which are located between cell body layer and central neuropil and extend processes around and into the neuropil; and (4) midline glia that are associated with commissural tracts at the midline. In PNS there is only one type of glial

cell, the peripheral glia. The peripheral glia are located at the transition zone between the CNS and PNS which wrap axon bundles within peripheral nerves. (Ito et al., 1995).

C. 3. iii. b. Guidance cues

Attractive and repellent signals are responsible for guiding axon growth cones to their final destination. Such cues can be provided by the cells located at proper points to direct axonal pathfinding along a particular path (Bentley and Caudy, 1983). Neurons extend axons toward the signaling source. They contact the cells and move toward the final target. Glia function as the signal source in insects and vertebrates.

C. 3. iii. b. 1. Attractive signals

In each segment of the *Drosophila* embryonic ventral nerve cord, some axons project across the midline to the contralateral side to form the commissures. Other axons remain within the ipsilateral half of the nerve cord. These axons receive the appropriate signal from midline cells. The midline is formed by the ventral unpaired median (VUM), MP1 neurons, and three pairs of glia. Both midline neurons and glia play separate roles in guiding axons by providing specific cues to growth cones (Jacobs, 2000). One of the major attractive signals is Netrin (Mitchell et al., 1996). In *Drosophila,* Netrin A and B are expressed by ventral midline glial cells as the first axons pioneer the longitudinal and commissural tracts of the CNS (Fig. 8a, 9, 10) (J.R. Jacobs, 2000). Netrins perform their attractive function through binding to their receptor, Frazzled, the *Drosophila* homolog of the guidance receptors deleted in colorectal cancer (DCC) in vertebrates and UNC-40 in

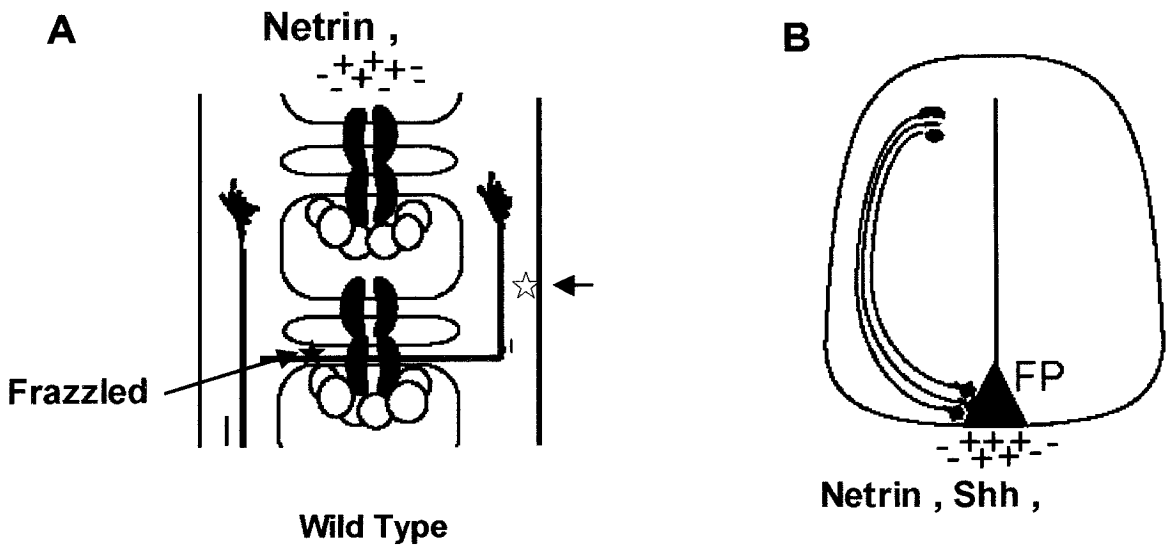


Figure 8. Attractive and repulsive signals in Drosophila midline and vertebrate floor plate. (A)Both midline neurons and glia provide the attractive guidance cue Netrin. Glia also secrete Slit, which acts as a repellent guidance cue. Netrin attracts commissural axons to the midline; Slit prevents Roundabout (Robo)-expressing ipsilateral axons from crossing and contralaterally projecting axons from re-crossing. (Adapted from Kidd et al., 1999). (B) Glia-like cells in the floor plate (FP) of the wild-type vertebrate spinal cord act as guidepost cells for commissural axons. They produce two attractive guidance cues, netrin and sonic hedgehog (Shh) as well as the repellent cue semaphorin (Sema)3B (Adapted and modified from Charron et al., 2003).

C. elegans (Kolodziej et al., 1996). Although netrins are expressed by glia, genetic studies suggest that signals from the midline neurons are initially required to attract pioneer growth cones. Loss-of-function of both Netrins in Drosophila leads to defects in commissural and longitudinal axons (Hummel et al., 1999).

C. 3. iii. b. 2. Repulsive signals

Midline glia also release the chemorepellent called Slit. Slit acts as a ligand for the Roundabout (Robo) receptor expressed by ipsilaterally and contralaterally projecting axons (Fig 8a, 9, 10) (Kidd et al., 1999). The Slit–Robo complex prevents ipsilateral growth cones from crossing and commissural growth cones that have already crossed the midline from re-crossing. Commissureless is a membrane-bound protein whose function is required for control of Robo expression in contralaterally projecting axons. Commissureless regulates the intracellular trafficking to the axonal membrane surface (Georgiou and Tear, 2002). Meanwhile, the transient expression of commisureless by midline glia brings up the idea of midline glia participating in regulating Robo levels (Georgiou and Tear, 2002).

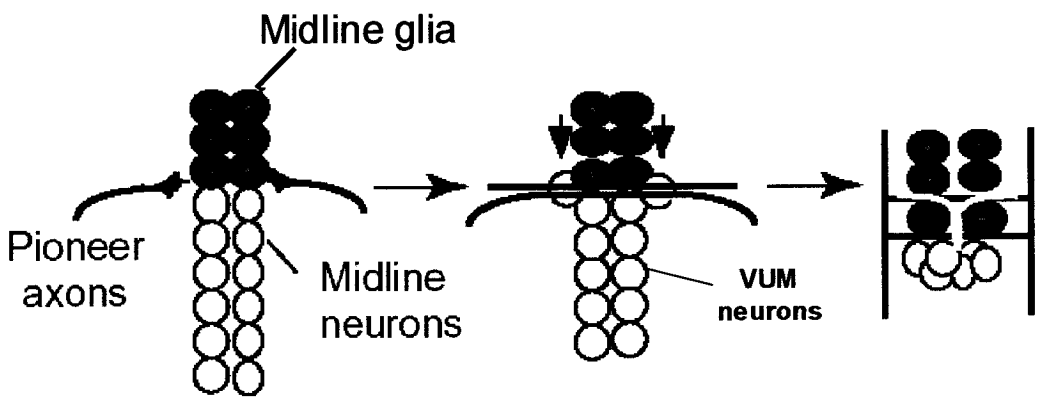


Figure 9. Glial cells and axonal outgrowth guidance. After midline cells have been specified, pioneer growth cones in each hemisegment project towards the most anterior midline neurons, the ventral unpaired median (VUM) neurons to establish the posterior commissures. Then VUM neurons extend axons and pioneer the anterior commissures. The two posteriorly located midline glia migrate between the anterior and posterior commissural tracts. Midline glia are crucial for separation of the commissures (Adapted from Hummel et al., 1999).

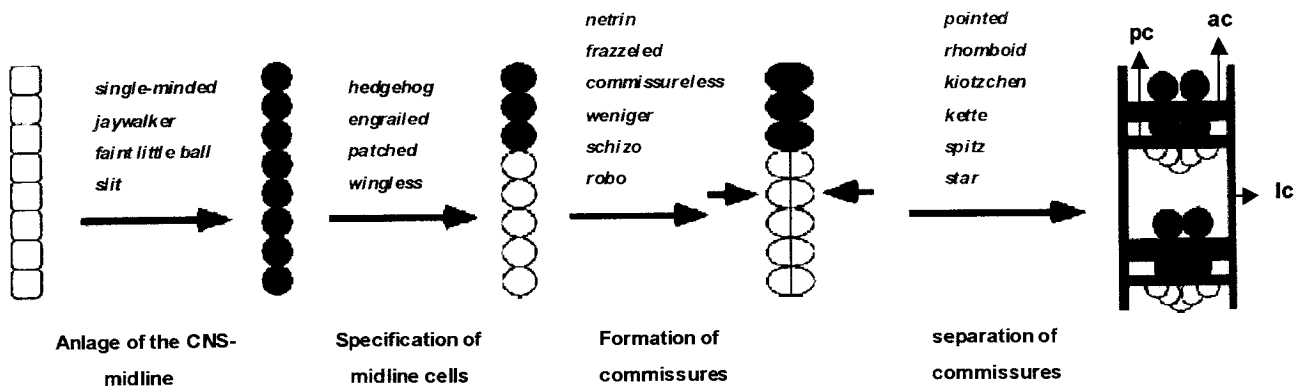


Figure 10. The development of the embryonic CNS axon pattern. Schematic view of the sequential steps of commissure formation. The midline glial cells are shown in blue. The VUM neurons are in yellow, the median neuroblast is in pale orange (the most posterior cell). Axons are shown in red, anterior is up. The process of commissure formation can be divided into four steps and genes involved in each step are indicated in red. ac, anterior commissure; pc, posterior commissure; lc, longitudinal connective (Adapted from Hummel et al., 1999).

C. 3. iii. c. Glial cells and longitudinal axons

There are three longitudinal fascicles in each hemisegment in the VNC of *Drosophila*. Longitudinal glia have a key role in the formation of longitudinal tract by guiding the growth cones of four pioneer neurons: dMP2, MP1, vMP2, and pCC. dMP2 and MP1 neurons extend axons posteriorly and contact the axons projecting from the vMP2 and pCC neurons (Hidalgo and Booth, 2000). Early removal of longitudinal glia by expressing the toxin ricin within the glioblast or by a mutation in *glial cells missing* (*gcm*), can affect the initial joining of ascending and descending pioneer axons. In addition, it can also affect further fasciculation and defasciculation steps of other axons (Fig 11) (Hidalgo and Booth, 2000).

C. 3. iii. d. Glial role in sensory and motor axon guidance

Glial cells guide the sensory and motor axons in the PNS of *Drosophila* (Sepp et al., 2000). In each segment, peripheral glia initially occupy regular positions at the transition between the CNS and PNS. They are arranged in a funnel-like array and guide the initial migration of sensory and motor axons to leave or enter the CNS. In the absence of peripheral glia, sensory neurons stall, exhibit abnormal fasciculation or enter the CNS at different positions, whereas motor pioneer axons exit the CNS and their pathfinding within the periphery is normal. It appears that the muscle targets guide motor axon migration. At later stages of embryonic and larval development, peripheral glia migrate from the transition zone along these axons into the PNS and, at the end, cover them. Similarly in vertebrates, motor neurons and sensory dorsal root ganglion cells extend

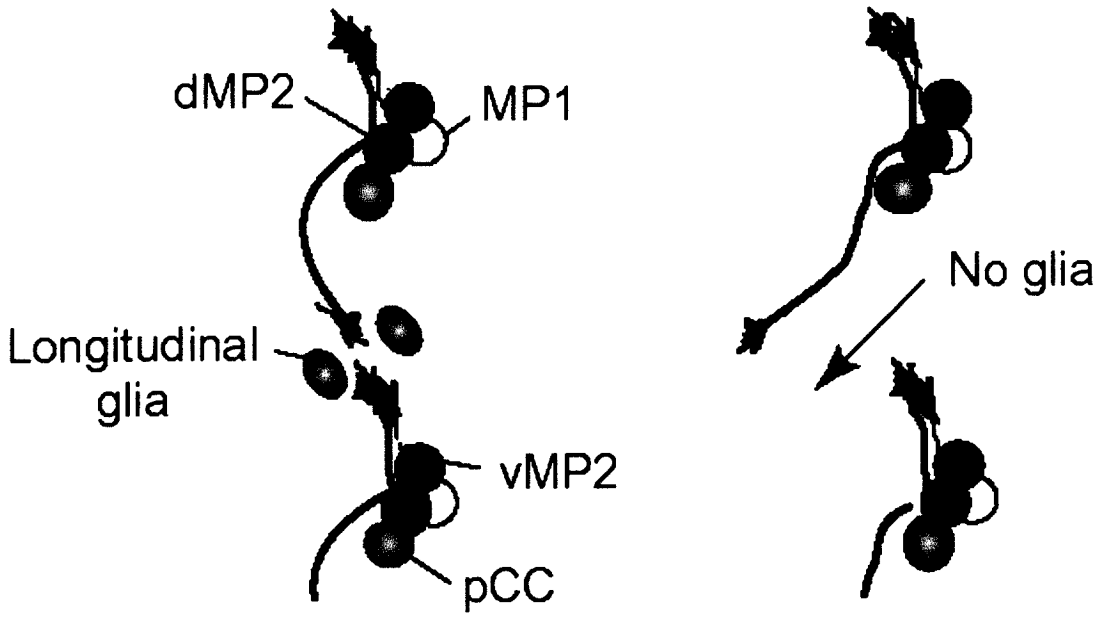


Figure 11. Formation of the longitudinal connectives. Longitudinal glia in the embryonic CNS of *Drosophila* function as guidepost cells for pioneer growth cones during the earliest stages of longitudinal tract formation. In each hemisegment, MP1 and dMP2 pioneer neurons extend axons posteriorly, whereas pCC and vMP2 axons project anteriorly. All growth cones encounter two glia at a choice point. If these glia are missing, growth cones fail to meet (Adapted from Hidalgo and Booth, 2000).

axons through ventral exit and dorsal entry points in the spinal cord, respectively. The position of these neurons is affected by neural-crest-derived glial-like boundary cap cells. However, genetic ablation of cap cells does not disturb axonal outgrowth (Golding and Cohen, 1997).

In the vertebrate spinal cord, glial-like cells in the floor plate play a role similar to that of guide cells at the ventral midline. Commissural axons project to the ventrally located floor plate and then to the contralateral side. Floor plate cells are the source of two attractive guidance cues to commissural axons, Netrin-1 and the morphogen Sonic hedgehog and also the source of repellent cues, such as Semaphorin 3B (Fig. 8b) (Charron et al., 2003; Salinas, 2003; Pascual et al., 2004).

C. 3. iii. e. Glial migration

It seems that glial cell movements can also be affected by chemoattractant and chemorepellent signals. In the *Drosophila* embryos, midline glia migrate along the axons of the VUM neurons to their appropriate position, allowing them to separate and enwrap the anterior and posterior commissures properly. Mutations in the *kette* gene which is necessary for axonal pathfinding of VUM neurons, disrupt midline glial migration and positioning (Hummel et al., 2000). Glia appear to respond to some of the same guidance cues used by neurons. For instance, *Drosophila* longitudinal glia transiently express the guidance receptor Robo and thus, become responsive to Slit which is provided by midline glia. This helps glia to stop migration and locate in the proper position from the midline

when interacting with axons pioneering longitudinal fascicle formation (Kinrade et al., 2001). Microarray studies have identified the repulsive type of *netrin* receptor, Unc-5, as a downstream target of the transcription factor Gcm. Unc-5 is expressed in peripheral glia and could direct their migration away from the midline towards the lateral edge of the CNS (Freeman et al., 2003). In the rodent optic nerve, glial precursors migrate from the brain to the retina and their migration is guided by the repulsive activity of netrin-1 and semaphorin 3A gradients, which are released at the lateral edges of the optic chiasm. In addition, localized *netrin-1* provided by glia at the junction between the retina and optic nerve might be required to prevent the migration of oligodendrocyte precursors into the retina. This process controls the proper location of the glia along the optic nerve, which leads to the correct levels of myelination (Sugimoto et al., 2001).

C. 4. Brain

The most complicated organ in *Drosophila*, the brain, develops from the head region of the embryo. Many of the developmental regulatory genes that are involved in early embryonic head development are also used for building the embryonic brain. Many of these genetic processes may be general and apply to vertebrates as well as insects (Thor, 1995).

The developing *Drosophila* brain shares a number of common features with the developing vertebrate brain. For example, early in development, a simple axonal scaffold develops in both systems. Similar to vertebrates, ascending and descending axons form a tract on each side of the midline resulting in the connection of the different regions of the *Drosophila* brain prior to the formation of the brain commissures (Easter et al., 1994). Migrating glial cells predict the brain commissures and connectives, which is similar to

what has been observed in early commissure formation in the mammalian brain (Silver et al., 1982). A second common feature is that the boundary regions between the different *Drosophila* brain segments express both the *engrailed (en)* and *wingless (wg)* genes. This is analogous to the expression of the homologous vertebrate genes *en-l, en-2*, and *wnt-3*, which are expressed at the hindbrain/midbrain boundary (Puelles and Rubenstein, 1993). In contrast to *Drosophila*, the vertebrate midbrain/forebrain border or other possible boundary regions in the anterior CNS do not express *en-l, en-2*, or *wnt-3*. Finally, both the *orthodenticle (otd)* and *empty spiracles (ems)* genes are expressed in the developing brain, however, in the most anterior part of the *Drosophila* brain. This restriction is analogous to the expression of the homologous Otx and Emx genes within the vertebrate brain (Simeone et al., 1992).

C. 4. i. Embryonic head development

The embryonic head region is composed of three anterior cephalic segments (labral, antennal, and intercalary) and three posterior gnathal segments (mandibular, maxillary, and labial) as well as an anterior procephalic region (also called acron) (Cohen and Jurgens, 1991; Rogers and Kaufman, 1996). Gene cascades similar to those that function in the development of the trunk, control embryonic development of the gnathal segments of the posterior head region (Cohen and Jurgens, 1991). In contrast, the developmental genetic mechanisms operating in the anterior head region differ from those in the trunk region in several respects (Finkelstein and Perrimon, 1991). Embryonic head development is under the control of only three of the maternal systems (anterior, terminal, and dorsoventral). These maternal genes regulate the expression of a class of head gap-like genes, including *tailless* (*tll*), *orthodenticle* (*otd*), *empty spiracles (ems),*

buttonhead (*btd*), and *sloppy paired* (*slp*) (Cohen and Jurgens, 1991; Finkelstein and Perrimon, 1990; Gao et al., 1996). During the early blastoderm stage, these head gap genes are expressed in a single circumferential stripe at the anterior end of the embryo, which includes the anlagen of cephalic segments (Schmidt-Ott and Technav, 1994).

C. 4. ii. Emryonic brain development

The specification of a cephalic neuroectodermal region in the anterior embryonic head region is a first step in the development of the brain. The maternal dorsoventral system is initially responsible for subdividing the embryo into mesodermal, neuroectodermal and nonneural ectodermal regions (Bier, 1997), and the zygotic genes support this subdivision. Transformation into neuroectoderm is repressed by the zygotic dorsoventral genes *twist* and *snail* in the ventral mesoderm and by *decapentaplegic* (*dpp*) and *tolloid* in the dorsal ectoderm (Skeath et al., 1992), whereas the zygotic dorsoventral gene *short gastrulation* is expressed in the neuroectodermal tissue and inhibits *dpp* from spreading into that region (Biehs et al., 1996). It has been suggested that a coordinate system produced by the expression of zygotic genes along the anteroposterior and the dorsoventral axes would provide a subdivision that allows for embryonic CNS localization. Thus, proneural genes are activated at the intersections of vertical bands of pair rule gene expression and the horizontal bands that do not have the repressing proteins of the dorsoventral system (like *dpp* or *snail*) (Skeath et al., 1992, Campos-Ortega, 1994; Younossi-Hartenstein et al., 1996).

In the head, four waves of NB delamination take place between embryonic stages 9 and 12, which generate 80 brain NBs (Younossi-Hartenstein et al., 1996). The NBs then undergo a series of asymmetrical divisions, producing a ganglion mother cell (GMC) in

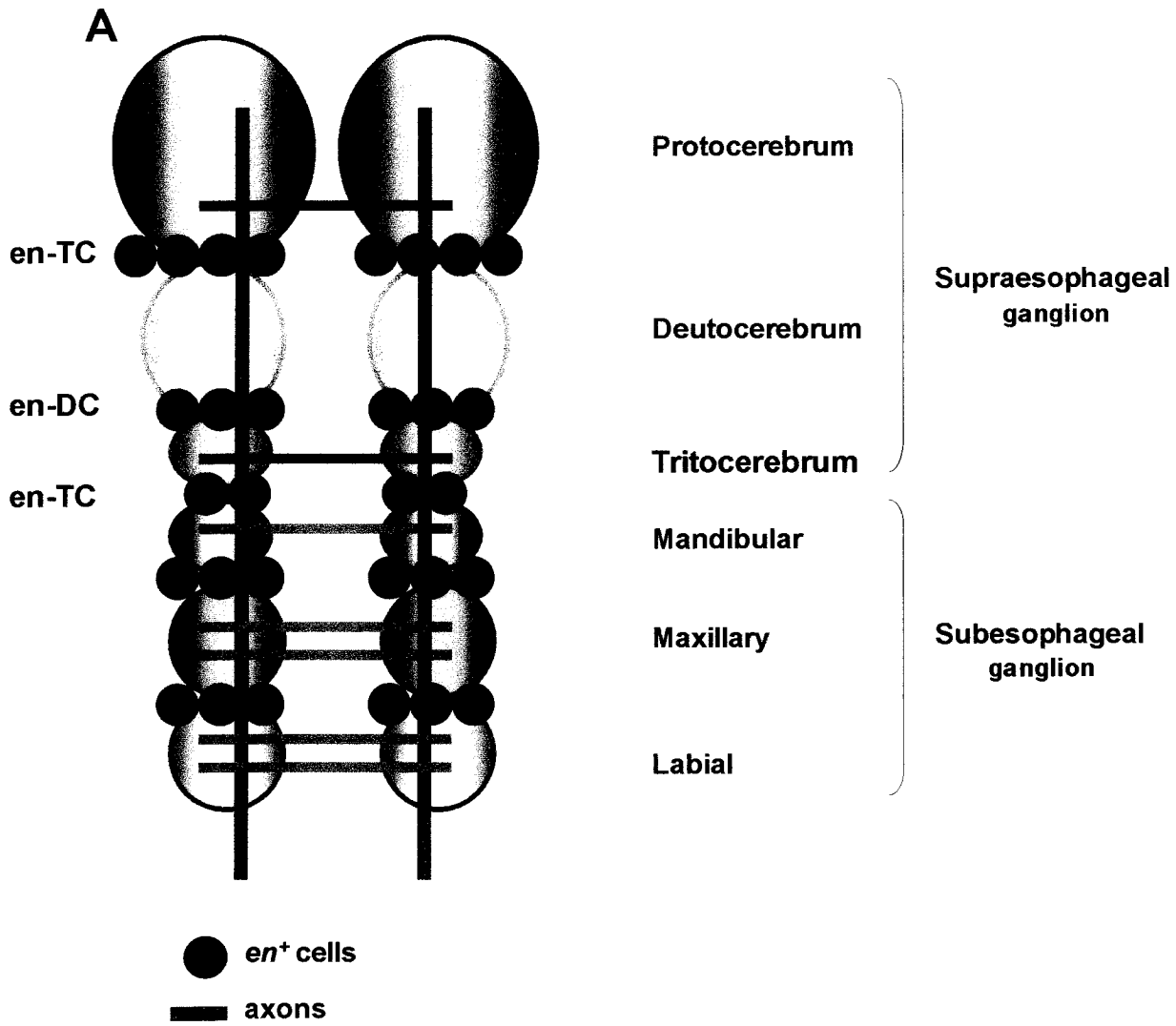


Figure 12. Schematic frontal view of the *Drosophila* embryonic brain. Supraesophageal anlage consists of protocerebrum, deutocerebrum and tritocerebrum, and subesophageal anlage is divided to mandibular, and maxillary labial neuromeres. At the posterior border of each neuromere, there are cells expressing the segmentation gene, *engrailed (en)*. The two halves of the protocerebrum are connected by the preoral commissure. Tritocerebral anlage are connected by the postoral commissure. The mandibular commissure connect mandibular neuromeres and the last two neuromeres are joined by the anterior and posterior commissure. Commissure located in the subesophageal section is also named subesophageal connectives. All six neuromeres are connected by longitudinal connectives that extend further posteriorly to the ventral nerve cord (Adapted and modified from Reichert et al., 1997).

each cell cycle. As more and more GMCs are produced they push each other in an elongating chain into the head. Each GMC then divides symmetrically, giving rise to two neurons. Through this process, the protocerebral (PC) brain anlage derives from the region of the labrum/acron, the deutocerebral (DC) brain anlage derives from the antennal segment, and the tritocerebral (TC) brain anlage derives from the intercalary segment. Together, these three neuromeres form the supraesophageal ganglion (Fig. 12).

The cephalic region of the *Drosophila* embryo is composed of various anatomical structures that undergo a series of complex morphogenetic movements as head involution occurs (Campos-Ortega and Hartenstein, 1985; Jurgens and Hartenstein, 1993). Between stages 11 and 12 of embryogenesis, three groups of *en*-expressing cells are observed in each hemisphere of the embryonic brain detected by antibody staining (*en-PC, en-DC and en-TC*) (Fig. 12). The existence of three distinct stripes of *en*-expressing cells in the embryonic brain indicates the metameric division of the embryonic *Drosophila* brain into three neuromeres (PC, DC and TC) (Fig. 12).

The subesophageal ganglion consists of the mandibular, the maxillary, and the labial neuromeres (Fig. 12). Its development is similar to that of the ganglia of the VNC of the embryo, which comprises three thoracic and nine abdominal neuromeres. Shortly after they have been generated, pioneering neurons extend axons and form a simple axon scaffold in the embryonic brain along which many follower axons project (Therianos et al., 1995). This leads to the formation of several major brain commissures and connectives, and two main longitudinal connectives, which interconnect the neuromeres of the brain (Fig. 12, 13a). A special feature in insects is the fact that the developing brain must form its axon scaffold around the ingrowing foregut. Three types of cellular

structures are involved in this process. First, the connectives grow along longitudinal rows of glial cells at the medial brain margin facing the foregut. Second, the preoral commissure (supraesophageal commissure), which links the two cephalic hemispheres, seems to be guided by cellular bridges across the midline composed of neuronal and glial somata. The third guiding structure could be the surface of the foregut epithelium itself, since the postoral tritocerebral commissure extends across the midline in close contact with the ventral epithelium of the foregut without the presence of glia or neuronal bridges there (Wildemann et al., 1997).

C. 4. ii. a. Formation of the axon scaffold in the embryonic brain

Several genes are involved in the formation of commissural and longitudinal axon pathways (Goodman and Doe, 1993; Reichert and Boyen, 1997). Two of these genes are *commissureless* (*comm*) and *single-minded* (*sim*). *comm* encodes for a surface protein and is expressed by midline glia (Tear et al., 1996). In *comm* mutants, the initial pioneering axons of the preoral brain commissure extend normally across the midline. Nonetheless, most of the follower fascicles, as well as all postoral commissures are missing (Therianos et al., 1995). *sim* acts as a master regulator of midline cell development (Crews et al., 1988; Nambu et al., 1990). *sim* mutants exhibit a gap in the longitudinal row of glial cells between the brain and the VNC. This gap cannot be crossed over by longitudinal pioneer axons, which results in the absence of the longitudinal connectives (Fig. 13b). Since similar defects are observed for other *spitz* group genes, it seems that the molecular genetic interactions among all of these regulatory genes are involved in controlling the development of the longitudinal connective pathways (Therianos et al., 1995).

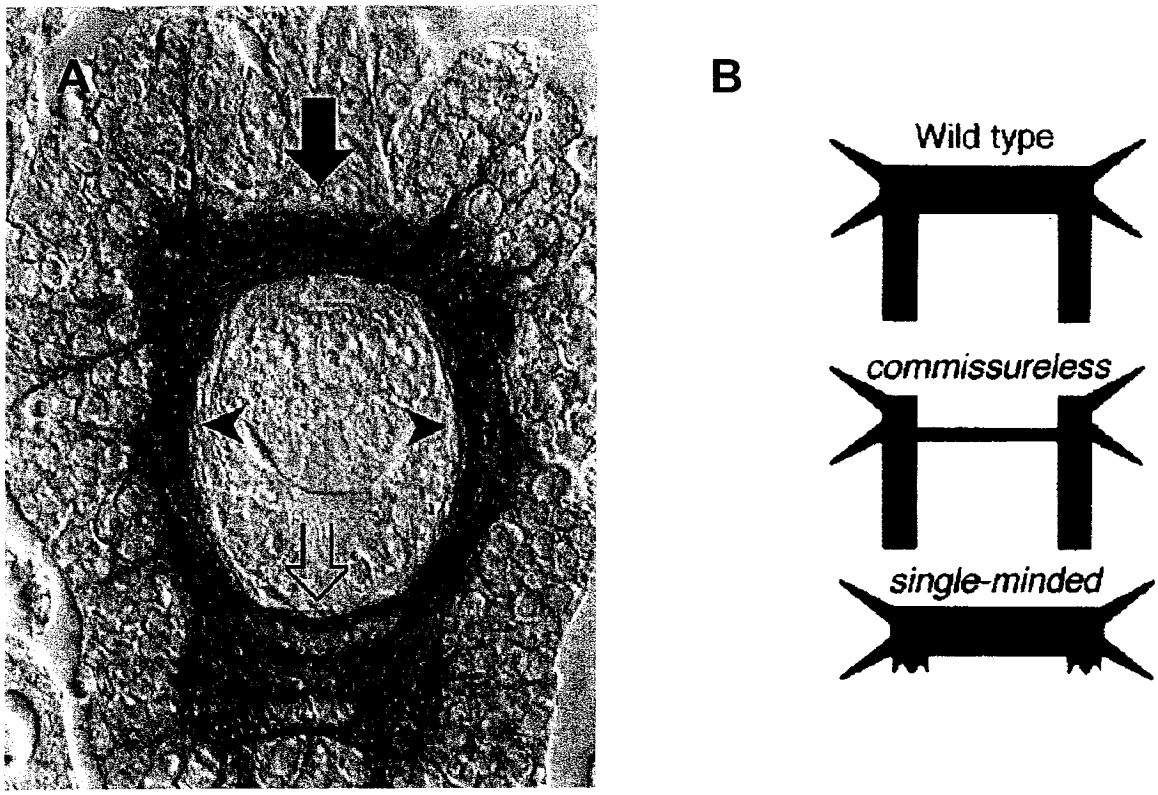


Figure 13. *Drosophila* embryonic brain axon scaffold. (A) Axon scaffold in the stage 16 embryonic brain stained with antibody BP102. Arrowheads show the longitudinal connectives, the preoral commissure is identified by the arrow and the open arrow indicates the postoral commissure and subesophageal connectives (Adapted from Therianos, 1995). (B) Schematic effects of *commissureless* and *single-minded* mutations on the embryonic brain axon scaffold patterning (Adapted from Reichert et al., 1997).

C. 4. ii. b. Brain neuromere formation

otx and *emx* are among the developmental regulatory genes with temporally and spatially restricted expression patterns in the vertebrate forebrain (Boncinelli et al., 1993). They are the vertebrate homologs of the genes *orthodenticle (otd)* and *empty spiracles (ems)* originally identified in *Drosophila*. The *otd* and *ems* homeobox genes are known to act as anterior gap genes during Drosophila embryogenesis (Jurgens et al., 1984; Dalton et al., 1989). The activity of the *otd* and *ems* genes is required in the development of the epidermal head segments, including antennal and pre-antennal structures. The *otd* gene is also required in ventral CNS development. Mutations in *otd* result in a malformation of the neuropil, the disappearance of midline-associated neurons, and fused commissures in the ventral nerve cord (Finkelstein et al., 1990; Klambt et al., 1991). The expression of *ems* in the embryonic CNS occurs in small clusters of lateral neuroblasts in the gnathal lobes and in several cells per segment at the lateral margins of the ventral nerve cord (Dalton et al., 1989; Walldorf and Gehring, 1992). Immunocytochemical double labeling experiments with an anti-otd antibody (Wieschaus et al., 1992) or an anti-Ems antibody (Walldorf and Gehring, 1992), in conjunction with a neuron-specific anti-HRP antibody (Jan and Jan, 1982) or an anti-En antibody (Patel et al., 1989), determined the expression domains of *otd* and *ems* in the embryonic brain. At embryonic stage 12 through stage 16, Otd immunoreactivity is seen throughout most of the neuronal cell body regions of brain neuromere PC, with the exception of the most anterior regions. Otd immunoreactivity is also seen at the border between neuromeres PC and DC and overlaps partially with the anterior part of neuromere DC. Similar consistent patterns were observed by in situ hybridization for the *otd* transcript using *otd* cDNA (Finkelstein et al., 1990) as a probe.

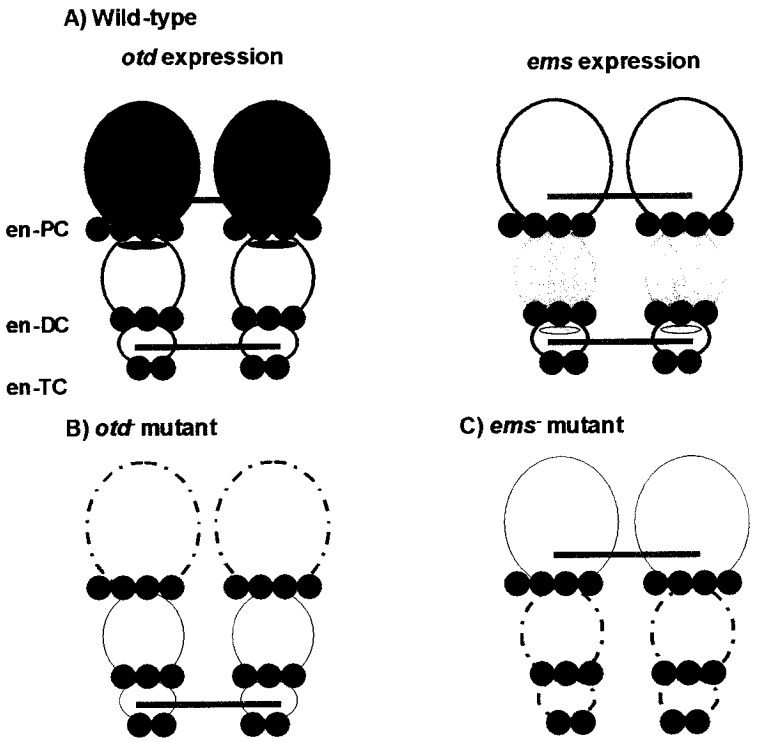


Figure 14. Schematic expression of otd and ems during *Drosophila* embryonic brain development. (A) At embryonic stage 16, *otd* expression (indicated in red) is seen throughout most of the neuronal cell body regions of brain neuromere PC, with the exception of the most anterior regions. Otd immunoreactivity is also seen at the border between neuromeres PC and DC and overlaps partially with the anterior part of neuromere DC . Expression of *ems* (indicated in green) in the embryonic brain is found in two neural cell body groups located in neuromeres DC and TC. (B) Mutations in the *otd gene* result in the absence or severe reduction of neuromere PC of the embryonic brain. In these embryos, the preoral brain commissure is completely absent, resulting in two separate rest hemispheres composed of neuromeres DC and TC. Mutation of the *ems* gene leads to complete absence of neuromeres DC and TC of the embryonic brain. In these mutants, the *en-DC* and *en-TC* stripes are missing. Neither the antennal nerve nor the tritocerebral commissure is maintained (Adapted and modified from Reichert et al., 1997).

Mutations in the *otd gene* result in absence or severe reduction of neuromere PC of the embryonic brain. Thus, in all the alleles of *otd* examined, more than 80% of the mutant embryos fail to develop neural structures anterior to the *en-PC* stripe. In these embryos, the preoral brain commissure is completely absent, resulting in two separate rest hemispheres composed of neuromeres DC and TC. Mutations in the *otd* gene do not show any morphological effects on the formation of brain neuromeres DC and TC, both of which appear to differentiate normally. Thus, the *en-PC* stripe is present in most mutant embryos (Fig. 14) (Hirth et al., 1995).

Ubiquitous overexpression of *otd* in a null mutant background is able to restore both the brain defects and the VNC defects (Leuzinger et al., 1998). Meanwhile, the overexpression of *otd* in a wild-type background results in the generation of ectopic or transformed neuronal structures such as dramatically increased brain lobes, ectopic head ganglionic structures, and fused and enlarged ventral ganglia (Leuzinger et al., 1998).

Expression of *ems* in the embryonic brain is found in two neural cell body groups located in neuromeres DC and TC. The larger, more anterior *ems*-immunoreactive cell body group is located in neuromere DC and extends from the *en-PC* stripe posteriorly to the *en-DC* stripe. With the exception of some of the cells that comprise the *en-PC* and *en-DC* stripes at the neuromere's borders, most of the neuronal cell bodies in neuromere DC appear to be ems^{+}. The smaller, more posterior ems^{+} cell body cluster is found in neuromere TC and extends from the *en-DC* stripe to the tritocerebral commissure. Mutation of the *ems* gene leads to the complete absence of neuromeres DC and TC of the embryonic brain. In almost 90% of the mutant embryos, a clear gap between brain neuromere PC and the first (mandibular) neuromere of the subesophageal ganglion is

observed. In these mutants, the *en-DC* and *en-TC* stripes are missing. Neither the antennal nerve nor the tritocerebral commissure is maintained (Fig. 14) (Hirth et al., 1995).

These expression patterns suggest that the *otd* homeobox gene might be important for the development of brain neuromere PC and that the *ems* homeobox gene is important for the development of brain neuromeres DC and TC.

C. 4. ii. c. Homeotic selector genes

Another family of genes required for embryonic brain development is the homeotic selector genes, which are arranged in two chromosomal complexes: the Antennapedia, and the Bithorax complexes (McGinnis and Krumlauf, 1992). The homeotic genes of the Antennapedia Complex are expressed both in the embryonic head and in the embryonic brain. However, spatial expression patterns and function of these genes are different in embryonic head development versus embryonic brain development. The members of the homeotic selector gene class specify segmental identity along the anteroposterior body axis of the early embryo. However, they are also expressed in the CNS later. The homeotic genes *lab* and *Dfd,* which are expressed in the embryonic brain anlage, are responsible for determining neuronal identity and cell fate. Although both genes are already expressed in the neuroectoderm, loss-of-function mutations show no apparent defects in neuroblast segregation or the generation of GMCs. The postmitotic cells in the mutant domain are generated and positioned correctly; but nevertheless, do not adopt a neuronal identity (McGinnis and Krumlauf, 1992).

As mentioned before, for most of the regulatory genes involved in embryonic brain development of *Drosophila,* there exist homologous genes in vertebrates, including

mammals. The similarities between the expression patterns and functions of orthologous genes between flies and vertebrates are significant for two important reasons. First, they suggest that both organisms are built according to a common basic plan, and second, it seems reasonable to assume that elucidating further the roles of regulatory genes in embryonic CNS development of *Drosophila* also helps us to understand the development of the mammalian nervous system.

In this study we have used molecular genetic analysis to identify roles of a gene, *jing,* in the differentiation of CNS midline and axon scaffold formation. The next tissue that seems to be affected by *jing* is the respiratory (tracheal) system.

D. Trachea

Epithelial tubes form the basic structure of many organs and tissues in *Drosophila melanogaster*, the nematode *Caenorhabditis elegans*, zebrafish, and mammals.. They usually constitute a single layer of cells that are frequently surrounded by additional layers of cells, which form barriers to separate the outside and inside compartments of the organism and regulate the transport of ions, gases, and liquids between these compartments. The formation of tubes involves interactions between different cell types and various environmental cues that result in tubular structures containing one or more cell types with potentially different functions. Understanding how tubes form is important in order to discover the fundamental aspects of development in addition to acquiring insights into the etiology of diseases and potentially their treatment by organ regeneration.

The question here is whether mechanisms regulating cell rearrangements during tube formation are evolutionarily conserved. Three levels of regulation appear to be involved in the formation of tubes; initiated with master regulation of gene transcription, followed by the localized expression of growth factors and their receptors involved in the transition of cells (e.g. from mesenchyme to epithelia), and by directed cell migration. The intracellular downstream effectors, such as cell adhesion molecules, play an important role in finalizing the tubule formation (Fig. 15, Nelson, 2003).

a) Tube formation without branching

b) Tube formation by inductive signaling from mesenchyme to epithelium, followed by induced branching

c) Reciprocal inductive signaling between mesenchyme and epithelium leads to epithelial tube formation, mesenchyme epithelium conversion, tube formation, and induced branching

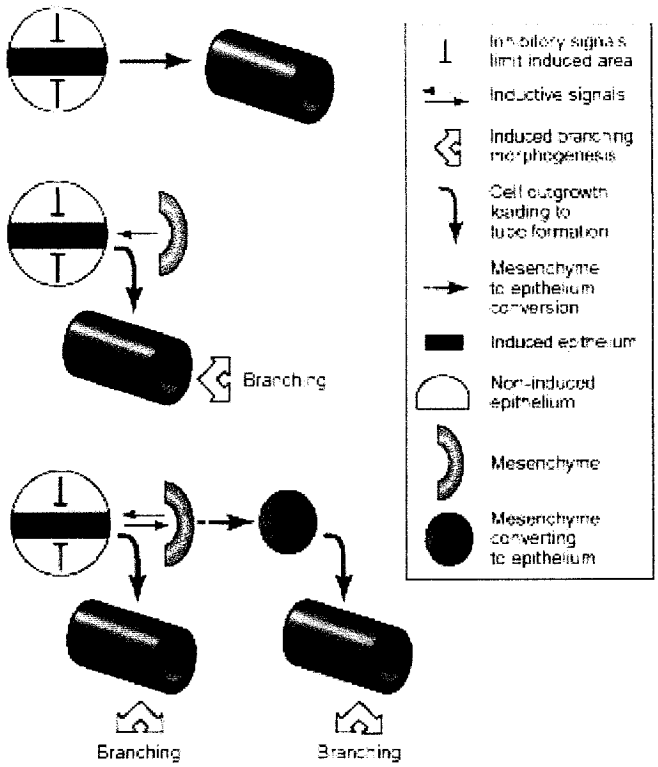


Figure 15. Schematic representation of different pathways leading to the formation of tubes. (a) A small group of cells in an epithelial primordium is induced by signals to invaginate and form a tube. The size of the induced group of cells is limited by inhibitory signals from the surrounding epithelium. Tube formation occurs without branching (e.g. in the salivary gland of *Drosophila*). (b) Inductive signaling from mesenchyme induces invagination of epithelial cells and formation of tubes. Stereotypic branching of the tube happens subsequently to form a tubular network (e.g. during the development of mammalian lung). (c) Reciprocal local signaling between adjacent epithelium and mesenchyme induces outgrowths of the epithelium leading to the formation of tubes and branches (e.g. during kidney development) (Adapted from Nelson, 2003).

D. 1. *Drosophila* embryonic tracheal development

The tracheal system of *Drosophila* consists of a branched network of epithelial tubes that provides oxygen from the environment to all tissues of the body. The interconnected network develops from individual clusters of ectodermal cells that invaginate into the underlying mesoderm and form 10 sacs (tracheal pits) on both sides of the embryo, each containing about 80 cells. Without further cell division, each sac forms five to six primary branches (dorsal branch, dorsal trunk anterior and posterior, lateral branch anterior and posterior, and visceral branch) by stereotypical, directed cell migration. Each of these branches has a defined identity that specifies tube size and the subsequent determination of specialized cell fates at precise positions and in the appropriate number (Fig. 16Ca, b; Ikeya and Hayashi, 1999).

Chemoattraction plays a crucial role in cell migration during the development of trachea (Fig. 16A), but in such an environment, migrating cells are faced with a number of additional factors. In many cases, cells first have to acquire the capability to detach from surrounding cells, invade other territories, and migration has to be initiated at precise developmental times. Ultimately, migrating cells have to stop their movement as they reach their final destination and differentiate into non-motile cells of distinct functions. The necessity of cells to coordinate their movement with their neighborhood in this system requires that motility as such is regulated by events that control cell adhesion, either between similar and/or different cells and the cellular matrix.

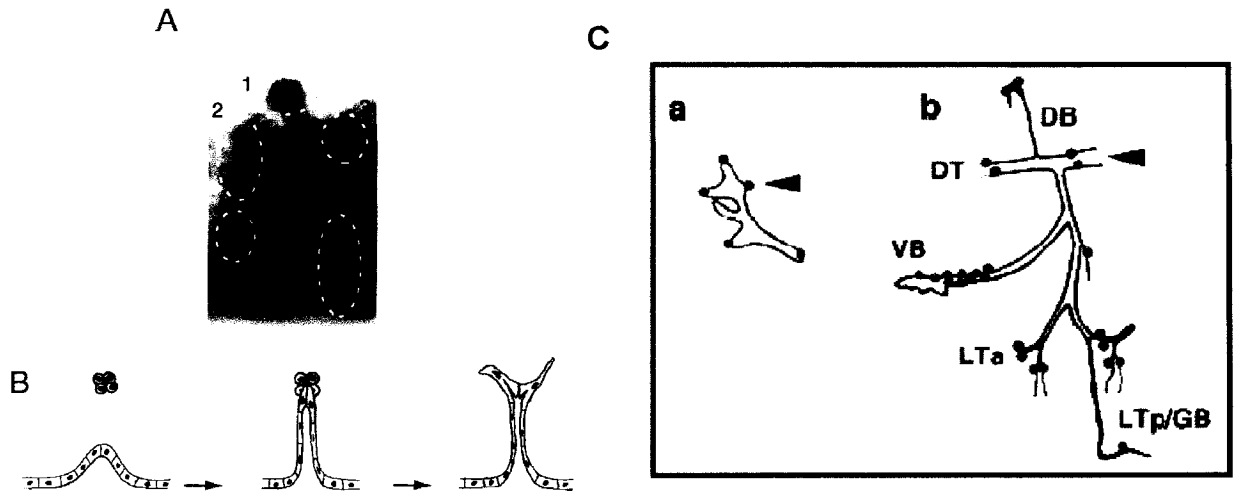


Figure 16. FGF control of branching morphogenesis. (A) Five domains of *branchless* mRNA expression (indicated in blue) surround a tracheal sac (*trachealess* expression in the center of the sac, indicated in brown) at the beginning of tracheal formation. Primary branches bud at these five positions and at a sixth position of *branchless* expression outside the focal plane. (B) Model of Branchless patterning during tracheal branching. Secreted Branchless FGF (blue) guides the migration of tracheal cells as they form primary branches. High levels of Branchless induce secondary branch genes such as *pointed* in the cells at the end of the primary branches (green), which guide these cells to form secondary branches. *Sprouty* encodes an FGF pathway inhibitor that limits the range of FGF signaling (green inhibitory arrows) (Adapted and modified from Metzger et al., 1999). (C) Schematic drawing of the branching pattern of the trachea. (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 terminal cells (blue) (Adapted from Ikeya and Hayashi, 1999).

D. 1. i. Primary branching

Two transcription factors belonging to the basic-helix-loop-helix (bHLH)-PAS protein family, *trachealess (trh)* (Isaac and Andrew, 1996) and the *Drosophila* homologue of ARNT *(tgo)* (Sonnenfeld, 1997) are essential for the development of the tracheal system. Trh::Tgo heterodimer complexes regulate the transcription of genes, including *btl*, required for the proper formation of the trachea.

The *Drosophila* fibroblast growth factor (FGF)-like gene, *branchless (bnl)* (Sutherland et al., 1996), has a critical role in tracheal branch patterning (Fig. 16A, B). Just before primary branching begins, *branchless* expression is initiated in clusters of cells around the tracheal sacs where primary branches will grow. Bnl binds the FGF receptor, *breathless (btl)* (Glazer and Shilo, 1991) on adjacent tracheal cells, stimulating the receptor's tyrosine kinase activity and downstream signal transduction cascades involving *Ras, Raf*, and *stumps* (*Dof*) (Vincent, 1998; Dossenbach, 2001). While each primary branch grows toward the adjacent cluster of *bnl*-expressing cells, expression of *bnl* ceases and the branch stops growing. Mis-expression of *bnl* causes ectopic branch migration toward the new sites. Therefore, the pattern of *branchless* expression establishes the pattern of primary branching (Fig. 17A, Metzger, 1999).

D. 1. ii. Secondary branching

Immediately after the beginning of the branch migration, single cells at the tip of branches express *escargot (esg)*, the zinc finger protein (Fuse et al., 1994). These cells

will become fusion cells and attach the branches through the formation of Cadherin-dependent intercellular adhesion, which is followed by changes in cell shape.

Several hours after primary branches begin, secondary branches begin to sprout. The secondary branching pattern is also controlled by *bnl* and *btl*, but through a different molecular mechanism. As primary branches extend toward the *bnl* signaling centers, cells at the growing end are exposed to high levels of the signal, which induces the expression of secondary branch genes such as *pointed (pnt)*, an ETS domain transcription factor (Klambt, 1993). The expression of *pnt* drives the formation of secondary branches. Meanwhile, *bnl* also induces the expression of an inhibitor, *sprouty (sty),* in the cells closest to the signaling center (Hacohen, 1998). Sty protein blocks *bnl* signaling to more distant tracheal cells and limits secondary branch sprouting to positions closest to the FGF signaling sources (Fig. 17A).

On the other hand, Bnl-Btl interactions lead to the stimulation of the mitogen-activated protein kinase (MAPK) pathway (Reichman-Fried et al., 1994). After the secreted Bnl activates its receptor, Btl, the signal is able to limit MAPK activation to the tip of the tracheal branches (Gabay, 1997). Notch is a transmembrane receptor involved in tracheal development and responsible for cell fate restriction (Greenwald, 1998). One of the well-known ligands of this receptor is Delta (Dl), a transmembrane protein (Kopczynski, 1988). The cross talk between the adjacent cells introduces a small difference in the level of Notch activity, resulting in a selection of one or a few cells taking a distinct developmental fate.

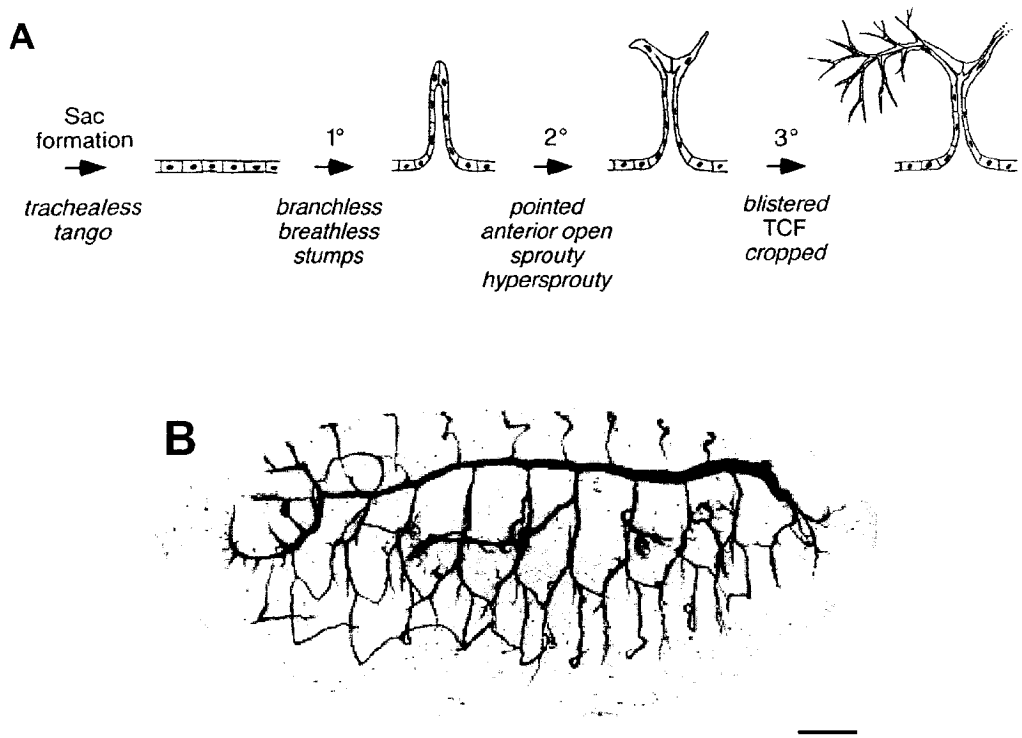


Figure 17. Structure and formation of branching networks. (**A**) Schematic of *Drosophila* tracheal development. A portion of an epithelial sac is shown sprouting primary (1°), secondary (2°), and terminal (3°) branches. Mutations in different genes can interrupt the process at the indicated steps. TCF, ternary complex factor. (**B**) Immunostaining with α-2A12 antibody shows the developing tracheal system in a *Drosophila* embryo at stage 15. There are 6 primary and approximately 25 secondary

branches in each segment, and hundreds of terminal (3°) branches will sprout during larval life. Bar is 15 μm (Adapted from Metzger et al., 1999).

Notch limits the expression of *escargot (esg)* (Tanaka-Matakatsu et al., 1996) to just one cell in each fusion branch. Notch signaling in the tracheal anlage is affected by the *bnl-btl* pathway, which stimulates *dl* expression at the tip of the primary branches in the place that *notch* appears to be suppressed. Meanwhile, Notch activation down regulates *bnl* expression followed by the repression of MAPK. This process leads to the differentiation of only a single cell at the tip of each branch to get involved in the fusion, later. During the early stages of primary branching formation, *bnl* up-regulates the expression of *dl,* which is essential to activate the Notch pathway. It seems that the pathway is affected by the exogenous FGF signal through the activation of *dl*. *pnt* mediates the activation by stimulating the transcription of dl (Ikeya and Andrew, 1999).

The increase in *dl* expression in the fusion cells located at the tip of branches activates Notch in adjacent cells resulting in the repression of *esg* transcription. However, in order to have the tracheal branching formed, Notch activity has to be low at the tip of fusion branches. Therefore, the fusion cell must have a mechanism that activates Notch in adjacent cells and represses Notch in itself at the same time. It seems that the high level of *dl* expression in the fusion cell is responsible for this mechanism. The fusion cells present Dl to Notch as an agonist but the over-expression of *dl* inhibits Notch activity in the trachea (Llimargas, 1999; Ikeya and Andrew, 1999). Thus, high-level of dl in fusion cells provides a cell-autonomous, dominant-negative effect on Notch within the cells to allow *esg* expression. It has been shown that activated *notch* is also able to repress the expression of *dl*. This common capability between these two proteins from a regulatory

circuit leads to the selection of just one cell among a group of cells to have high level of *dl* expression.

D. 1. iii. Terminal branching

Terminal branches start growing several hours after the secondary branches and continue throughout larval stages. The structure of terminal branches is completely different from that of primary and secondary branches. The pattern is not completely conserved and varies depending on the amount of the oxygen each tissue needs. The *Drosophila* serum response factor, *blistered* (*pruned*), is a MADS domain protein (Fristrom et al., 1994; Nussbaumer et al., 2000) and part of an FGF-activated transcription complex that regulates other terminal branch genes. FGF signaling in primary and secondary branching triggers this gene right before the initiation of the terminal branching (Fig. 17A, B).

Therefore, the FGF pathway is necessary for the patterning of each generation of tracheal branches. Moreover, what makes it act differently is the influence of different genes on the ligand activity, which results in various and proper branch patterning. Furthermore, it seems that mutation in *jing* has an effect on cell fusion fates and adhesion molecules, which are components of epithelial cell junctions, during tubule formation. In the following section, the epithelial cell junctions in *Drosophila* and their molecular components will be described.

D. 2. Regulation of junction formation in *Drosophila* epithelial cells

Epithelial cells separate the outside world from the inside of the multicellular organism body. Division of the epithelial plasma membrane is important in the appearance of the

epithelial cells. These divisions occur in at least two separate domains, the apical domain, which is facing the outside, and the basolateral domain, where it is in contact with the other cells as well as the internal medium. Cell-cell contacts allow adhesion molecules to work together and gather other scaffold proteins that will lead to reinforcement of other transmembrane proteins (Yeaman et al., 1999). At this point, junctions are formed and recruited to separate the plasma membrane domains. The extracellular cell surface organizers give signals to the cells to reach to the right position followed by choice of their future polarity (Mostov et al., 2000; Knust, 1994).

In the mammalian system, integrins and cadherins are responsible for establishing and maintaining epithelial polarity (Hynes, 1992). *Drosophila* provides a powerful complementary system to study epithelial polarity regulation since epithelial cell organization in *Drosophila* is similar to that of mammalian cells.

D. 2. i. Epithelial cell junctions

Epithelial cell junctions serve the adhesion, communication, directed transport, and morphogenetic properties of the epithelial system. One of the most important functions of the epithelial cells is to make a barrier between two biological compartments with the ability of transporting certain molecules. Cell junctions in vertebrates and invertebrates have several features in common.

In vertebrates, the transport of the molecules through the epithelial layer as well as transport in between the individual cells, are provided by tight junctions (TJs) (Dragsten et al., 1981; Tsukia et al., 2001). In invertebrates, including *Drosophila,* this transportation is mediated by septate junctions (SJs) (Wood, 1990; Genova and Fehon, 2003). In both vertebrates and invertebrates, adherens junctions form a circumferential

belt close to the apical-lateral border of the membrane domains; this structure is called zonula adherens (ZA). The Cadherin/catenin cell adhesion system is the major component of the ZA. The ZA is also required for coordinated movement of the epithelial tissues. SJs and TJs are different in their ultrastructural appearance, the ZA localizes in different positions in vertebrates compared with invertebrates: in the former, TJs are localized apical to the ZA, but in the latter SJs are located basal to the ZA (Fig. 18) (Tepass et al., 2001).

D. 2. ii. Molecular components of *Drosophila* epithelial polarity complexes

The ZA junctions are well conserved among the species and composed of a calcium-dependent adhesion transmembrane protein E-cadherin (DE-Cad in Drosophila and E-Cad in vertebrates), connected to the actin cytoskeleton through α- and β-catenin (D α-Cat and Armadillo in *Drosophila* and α-Cat and β-Cat in vertebrates) (Vasioukhin and Fuchs, 2001). In *Drosophila* the sub-apical complex (SAC) (Tepass et al., 2001) is apical to the ZA, whereas in mammals the tight junction (TJ) is located at this position. In the SAC, the transmembrane protein Crumbs (Crb) (Jurgen et al., 1984) has been shown to be essential for the ZA morphogenesis. *crb* loss-of-function is embryonic-lethal after gastrulation and it seems that it is involved in epithelial movement and reorganization (Tepass et al., 1990). The ZAs which are normally formed as spots on the lateral membrane and transform to a belt-like structure around the cell cannot proceed through this transition in *crb* mutants.

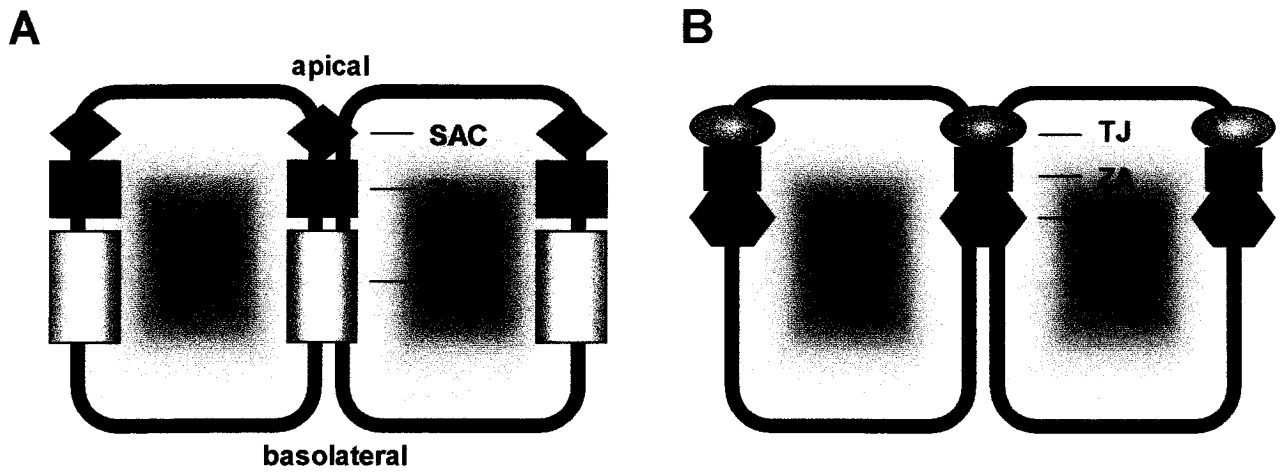


Figure 18. Cell junctions in invertebrate and vertebrate epithelia. (A) The invertebrate junctional complex consists of the sub-apical complex (SAC), the zonula adherens (ZAs), and a lateral septate junction (SJ). (B) In vertebrates, the epithelial junctional complex consists of tight junctions (TJs), ZAs, and desmosomes (Ds) (Adapted and modified from Tepass et al., 2001).

It seems that *crb* function is necessary for the proper positioning of the ZAs (Tepass, 1996; Wodarz et al., 1995). This epithelial cell surface organizer plays a crucial role in epithelial morphogenesis through its interaction with Stardust (Sdt) and Discs lost (Dlt), and is linked to the cortical cytoskeleton through DMoesin (Dmoe). *sdt* is an important regulator of ZA formation and epithelial polarity (Bachmann et al., 2001). Immunostaining studies have shown the co-localization of Sdt with Crb and one of the four Sdt proteins can bind directly to the cytoplasmic domain of Crb; Crb is also associated with Dlt (Tepass and Knust, 1993; Bachmann et al., 2001). Overall, the Crb complex is necessary for the specification of the apical membrane.

In mammals the homologues of the SAC components are located at the TJ level and are in close relation with the TJ components (the transmembrane proteins Claudin (Clau) and Occludin (Occ), and the MAGUKs ZO-1/ZO-2/ZO-3). In *Drosophila,* the septate junction (SJ) lies basal to the ZA and plays a role in controlling paracellular diffusion. This junction is formed by the transmembrane protein Neurexin IV (NrxIV) and several other proteins important for epithelial polarity such as Discs large (Dlg), Lethal giant larvae (Lgl), and Scribble. In mammals the Dlg, Lgl, and Scribble homologues are also found below the ZA and co-localize with the HER2/Erbin complex (Muller and Bossinger, 2003).

E. Statement of problem

Previous studies have shown that during the embryonic development of *Drosophila*, a variety of cell types undergo temporally and spatially controlled cell migrations in addition to dynamic changes in cell behavior. When these processes are not properly controlled, various tissues are affected, resulting in the development of abnormal or non-functional organs. Transcription factors play a major role in these essential processes. Specifically, bHLH-PAS proteins have been shown to be required for neuronal, glial, and tracheal development. However, the numerous downstream targets mediating these effects are poorly understood.

My main goal is to elucidate the mechanisms required in the regulation of cell differentiation and survival in the *Drosophila* embryonic central nervous system and tracheal system. Based on the initial examination of the *sim/tgo* heterozygous line in a genetic screen, mutations in the *jing* gene were found to be dominant enhancers of *sim/tgo* during CNS axon formation. Embryos with mutations in *jing* also demonstrated defects in tracheal tubule patterning.

Genetic analysis of axon patterning in the *Drosophila* CNS has revealed the important role of neuronal and glial function. Mutations leading to reductions in midline neuron numbers correlate with a reduction in the number of commissural tracts, whereas mutations leading to reductions in midline glia numbers show fused commissure phenotypes. Therefore, midline neurons (such as the VUMs) are required to attract commissural growth cones initially to the CNS midline, whereas midline glia are required subsequently for the organization of commissural axons. There are questions which remain to be answered; whether *jing* phenotype is the consequence of midline neuron

and/or midline glial dysfunction, and if *jing* regulates the transcription of important survival factors including those of the Egfr pathway? It has been shown that proper function of Egfr pathway genes is required for the survival of cells most highly affected by *jing* mutations, including the CNS midline, the tracheal dorsal trunk and visceral branches.

The *jing* locus was also identified in a screen for mutations that cause border cell migration defects in the ovaries. It was identified as a downstream target of *slbo*, an important factor required for border cell migration. An interesting parallel between the ovarian and embryonic pathways with the possible involvement of *jing,* is the activation of *btl, Drosophila* FGF receptor. *btl* is expressed in embryonic tracheal and midline glial cells, as well as border cells of the ovary, *btl* is involved in differentiation of trachea cells and central nervous system glial cells. In both cases, interruption of *btl* function results in altered cell mobility. *btl* is a direct target of C/EBP and Trh::Tgo heterodimers *in vitro.* Therefore, it would be necessary to study the possible affect of *jing* on *btl* expression. *jing* expression itself, must also be controlled during embryonic development. Studying *jing* 5′ flanking region could identify the presence of potential binding site for transcription factors that are involved in the regulation of *jing* expression.

Chapter Two

Sedaghat et al. (2002). The *jing* Zn-finger transcription factor is a mediator of cellular differentiation in the *Drosophila* CNS midline and trachea.

The following outlines the experimental contributions of the authors of this manuscript. Contribution of Y. Sedaghat: (1) The sequencing of the genomic DNA surrounding the P element insertions in $jing^{01094}$ and $jing^{K03404}$ flies including the *jing*-coding sequences; (2) The sequencing and comparison of LD36562 and LD10101 to $jing^{01094}$ and $jing^{K03404}$; (3) The sequencing of *jing* EMS-induced alleles; (4) The cloning of the *jing* full-length cDNA (LD36562) into pUAST and the selection of the transgenic UAS-*jing* lines; (5) Immunostaining of embryos with α-BP102, α-2A12, α-β-gal, α-Jing and α-Odd; (6) Y. Sedaghat also established and performed in situ hybridization; (7) Confocal microscopy was done in part by Y. Sedaghat; and (8) TUNEL staining was established by W. Miranda and Y. Sedaghat.

EMS-induced *jing* mutations were made and screened by W. Miranda. The Majority of confocal microscopy and immunostaining of embryos with α-Trh, α-Odd, α-Sli, α-Sim, α- β-gal and TUNEL were performed by W. Miranda and analyzed by W. Miranda and Dr. M. Sonnenfeld . The *jing* gene was originally found in a screen for CNS axon phenotype by Dr. M. Sonnenfeld. Dr. Sonnenfeld wrote the manuscript.

The *jing* Zn-finger transcription factor is a mediator of cellular differentiation in the *Drosophila* CNS midline and trachea

Yalda Sedaghat*, Wilson F. Miranda* and Margaret J. Sonnenfeld†

Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

* These authors contributed equally to this work

Development 129, 2591-2606 (2002)

Abstract

We establish that the *jing* zinc-finger transcription factor plays an essential role in controlling CNS midline and tracheal cell differentiation. *jing* transcripts and protein accumulate from stage 9 in the CNS midline, trachea and in segmental ectodermal stripes. JING protein localizes to the nuclei of CNS midline and tracheal cells implying a regulatory role during their development. Loss of *jing-lacZ* expression in homozygous *sim* mutants and induction of *jing-lacZ* by ectopic *sim* expression establish that *jing* is part of the CNS midline lineage. We have isolated embryonic recessive lethal *jing* mutations that display genetic interactions in the embryonic CNS midline and trachea, with mutations in the bHLH-PAS genes *single-minded* and *trachealess*, and their downstream target genes (*slit* and *breathless*). Loss- and gain-of-function *jing* is associated with defects in CNS axon and tracheal tubule patterning. In *jing* homozygous mutant embryos, reductions in marker gene expression and inappropriate apoptosis in the CNS midline and trachea establish that *jing* is essential for the proper differentiation and survival of these lineages. These results establish that *jing* is a key component of CNS midline and tracheal cell development. Given the similarities between JING and the vertebrate CCAAT-binding protein AEBP2, we propose that *jing* regulates transcriptional mechanisms in *Drosophila* embryos and promotes cellular differentiation in ectodermal derivatives.

Key words: *jing*, Zinc finger, CNS midline, Trachea, *Drosophila*

Introduction

Transcriptional regulatory mechanisms play an important role in cell fate determination in both vertebrates and invertebrates. Transcription factors have multiple roles in controlling cellular competence or determination and also in regulating differentiation (Cau et al., 2000; Hallam et al., 2000; Moran-Rivard et al., 2001; Pierani et al., 2001; Portman and Emmons, 2000). The combinatorial nature of transcriptional regulation during cellular specification is emerging from studies of diverse embryonic tissues including the nervous system and respiratory system (trachea) (Boube et al., 2000; Jurata et al., 2000; Ma et al., 2000; Zelzer and Shilo, 2000). In addition, different categories of transcription factors can function at successive steps during cellular development. In this way, cellular responses may be dictated by the temporal and spatial characteristics of multiple and/or diverse types of transcription factors.

In *Drosophila*, the ventral midline and respiratory system (trachea) are ectodermal derivatives patterned by positional cues present in embryos. The ventral midline is patterned by the combinatorial actions of dorsal/ventral (D/V) and neurogenic genes that confine the expression of the bHLH-PAS transcription factor *single-minded* (*sim*) to the mesectoderm (Crews, 1998; Morel and Schweisguth, 2000). Development of the entire CNS midline requires the regulatory functions of *sim* and in the absence of *sim* function midline cells take on lateral neuroectodermal fates (Crews, 1998; Estes et al., 2001; Nambu et al., 1991; Xiao et al., 1996). The midline-inducing capabilities of *sim* were discovered by ectopic expression experiments (Nambu et al., 1991). Subsequent CNS midline gene regulation involves the combinatorial functions of 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 UMI neurons, the MNB and VUMs (Klämbt et al., 1991; Bossing and Technau, 1994; Schmid et al., 1999).

The tracheal placodes are specified by TGFß signaling along the dorsoventral axis and Wingless (WG) signaling along the anteroposterior axis (Affolter et al., 1994; de Celis et al., 1995; Wilk et al., 1996). These cues are responsible for independently activating primary genes such as the bHLH-PAS transcription factor *trachealess* (*trh)* and the POU domain transcription factor *ventral veinless* (*vvl*) (previously known as *drifter*), which are required in a combinatorial fashion for subsequent tracheal development (Boube et al., 2000; Isaac and Andrew, 1996; Llimargas and Casanova, 1997; Wilk et al., 1996; Zelzer and Shilo, 2000). In the absence of *trh* and *vvl* function tracheal cells fail to invaginate and tracheal tubules do not form (de Celis et al., 1995; Isaac and Andrew, 1996; Wilk et al., 1996). Ectopic *trh* expression can induce tracheal pits and therefore it has been considered an inducer of cell fates (Wilk et al., 1996). Within the trachea, the DPP pathway specifies the fates of branches that will give rise to the dorsal branch and lateral anterior and posterior branches (Affolter et al., 1994; Vincent et al., 1997; Wappner et al., 1997). Activation of the EGF receptor pathway is required for specifying the dorsal trunk and visceral branch (Wappner et al., 1997). In addition, the WNT pathway is required for specification of the dorsal trunk (Llimargas, 2000; Chihara and Hayashi, 2000).

The invertebrate ortholog of the aryl hydrocarbon nuclear translocator, known as *tango* (*tgo*), encodes a common partner for SIM and TRH (Oshiro and Saigo, 1997; Sonnenfeld et al., 1997; Zelzer et al., 1997). TGO is present in the cytoplasm and translocates to the

nucleus upon expression of a dimerization partner such as *sim*, *trh* or *Spineless-Aristapedia* (*ss*) (Emmons et al., 1999; Ward et al., 1998). Therefore, the precise regulation of lineage-specific transcriptional regulators such as *sim*, *trh* and *ss* is critical. In both the CNS midline and trachea, TGO::SIM and TGO::TRH heterodimers activate common target genes containing asymmetrical E-box sites with an ACGTG core (Crews, 1998; Zelzer and Shilo, 2000). These E-box sequences are sufficient to drive both midline and tracheal expression and are required for the expression of known target genes including the *breathless* fibroblast growth factor receptor and the repulsive guidance molecule *slit* (Battye et al., 1999; Glazer and Shilo, 1991; Kidd et al., 1999; Sonnenfeld et al., 1997; Wharton et al., 1994).

In this study, we have used genetic and cellular analysis to establish novel roles for the *jing* zinc-finger transcription factor in the differentiation of CNS midline and tracheal cells. A genetic approach to identify molecules required for the commitment of CNS midline and tracheal cells led to the identification and characterization of the *jing* locus during embryogenesis. The *jing* locus has been previously identified in genetic screens and recently characterized for its role in border cell migration in *Drosophila* ovaries (Karpen and Spradling, 1992; Liu and Montell, 2001). During embryogenesis, *jing* transcripts and protein are detected in the CNS midline, trachea and segmental ectodermal stripes. Gene dosage and overexpression experiments reveal that appropriate levels of *jing* in the CNS midline and trachea are crucial for formation of CNS commissural and longitudinal axons as well as tracheal tubules, respectively. Loss-of-function mutations in *jing* are associated with reductions in cell-type gene expression and inappropriate apoptosis of CNS midline and tracheal precursors. These results therefore establish that

jing is required in a positive manner to promote cellular differentiation and survival in embryonic ectodermal lineages.

Materials and Methods

Drosophila strains and genetics

The wild-type strain was w^{118}. The *tango*^{1} (*tgo*^{1}) allele and the *tgo*^{1}*trh*^{8} double mutant strain have been previously described (Sonnenfeld et al., 1997). Double mutant stocks were generated by standard genetic procedures using P element *jing* alleles and null alleles of *single-minded* (*sim*^{H9}) and *trachealess* (*trh*^{1}) (Isaac and Andrew, 1996; Nambu et al., 1990). The second and third balancer chromosomes in these stocks carried the *lacZ*-markers *CyO*P[*wg-lacZ*] and TM3P[*ubx-lacZ*]. The *slit* (*sli*) reporter P[*sli* 1.0 HV-*lacZ*] was used to assess transcription of the *sli* gene (Ma et al., 2000; Wharton et al., 1994). The *breathless*$^{H82\Delta 3}$ P-element excision allele (Klämbt et al., 1992) and the null *sli*^{1} allele (Nüsslein-Volhard et al., 1984; Rothberg et al., 1990) were used to assess genetic interactions with multiple *jing* alleles.

P-element lethal stocks were obtained from the Indiana University *Drosophila* Stock Center (Bloomington, Indiana). The *jing-lacZ* enhancer trap strain (*jing*^{01094}) contains an embryonic recessive lethal insertion of the P-element P[PZ] originally designated l(2)*01094* (BDGP) (Karpen and Spradling, 1992; Spradling et al., 1999). l(2)*01094*^{K03404} is a second embryonic lethal insertion of the P[*lacW*] P-element in the *jing* gene (from I. Kiss, T. Laverty and G. Rubin) (Liu and Montell, 2001) and we refer to this allele as

jing^{K03404}. Both alleles do not complement the lethality of a *jing* deficiency Df(2R)ST1 (Liu and Montell, 2001).

The P[*sim*-UAS] and P[*prd*-Gal4] strains were used to ectopically express *sim* under control of a *prd* enhancer in a background heterozygous for the *jing-lacZ* enhancer trap (Brand and Perrimon, 1993; Ward et al., 1998; Xiao et al., 1996). P[*sim*-GAL4] and P[*btl*-GAL4] were used as drivers to overexpress *jing* in the CNS midline and trachea, respectively (Shiga et al., 1996; Ward et al., 1998).

Molecular analysis of *jing*, P[UAS-*jing*] construction and antibody production

Genomic DNA surrounding the P element insertions in *jing*^{01094} and *jing*^{K03404} flies, and including *jing*-coding sequences, was sequenced and deposited into GenBank (AF285778). Expressed sequence tags (ESTs) LD10015, LD36562 and LD10101 were identified by database searching, obtained from Research Genetics (Birmingham, AL) and subjected to DNA sequence analysis. Gel-purified fragments of PCR-generated genomic and EST DNA were sequenced on both strands by dye terminator cycle DNA sequencing (Perkin Elmer) using an ABI PRISM Genetic Analyzer. LD36562 and LD10101 sequences were identical to FlyBase Genome Annotation Database (GadFly) identifier CG9403 (http://flybase.bio.indiana.edu/annotl) (Adams et al., 2000).

The *jing* full-length cDNA (LD36562) was cloned into pUAST (Brand and Perrimon, 1993) and together with pUChspΔ2,3 P element helper plasmid was injected into *y w* embryos and w^+ transformants were selected (Spradling, 1986). Three transgenic UAS-*jing* lines produced similar results in overexpression experiments using P[*sim*-GAL4] and P[*btl*-GAL4] as drivers (Shiga et al., 1996; Ward et al., 1998).

For antibody production, the JING peptide VPAASANKNKRTAAG (amino acids 81-95) was synthesized (Eastern Quebec Proteomics Core Facility), coupled to KLH (Sigma) and used to generate anti-JING rat antisera (PRF&L). JING antibody specificity was confirmed by examining embryos homozygous for a deficiency in *jing* (Df(2R)ST1) and after ectopic expression of *jing* (*prd*-GAL4/*jing*-UAS).

Antibodies

The following antibodies were used: rat anti-JING (1:100; this work); mAb anti-ß-galactosidase (anti-ß-gal) (Promega); rabbit polyclonal anti-ß-gal (Promega); rat polyclonals anti-Single-minded (anti-SIM) and anti-Trachealess (anti-TRH) (Sonnenfeld et al., 1997; Ward et al., 1998); anti-Wrapper and mAb 1D4 anti-FASCICLIN II (Noordemeer et al., 1998; Lin et al., 1994) (gifts from C. S. Goodman); mAb anti-Slit (a gift from Spyros Artavanis-Tsakonas); mAb 2A12 (a gift from N. Patel); and rabbit anti-Odd-skipped (ODD) (Skeath and Doe, 1998; Spana et al., 1995) (a gift from Jim Skeath). The following antibodies were obtained from the Developmental Studies Hybridoma Bank: mAb BP102; 22C10/FUTSCH (Fujita et al., 1982; Hummel et al., 2000); and mAb 4D9 anti-Engrailed/Invected (Patel et al., 1989).

Immunohistochemistry, in situ hybridization and TUNEL labeling

Embryo staging was carried out according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985) and processing for light microscopy was undertaken according to standard protocols (Patel, 1994). JING protein distribution was determined by staining whole-mount embryos with rat anti-JING at 1:100 dilution. Antibody staining was visualized using HRP- or rhodamine-conjugated secondary antibodies (Jackson).

Balancer chromosomes carrying *lacZ* for the second (*CyO*P[*wg-lacZ*]) and third chromosomes (*TM3*P[*ubx-lacZ*]) were used to identify homozygous mutant embryos after anti-ß-gal staining. HRP-labeled embryos were analyzed by light microscopy using a Zeiss Axioskop.

In situ hybridization was performed on w^{118} whole-mount embryos as described (Tautz and Pfeifle, 1989). DNA probes were generated by random priming using wild-type *Drosophila* genomic DNA (GenBank Accession AF285778) and *jing* cDNA (LD36562) as templates. All probes showed identical expression patterns. *jing* DNA probes were labeled with dig-11-dUTP (Boehringer Mannheim) and their specificity determined by in situ hybridization to embryos carrying the Df(2R)ST1 deficiency. Embryos were analyzed by light microscopy.

jing enhancer trap expression was analyzed after staining heterozygous $jing^{01094}$ embryos with anti-ß-gal and a secondary antibody conjugated with FITC or rhodamine at 1/250. TUNEL (TMR red; Roche) staining was carried out according to previous procedures and was double stained with anti-SLI, -TRH or -Odd-skipped (Booth et al., 2000). Fluorescently labeled embryos were mounted in 4% n-propyl gallate to inhibit photobleaching and analyzed on a Zeiss Axiovert 100 TV confocal microscope. Optical sections of 1 µm were recorded in line average mode. All figures were processed using Adobe Photoshop software.

Mutagenesis

An F_2 lethal complementation screen using ethylmethane sulfonate (EMS) was performed as described (Grigliatti, 1986; Sonnenfeld et al., 1997). Three lethal EMS-induced *jing*

mutations were tested for genetic complementation inter se and with deficiency Df(2R)ST1 (Lui and Montell, 2001). All mutant chromosomes were balanced over *CyO*P[*wg-lacZ*] marked balancers. The *jing*^{3} EMS-induced allele was sequenced according to previous procedures (Sonnenfeld et al., 1997). Embryonic lethal and viable excision (reversion) *jing*^{01094} and *jing*^{K03404} alleles were obtained by standard procedures (Bellen et al., 1989; Robertson et al., 1988). Lethal and viable excisions were isolated by loss of eye color and by genetic complementation and were mapped by PCR using *jing*-specific primers and DNA sequence analysis.

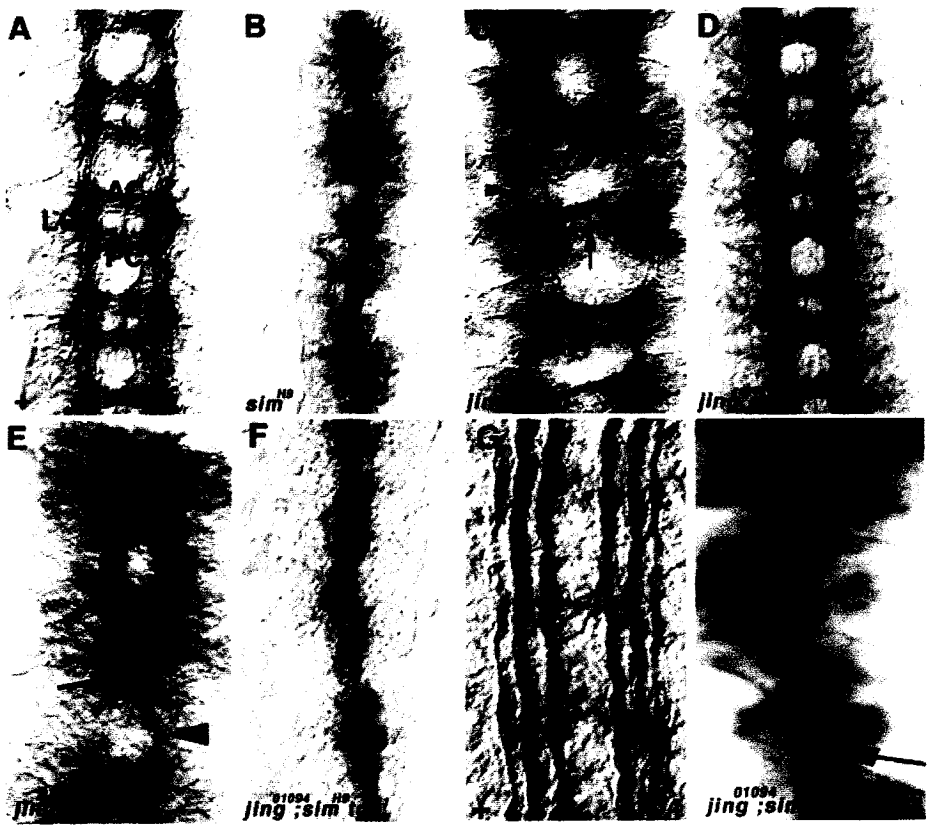
Results

jing mutations display dominant genetic interactions in the CNS with mutations in *sim*, *tgo* and their target *sli*

Dosage-sensitive genetic interactions between two loci are a good indicator that two gene products are functionally related. We identified the *l(2)01094* gene (BDGP) in a search for P-element-induced mutations displaying severe CNS axon phenotypes in double heterozygous combination with null mutations in *sim*. The *l(2)01094* gene has been previously isolated in genetic screens and recently characterized for its role in border cell migration in *Drosophila* ovaries (Karpen and Spradling, 1992; Liu and Montell, 2001). *l(2)01094* encodes a zinc-finger transcription factor called *jing* and we refer to this P-element-induced allele as *jing*^{01094} (Liu and Montell, 2001).

To address whether *jing* dosage is important for CNS midline development, *jing* P-element insertion mutant alleles were placed in heterozygous combination with null mutations in genes whose primary effects arise from the CNS midline, including *sim* and *sli* mutations. We also tested hypomorphic *tgo* mutations. CNS axon and midline cell development were assessed in double heterozygous embryos by BP102, anti-SIM or anti-SLI staining (Klämbt et al., 1991; Rothberg et al., 1990; Ward et al., 1998). $jing^{01094}$ alleles perturb CNS axon formation and midline cell development in double heterozygous combination with sim^{H9} (Fig. 1C,I; Fig. 2C), tgo^{1} (Fig. 1E,I), and sli^{1} (Fig. 1I; Fig. 2I,K). For example, 54% of *jing* and *sim* double heterozygotes show improper commissural and longitudinal axon formation ('stalled axons'). A smaller percentage of $jing^{01094}$ and sim^{H9} double heterozygotes (7.7%) show 'collapsed axon' phenotypes similar to those of *sim* or *sli* homozygotes (Fig. 1B,I) (Nambu et al., 1990; Rothberg et al., 1990). The phenotypes of *jing* and *sim* double heterozygotes are insertion dependent as they revert to wild- type after precise excision of the P element in $jing^{01094}$ flies (Fig. 1D,I; not shown).

CNS axon and midline cell development are perturbed in embryos triple heterozygous for *jing*, *sim* and *tgo* (Fig. 1F,I; Fig. 2B,E,G). However, unlike in homozygous *sim* mutants, the midline cells in *jing*, *sim* and *tgo* triple heterozygotes are specified but then fail to differentiate properly, as determined by their displacement from the ventral nerve cord (a feature characteristic of apoptotic cells) and loss of Sim immunoreactivity by stage 15 (Fig. 1B; Fig. 2B,E,G) (Sonnenfeld and Jacobs, 1995). The ventral displacement of the


A
B
D
E
F
G
H
sim^H9
jing^01094;sim^H9

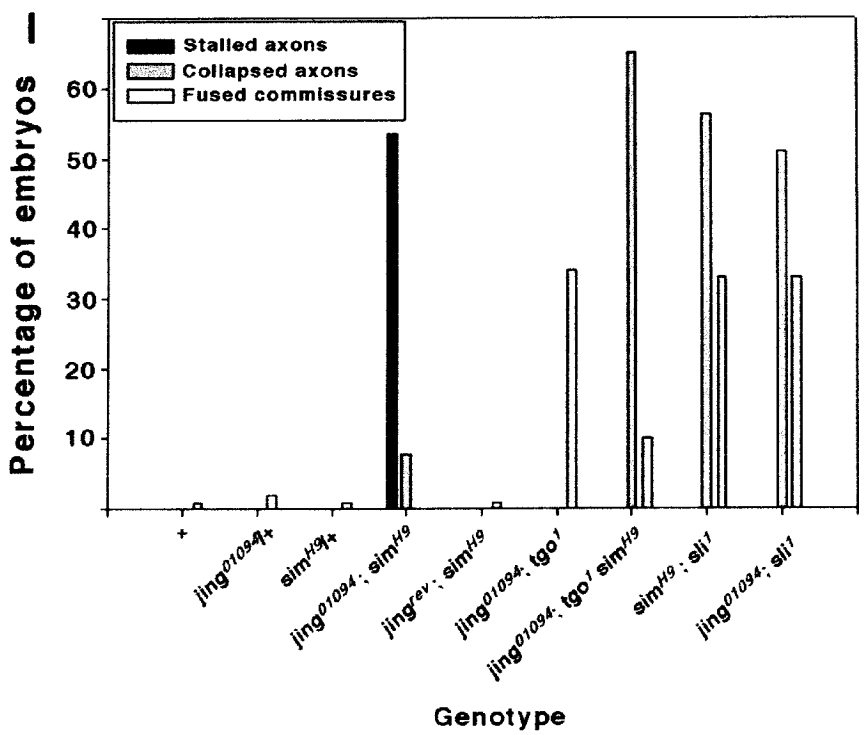

Stalled axons
Collapsed axons
Fused commissures
Percentage of embryos
60
50
40
30
20
10
+
jing^01094/+
sim^H9/+
jing^01094; sim^H9
jing^rev; sim^H9
jing^01094; tgo^1
jing^01094; tgo^1 sim^H9
sim^H9; sli^1
jing^01094; sli^1
Genotype

Fig. 1. Genetic interactions in the CNS. (A-F) Preparations of stage 14 ventral nerve cords stained with mAb BP102. Anterior is upwards. (G,H) Stage 15 embryos stained with mAb 1D4 (anti-Fasciclin 2). (A) Wild-type CNS axon scaffold with anterior and posterior commissures (AC and PC) separating longitudinal connectives (LC). (B) Collapsed axon phenotype of a sim^{H9} homozygote. (C) 'Stalled axon' phenotype of $jing^{01094}$; sim^{H9} double heterozygote. Arrow and arrowhead indicate improper commissural and longitudinal formation, respectively. (D) The axon scaffold develops properly in embryos heterozygous for sim^{H9} and a $jing^{01094}$ P element revertant ($jing^{rev}$). (E) The phenotype of embryos heterozygous for tgo^{1} and $jing^{01094}$ resembles weak *sim* phenotypes (arrow) and *spitz* group phenotypes (arrowhead). (F) Severe 'collapsed axon' phenotype in $jing^{01094}$, tgo^{1} sim^{H9} triple heterozygote. (G) Three wild-type 1D4-positive longitudinal fascicles run parallel to the midline. (H) Fusion of longitudinal fascicles in $jing^{01094}$, tgo^{1} sim^{H9} triple heterozygotes. (I) Quantification of *jing* genetic interactions. Double and triple heterozygotes (genotype) were examined for CNS axon scaffold formation using mAb BP102. Data are presented as the percentage of embryos with stalled, collapsed and fused axon phenotypes (n>50 embryos scored in all cases). Note that reduction of one copy of *tgo* in a $jing^{01094}$; *sim* double heterozygote causes a shift in distribution from embryos with stalled axon phenotypes toward those with collapsed axon phenotypes ($jing^{01094}$, tgo^{1} sim^{H9}). $jing^{01094}$ and sim^{H9} mutations show similar dose sensitivities to sli^{1}.

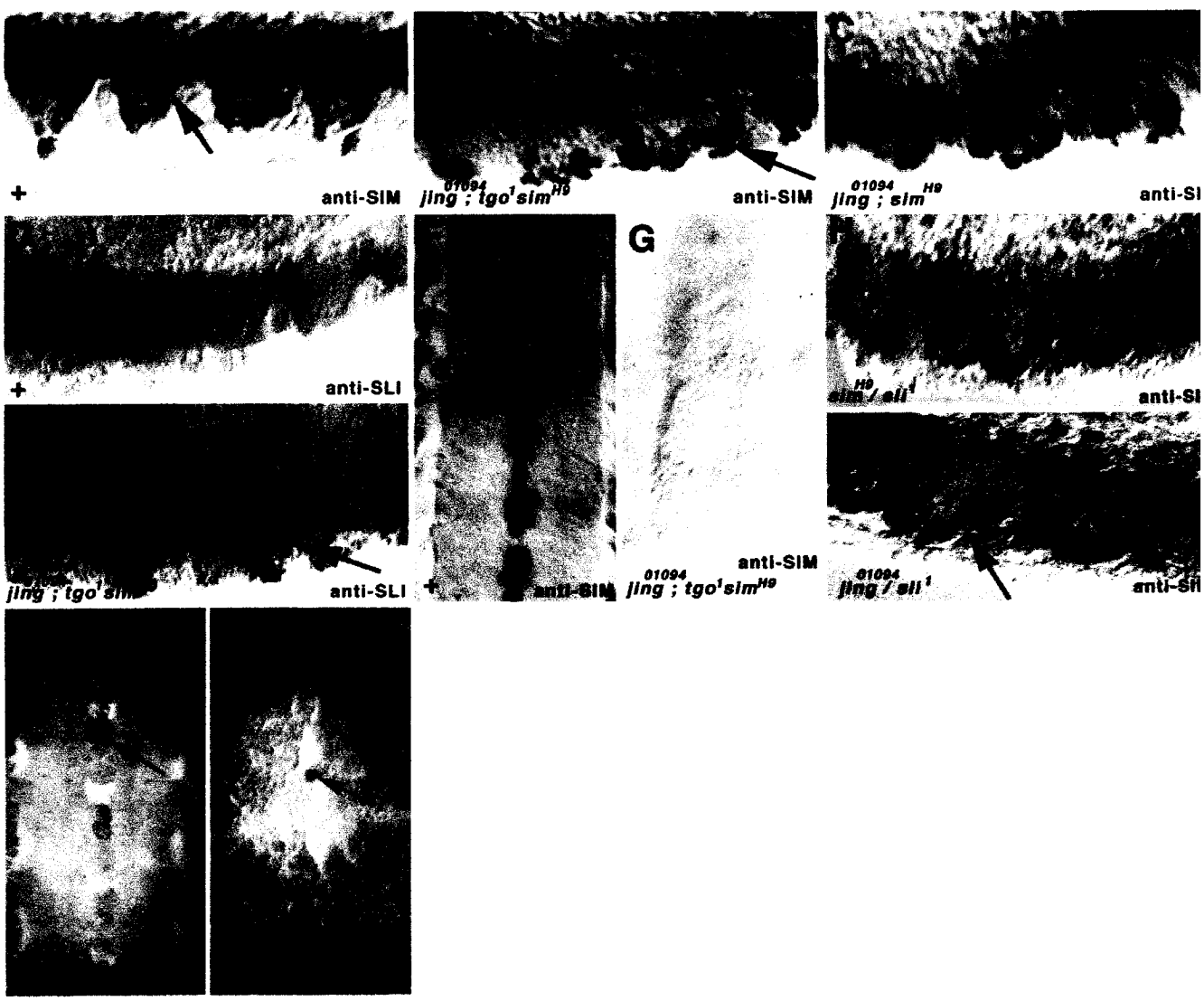

+
anti-SIM
anti-SIM
anti-SIM
+
anti-SLI
anti-SLI
G
anti-SIM
anti-SIM

Fig. 2. Genetic interactions alter CNS midline cell development. Whole-mount embryos stained with anti-SIM (A-C,F-I) and anti-SLI (D,E,J,K). (A-E,H,I) Sagittal section with anterior towards the left and (F,G,J,K) frontal views with anterior upwards. (A) In stage 13 wild-type embryos, SIM-positive cells are distributed ventrally to dorsally (arrow) and are only occasionally outside the nerve cord (arrowhead). (B) In stage 13 $jing^{01094}$, tgo^{1} sim^{H9} triple heterozygotes, the midline cells are displaced dorsally (arrowhead) and ventrally (arrow). (C) In stage 14 $jing^{01094}$, sim^{H9} double heterozygotes, midline cells are displaced ventrally. Arrow denotes absence of dorsal midline cells. (D) In stage 13 wild-type embryos, SLI-positive glia are present dorsally. (E) In stage 13 $jing^{01094}$, tgo^{1} sim^{H9} triple heterozygotes, SLI immunoreactivity is displaced to segment boundaries (arrow) and ventrally. (F) Stage 15 wild-type embryo showing SIM^{+} cells. (G) Absence of SIM immunoreactivity in stage 15 $jing^{01094}$, tgo^{1} sim^{H9} triple heterozygotes. (H) SIM^{+} midline cells are displaced ventrally in stage 14 sim^{H9}/sli^{1} double heterozygotes. (I) Midline cells are displaced ventrally (arrow) in $jing^{01094}/sli^{1}$ double heterozygotes. Arrowhead denotes absence of cells. (J) Wild-type midline SLI immunoreactivity. Stage 14. (K) Reduced SLI immunoreactivity in stage 14 $jing^{01094}/sli^{1}$ double heterozygotes. Arrows in J,K indicate SLI immunoreactivity.

midline cells occurs after reduction of one copy of *jing* and *sim*, suggesting that these effects are specific for the midline (Fig. 2C). Triple heterozygotes also show alterations in repulsive signaling mechanisms. Fasciclin 2-positive longitudinal axons collapse into a single tract along the midline in $jing^{01094}$; tgo^{1} sim^{H9} triple heterozygotes stained with 1D4 monoclonal antibody (Fig. 1G,H) (Van Vactor et al., 1993). These phenotypes are, therefore, similar to those of homozygous mutations in sli^{1}, which affect midline repulsion mechanisms and cause the ventral displacement of midline cells (Sonnenfeld and Jacobs, 1994; Battye et al., 1999; Kidd et al., 1999).

To characterize the relationship between *jing* and CNS midline further, we removed one copy of both *jing* and *sli* and analyzed the development of the CNS axons and midline cells. Reducing one copy of both *jing* and *sli* is associated with collapsed axons (55%), the ventral displacement of SIM^{+} midline cells (38%) and reductions in SLI immunoreactivity (40%) in stage 14 embryonic nerve cords compared with wild type (Fig. 1I; Fig. 2I-K). By comparison, 57% of sim^{H9} and sli^{1} double heterozygotes have collapsed axons (Fig. 1I) and ventrally displaced midline cells (45%; Fig. 2I), which is consistent with the established regulatory role of *sim* (Fig. 2H) (Ma et al., 2000; Wharton et al., 1994). Comparison of SIM and SLI immunoreactivity in *jing* and *sli* double heterozygotes therefore reveals that although midline cells are present in these embryos, they do not adequately express *sli*. In summary, these results imply that *jing* dosage may be important for the regulation of *sli*.

jing mutations interact genetically with mutations in _trh_ and its target _breathless_

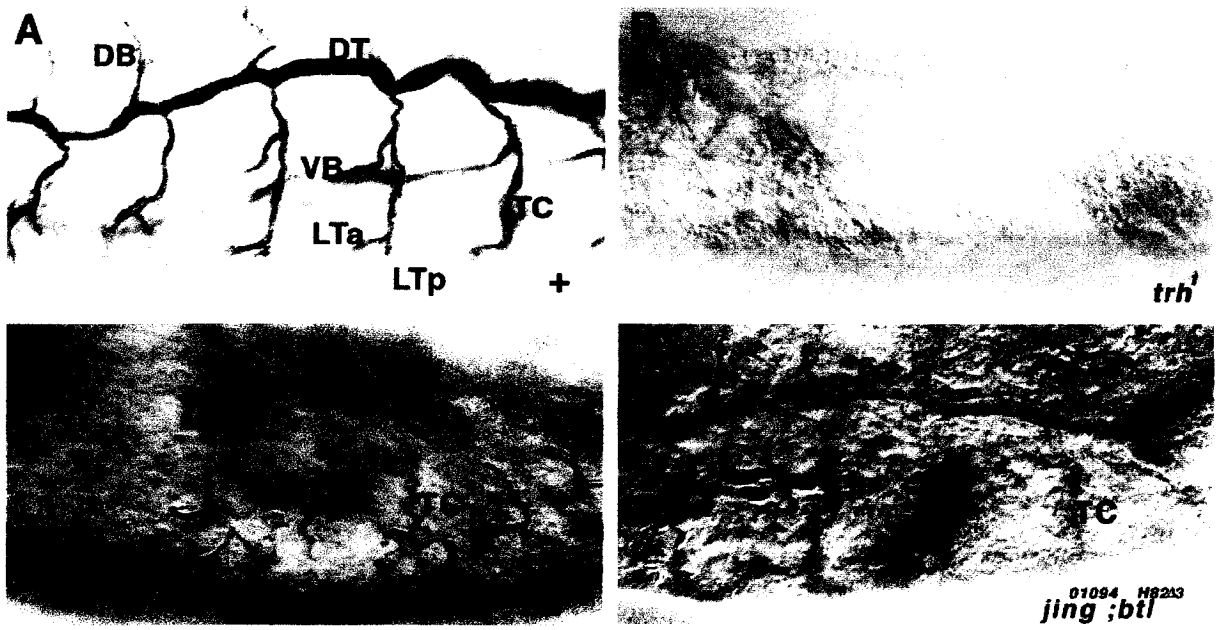
We next assessed whether *jing* dosage is important for tracheal development by analyzing

jing in trans-heterozygous combination with mutations in genes whose function is specific for the embryonic trachea. Tracheal tubule development was analyzed in double heterozygous embryos by staining with mAb 2A12, which in wild-type embryos stains the lumen of all tracheal tubules (Fig. 3A). Tracheal tubules do not form in homozygous *trh* mutants (Fig. 3B) (Isaac and Andrew, 1996; Wilk et al., 1996; Sonnenfeld et al., 1997). Tracheal tubule formation is defective after both *trh* and *jing* are reduced by only one copy each. For example, 51% of embryos double heterozygous for $jing^{01094}$ and trh^{1} show a significant loss of most tracheal branches by stage 15 (Fig. 3C,E). In addition, $jing^{01094}$ and trh^{1} double heterozygotes are sensitive to the dose of *tgo*, as 69% of embryos triple heterozygous for these mutations ($jing^{01094}$; trh^{1} tgo^{1}) show tracheal phenotypes (Fig. 3E). *jing* mutations also show dominant interactions with a direct target of TGO and TRH heterodimers, the fibroblast growth factor receptor known as *breathless* (*btl*) (Klämbt et al., 1992; Oshiro and Saigo, 1997). Ninety eight percent of $jing^{01094}$and $btl^{H82\Delta3}$ double heterozygotes show tracheal phenotypes that affect the formation of transverse connectives and visceral branches (Fig. 3D,E).

tubule patterning. If *jing* functions in a parallel pathway to that of *trh* and *btl* these results would then indicate that the pathways must converge on a common component that is necessary for tracheal tubule formation.

Molecular and genetic analysis of the *jing* locus

The *jing* locus encodes a transcription factor with homology to the mouse transcription factor AEBP2 (Lui and Montell, 2001). *jing*-coding sequence corresponding to the


A
DB
DT
VB
TC
LTa
LTp
+
trh1
TC
01094 H82Δ3
jing ;btl

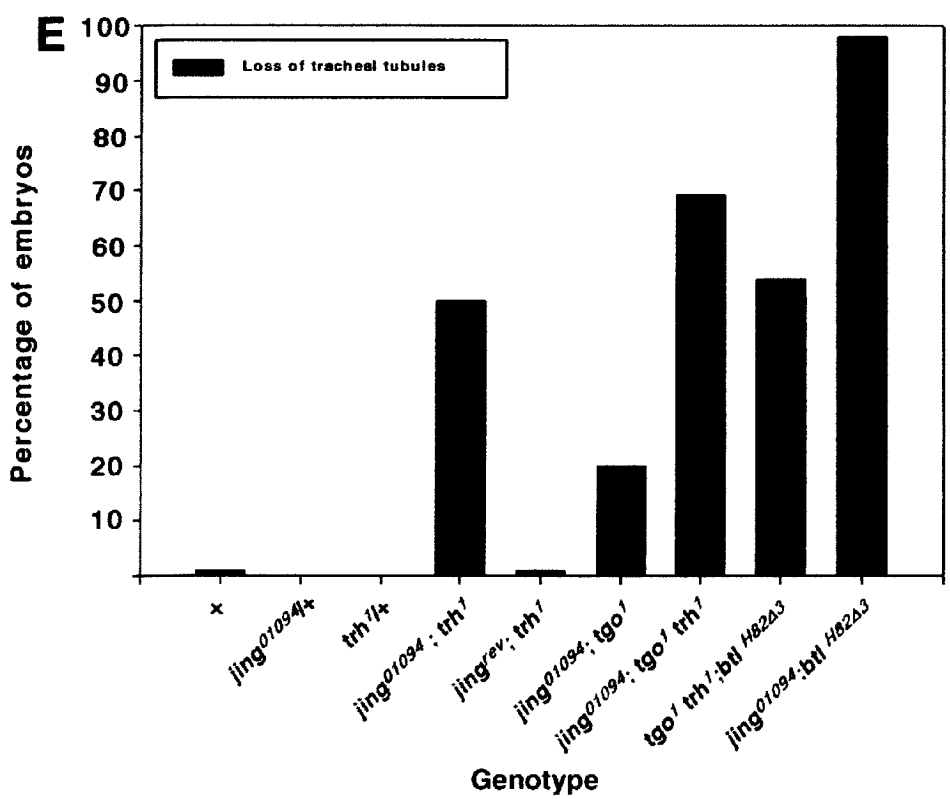

E
Loss of tracheal tubules
Percentage of embryos
100
90
80
70
60
50
40
30
20
10
x
jing01094/+
trh1/+
jing01094 ; trh1
jingrev; trh1
jing01094; tgo1
jing01094; tgo1 trh1
tgo1 trh1;btl H82Δ3
jing01094;btl H82Δ3
Genotype

Fig. 3. Genetic interactions in the trachea. Stage 15 embryos stained with mAb 2A12 to visualize tracheal tubules and shown with anterior left in sagittal view. (A) Wild-type embryo. (B) Absence of tracheal tubules in homozygous trh^{1} mutants. (C) Severe loss of tracheal tubules in $jing^{01094}$; trh^{1} double heterozygotes. (D) Reductions in tracheal branches in $jing^{01094}$; $btl^{H82\Delta 3}$ double heterozygotes. (E) Quantification of *jing* genetic interactions in the trachea. Double and triple heterozygous combinations (genotype). Data show the percentage of embryos with loss of tracheal branches. Tracheal development in $jing^{01094}$; trh^{1} double heterozygotes is sensitive to the dose of *tgo* ($jing^{01094}$; tgo^{1} trh^{1}). $jing^{01094}$; $btl^{H82\Delta 3}$ double heterozygotes show the highest percentage of embryos with tracheal tubule defects. In all cases more than 65 embryos were scored. DB, Dorsal branch; VB, visceral branch; DT, dorsal trunk; TC, transverse connective; LTa, lateral trunk anterior; LTp, lateral trunk posterior.

expressed sequence tag (EST) LD36562 rescues *jing* mutant effects in the ovary, confirming the identity of this gene (Lui and Montell, 2001). Fig. 4A shows the proximity of embryonic lethal *jing* P element insertions to a transcription unit (LD10015) adjacent to the *jing* 5' regulatory region. Given the proximity of the *jing* P elements to the LD10015 transcription unit, it was important to determine whether the latter was affected by these insertions. We therefore performed in situ hybridization on embryos homozygous for *jing* P element insertional mutations ($jing^{01094}$ and $jing^{K03404}$) using digoxigenin-labeled LD10015 EST as a probe. LD10015 mRNA was detected in embryos homozygous for either $jing^{01094}$or $jing^{K03404}$ and therefore we conclude that LD10015 transcription is unaffected by lethal *jing* P element insertions (data not shown).

Analysis of genomic DNA sequence (GenBank Accession Number, AF285778) surrounding two lethal P-element insertions in *jing* reveals that there are three putative DNA binding sites for Tgo::Sim and Tgo::Trh (CMEs), and one for the HMG SOX protein called Fish-hook (also known as Dichaete, D – FlyBase) (TACAAT) in the 5' regulatory region of *jing* (Fig. 4A) (Ma et al., 2000; Oshiro and Saigo, 1997; Sonnenfeld et al., 1997; Wharton et al., 1994). This raises the possibility that *jing* may be a direct transcriptional target of bHLH-PAS heterodimers and SOX proteins including TGO:SIM, TGO:TRH or Fish-hook (Crews, 1998; Ma et al., 2000).

Point mutations in *jing* were isolated by a chemical mutagenesis. From a screen of 6344 EMS-mutagenized second chromosomes, three novel *jing* mutations were isolated for failure to complement the embryonic lethality of $jing^{K03404}$ genetically, therefore defining a single complementation group. *jing* EMS-induced mutations are homozygous

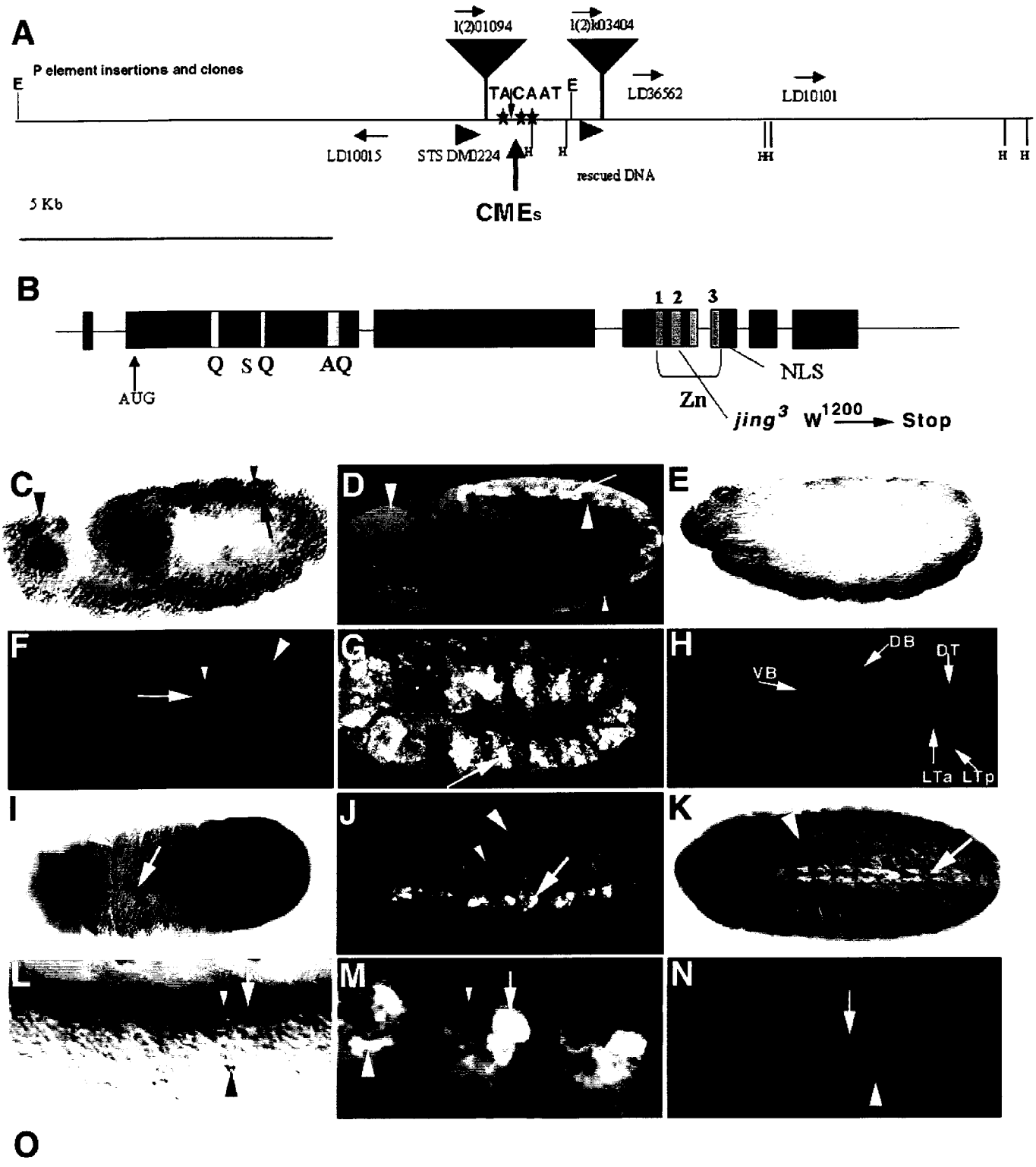

A
P element insertions and clones
E
l(2)01094
l(2)k03404
TACAAT
E
LD36562
LD10101
LD10015
STS DMD224
H
H
HH
H H
rescued DNA
5 Kb
CMEs
B
1 2 3
Q SQ AQ
AUG
NLS
Zn
jing3 W1200 Stop
C
D
E
F
G
H
DB
DT
VB
LTa LTp
I
J
K
L
M
N
O

Fig. 4. *jing* genomic structure and embryonic expression. (A) Genomic interval containing *jing* ESTs, lethal P element insertions and adjacent gene (LD10015). DNA-binding sites of bHLH-PAS (CMEs) and SOX HMG protein Dichaete (TACAAT) are present in the *jing* 5' regulatory region. (B) *jing* exon/intron structure and point mutation. Exons (filled boxes), introns (lines) and protein motifs (colored boxes). Exon 2 contains repeats of poly-glutamine (Q), poly-serine (S) and alanine-glutamine (AQ). C_2H_2-type zinc fingers (Zn). The $jing^3$ mutation is a G-to-A change at nucleotide 3806 of cDNA LD36562 converting W^{1200} to a stop codon. (C-O) Embryonic expression of *jing*. (C,I,L) In situ hybridization of *jing* digoxigenin-labeled DNA probes to wild-type embryos. (D,G,J,M) *jing-lacZ* enhancer trap expression detected via anti-ß-gal staining and confocal microscopy. (E,F,H,K,N,O) Anti-JING immunostaining of wild-type embryos. Confocal microscopy (F,H,N), light microscopy (E,K,O). (C-E) By stage 9, *jing* transcripts and protein product are present in the CNS midline (arrows) and neuroectoderm (small arrowheads) and supraesophageal ganglion (arrowheads). (D) Colocalization of *jing-lacZ* product (anti-ß-gal, green) and SIM (red) in a subset of CNS midline precursors at stage 9, as detected via double-label immunostaining (arrow). (F) Stage 10 embryo showing JING localization in tracheal placodes (arrow) and two adjacent cells (small arrowhead). In this focal plane, JING is observed in three MP neurons in the CNS (larger arrowhead). (G) Merged confocal image showing co-localization of *jing-lacZ* product (anti-ß-gal, green) and TRH (red) in tracheal placodes (arrow) detected via double-label immunostaining of wild-type stage 10 embryos. (H) Localization of JING to all tracheal branches in a stage 15 wild-type embryo. Note nuclear localization of JING. (I-K) At stage 12/3, *jing* transcription occurs in the CNS midline (arrows) and segmental ectodermal stripes (arrowheads). Small arrowhead in J indicates Sim^+ muscle cells. *jing* transcription (L,M) and protein product (N) are highest in midline glia (arrows), as shown in a stage 15 wild-type embryo. Large arrowheads in L-N show midline neurons; small arrowheads in L,M show *jing*-negative midline glia. (O) anti-JING immunostaining of homozygous embryos carrying a deletion in the *jing* gene (Df(2R)ST1) to reveal the specificity of the antisera.

embryonic lethal and are lethal in *trans* to *jing* P element-induced mutations and a deficiency Df(2R)ST1 covering the *jing* locus (Liu and Montell, 2001). Based on phenotypic analysis of the CNS and trachea, the *jing* EMS-induced alleles were placed in the following allelic series of phenotypic severity: $jing^{3}>jing^{2}>jing^{1}$. Molecular analysis of $jing^{3}$ reveals a single nucleotide change in the coding region of this gene, confirming the identity of this complementation group (Fig. 4B). The $jing^{3}$ mutation results in the conversion of tryptophan^{1200} (w^{1200}) to a premature stop codon located in the middle of the second zinc-finger motif (Fig. 4B). Given the importance of the zinc-finger motifs and a nuclear localization signal to DNA binding, the molecular nature of the $jing^{3}$ mutation is consistent with its strong loss-of-function and hemizygous phenotypes. The phenotype of $jing^{3}$ mutant embryos is therefore shown in phenotypic analyses.

jing embryonic expression

The expression pattern of *jing* was studied throughout embryogenesis with a *jing-lacZ* enhancer trap line ($jing^{01094}$), digoxigenin-labeled *jing* DNA probes and a rat JING antibody. *jing* mRNA and protein product are first detected during precellular blastoderm stages, suggesting that *Drosophila* embryos contain a maternal supply of *jing* (data not shown). A discernable *jing* expression pattern is apparent from stage 9, as *jing* transcripts and protein accumulate in the CNS midline, neuroectoderm and trachea (Fig. 4C-E).

In the wild-type stage 9 CNS, *jing* mRNA is distributed in a dorsoventral pattern that is not continuous between segments (Fig. 4C). To determine the identity of the *jing*-expressing CNS cells, co-localization studies were performed using a *jing-lacZ* enhancer

trap and confocal microscopy. Embryos carrying the *jing-lacZ* enhancer trap and stained with anti-ß-gal and anti-SIM show co-localization in subsets of CNS midline cells during stage 9 (Fig. 4D, arrow). As SIM localizes only to midline cells in the CNS, this result confirms the midline expression of *jing* (Crews, 1998). During stage 9, *jing* transcription also occurs in the neuroectoderm and in the supraoesophageal ganglion (Fig. 4C,D).

During stage 10, JING protein is present in the tracheal placodes (Fig. 4F). A pair of JING-positive cells flank the tracheal placodes dorsally (Fig. 4F). The *jing-lacZ* enhancer trap is also expressed in TRH-positive tracheal cells in the anterior of each placode (Fig. 4G). The *jing-lacZ* enhancer trap is co-expressed with *trh* and *tgo* from stage 10 until stage 16 of embryogenesis (data not shown). JING protein is detected in all tracheal branches throughout embryonic tracheal development, consistent with a role for *jing* throughout tracheal tubulogenesis (Fig. 4H).

During stage 12/3, *jing* transcripts and protein product are present in CNS midline cells and segmental ectodermal stripes (Fig. 4I-K). By stage 14, *jing* is strongly expressed in midline glia that occupy a characteristic dorsal position in the ventral nerve cord (Fig. 4L,M). Weaker *jing* expression is detected in ventrally positioned midline neurons (Fig. 4L, black arrowhead). To determine the subcellular localization of JING in the CNS, wild-type embryos were stained with anti-JING and analyzed by confocal microscopy. By this method, JING protein can be detected within the nuclei of the midline glia (Fig. 4N, arrow) and to a lesser degree in midline neurons (Fig. 4N, arrowhead). JING protein is not detectable by confocal microscopy in cells of the lateral neuroectoderm, as opposed to *jing-lacZ* expression (see Fig. 5A) (data not shown).

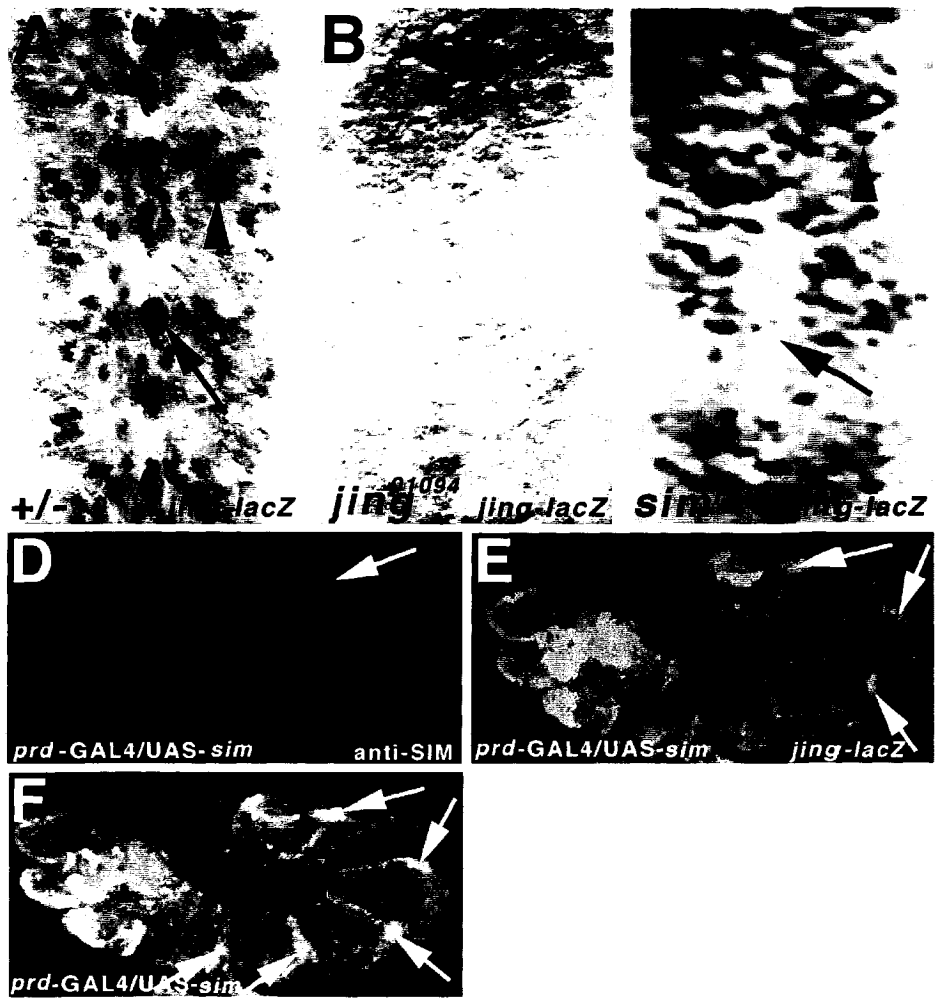


Fig. 5. The *jing-lacZ* enhancer is expressed in the midline. *lacZ* expression in whole-mount embryos heterozygous for the *jing-lacZ* enhancer trap and stained with anti-ß-gal. (A) Strong midline *jing-lacZ* expression (arrow) and weaker neuroectodermal expression in stage 14 $jing^{01094}$ heterozygotes (arrowhead). (B) Reduced *jing-lacZ* expression in the midline and neuroectoderm of $jing^{01094}$ homozygotes suggests autoregulation. (C) In sim^{H9}/sim^{H9} null mutants, *jing-lacZ* midline expression is not detectable (arrow) but *lacZ* expression still occurs in the neuroectoderm (arrowhead). (D-F) Embryos heterozygous for the *jing-lacZ* enhancer trap, P[*prd*-Gal4] and P[UAS-*sim*] and stained with anti-SIM (D, red), anti-ß-gal (E, green). (E) Ectopic *jing-lacZ* activation (arrows). (F) Merged images of D,E. (F) Activation of *jing-lacZ* in the ventral region of *prd* pair-rule stripes (arrows).

Midline specificity of *jing-lacZ* expression

To further analyze *jing-lacZ*, we assessed its expression in homozygous *jing* and *sim* mutants using monoclonal anti-ß-gal. During stage 14, the *jing-lacZ* enhancer shows strong expression in CNS midline cells (Fig. 5A, arrow) and weaker expression in lateral CNS cells (Fig. 5A, arrowhead). In homozygous $jing^{01094}$ mutants carrying the *jing-lacZ* P element insertion, *lacZ* expression is reduced in the entire CNS suggesting that this insertion affects *jing* gene expression and that *jing* expression may be controlled by autoregulation (Fig. 5B). By contrast, in stage 15 sim^{H9} homozygotes, *jing-lacZ* enhancer expression is absent in the CNS midline (Fig. 5C, arrow) but still present in the lateral CNS (Fig. 5C, arrowhead) and other areas of embryonic *jing* expression (data not shown). This result confirms the midline identity of the *jing-lacZ*-expressing cells.

To assess the midline identity of *jing-lacZ* enhancer expression further, we determined whether *sim* activates the *jing-lacZ* enhancer by in vivo ectopic expression experiments. The ability of *sim* to induce midline gene expression ectopically has been established (Nambu et al., 1991; Wilk et al., 1996; Xiao et al., 1996; Zelzer et al., 1997). *sim* expression was targeted to the pair-rule ectodermal stripes of the *paired* (*prd*) gene using GAL4/UAS (Brand and Perrimon, 1993) and by crossing flies containing the P[*prd*-GAL4] driver, and heterozygous for the *jing-lacZ* enhancer, with flies containing P[UAS-*sim*] (Ward et al., 1998). The progeny were stained with anti-SIM to confirm ectopic expression (Fig. 5D) and with anti-ß-gal to identify ectopic *jing-lacZ* expression (Fig. 5E). Ectopic expression of *sim* is sufficient to activate *jing-lacZ* in ventrally positioned cells in pair-rule ectodermal stripes (Fig. 5E,F, arrows). The ventral activation of *jing-*

lacZ by *sim* is consistent with previous results showing the activation of midline-specific genes by ectopic *sim* expression (Xiao et al., 1996). In summary, the results shown here provide strong evidence that *jing* expression occurs in CNS midline cells.

jing loss- and gain-of-function disrupts CNS axon and tracheal tubule development

The *jing* expression pattern and gene dose effects in the CNS midline and trachea suggest that *jing* function may be important for the development of both systems. Therefore, CNS axon and tracheal tubule development was assessed in *jing* homozygous mutant embryos stained with monoclonal antibodies BP102 and 2A12, respectively. In $jing^{3}$ homozygous mutant embryos, commissural growth cones are often absent in the midline at stage 12 when compared with wild type (Fig. 6A,B). By stage 14, homozygous $jing^{3}$ mutants show losses of longitudinal connections and reduced commissures compared with wild type (Fig. 6C,D). Embryos double mutant for *jing* and *sim* display phenotypes similar to those of *sim* homozygotes (Fig. 6E). Therefore, the *sim* embryonic CNS axon phenotype is epistatic to that of *jing*, implying that *jing* functions downstream of *sim* (Avery and Wasserman, 1992).

The GAL4/UAS system was used to determine the effects of overexpressing *jing* in the CNS midline (Brand and Perrimon, 1993). Flies containing P[*sim*-GAL4] were crossed to flies containing P[*jing*-UAS] and their progeny stained with BP102 to assess CNS axon formation. Expression of one copy of P[*jing*-UAS] specifically in the CNS midline is sufficient to inhibit commissural and longitudinal axon formation (Fig. 6F). Therefore, the *jing* midline overexpression phenotype is similar to that resulting from *jing* loss of function (Fig. 6D), and phenotypes of *jing* and *sim* double heterozygotes (Fig. 1C).

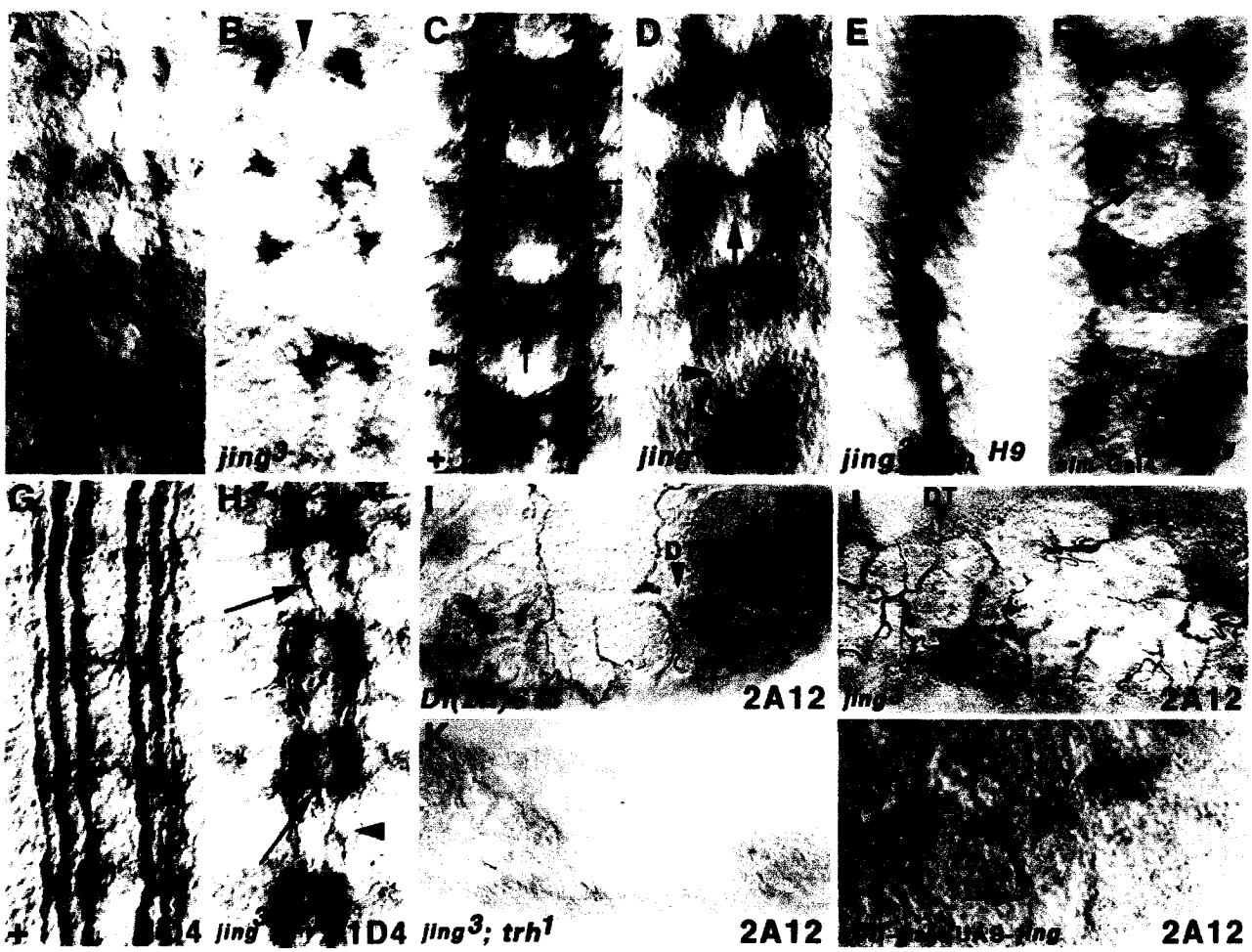

A
B
C
D
E
F
H9
G
H
I
J
D
2A12
2A12
1D4
2A12
2A12

Fig. 6. *jing* loss- and gain-of-function phenotypes in the CNS and trachea. Frontal views showing CNS axon scaffolds (BP102 stain) (A-F) and longitudinal fascicles (mAb 1D4 stain; anti-Fasciclin 2) (G,H). Sagittal views of stage 15 whole-mount embryos stained with mAb 2A12 to visualize tracheal tubules (I-L). (A,B) Pioneering growth cones do not approach the midline during stage 12 in homozygous $jing^3$ mutants (B) as they do in wild-type (A) (arrowheads). (C,D) In stage 14 $jing^3$ mutants, there are variable absences of either anterior or posterior commissures (arrow) and longitudinal connectives (arrowhead) (D) compared with wild type (C). (E) $jing^3$;sim^{H9} double mutants show collapsed axon phenotypes. (F) Overexpression of *jing* in the CNS midline (*sim*-GAL4/*jing*-UAS) disrupts commissural (arrow) and longitudinal axon formation (arrowhead). (G) Ipsilateral projection of wild-type Fasciclin 2-positive longitudinal bundles. (H) In $jing^3$ homozygotes, longitudinal fascicles inappropriately cross the midline (arrows). Longitudinal fascicles stall within segments (arrowhead). (I,J) Embryos homozygous for a deficiency covering *jing* (Df(2R)ST1) and $jing^3$ mutations show defects in the formation of all tracheal branches, including the dorsal trunk (arrowhead) and transverse connectives (TC) (arrow). Note absence of the visceral branch (compare with Fig. 3A). (K) $jing^3$; trh^1 double mutants show loss of all tracheal tubules. (L) Overexpression of *jing* in the trachea (*btl*-GAL4/*jing*-UAS) disrupts all aspects of tracheal tubule development. Branch fusion in the dorsal trunk does not occur (arrowhead) and the dorsal branch and transverse connectives are reduced (arrows). The visceral branch is absent. DB, Dorsal branch; VB, visceral branch; DT, dorsal trunk; TC, transverse connective; LTa, lateral trunk anterior; LTp, lateral trunk posterior.

These results demonstrate that appropriate *jing* dose is a requirement for proper CNS axon development in the CNS midline. Interestingly, we observe a similar CNS axon phenotype after overexpression of *sim* in the CNS midline (data not shown).

The homozygous *jing* CNS phenotype suggests an alteration in the mechanisms that guide CNS axons. Fasciclin 2 staining using 1D4 mAb, shows that longitudinal fascicles stall within segment boundaries causing breaks in the longitudinal tracts in 95% of $jing^3$ mutant segments (Fig. 6H, arrowhead; n=210 segments) (Van Vactor et al., 1993). A subset of normally ipsilateral axons of the most medial fascicle project instead contralaterally in $jing^3$ mutants (Fig. 6H, arrows; n=210 segments). As ipsilateral fascicles are prevented from crossing the midline in wild-type embryos (Fig. 6G), these results suggest that midline repulsive mechanisms are perturbed in *jing* mutant embryos (Hummel et al., 1999b; Kidd et al., 1999).

We next wanted to determine whether *jing* is involved in tracheal patterning due to its expression pattern and its dose-sensitive effects with mutations in genes controlling tracheal development. Embryos homozygous for a *jing* deficiency (Df(2R)ST1) and $jing^3$ mutations are associated with losses of the dorsal trunk, severely disrupted transverse connectives and absences of the visceral branch (Fig. 6I,J) compared with wild-type (see Fig. 3A). Embryos doubly mutant for *jing* and *trh* lack all tracheal tubules and display phenotypes identical to *trh* homozygous mutants (Fig. 6K). Therefore, *trh* loss-of-function is epistatic to *jing* loss-of-function, implying that *jing* functions downstream of *trh*.

To determine the effects of overexpressing *jing* in the trachea, flies containing the P[*breathless* (*btl*)-GAL4] driver were crossed to those containing P[*jing*-UAS]. Progeny from this cross were stained with 2A12 antibody and tracheal tubule development was analyzed by light microscopy. 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 (Fig. 6L). Therefore, *jing* overexpression tracheal phenotypes are similar to *jing* loss-of-function tracheal phenotypes.

jing CNS midline phenotype

Cell type-specific markers were used to follow CNS midline development in homozygous *jing* mutant embryos. Midline cells were identified using anti-SIM and the glial-specific marker anti-Slit (Nambu et al., 1990; Rothberg et al., 1990). Expression of *sli* was assessed in homozygous *jing* mutant embryos using the *lacZ* reporter P[1.0 HV, *sli-lacZ*] (Ma et al., 2000; Wharton and Crews, 1993).

There are reductions in the number of SIM-positive and *sli-lacZ* expressing midline cells in homozygous $jing^3$ mutants compared with wild-type embryos during stage 9 and 11, respectively (Fig. 7A,B,E,F). This clearly demonstrates that the early differentiation of midline lineages requires *jing* function. By later stages of embryogenesis (stage 15), SIM and SLI immunoreactivity is drastically reduced in *jing* mutant nerve cords (Fig. 7D,H) compared with wild-type (Fig. 7C,G). The presence of SLI-positive cellular profiles in macrophages outside the VNC suggests that midline lineages are lost by cell death.

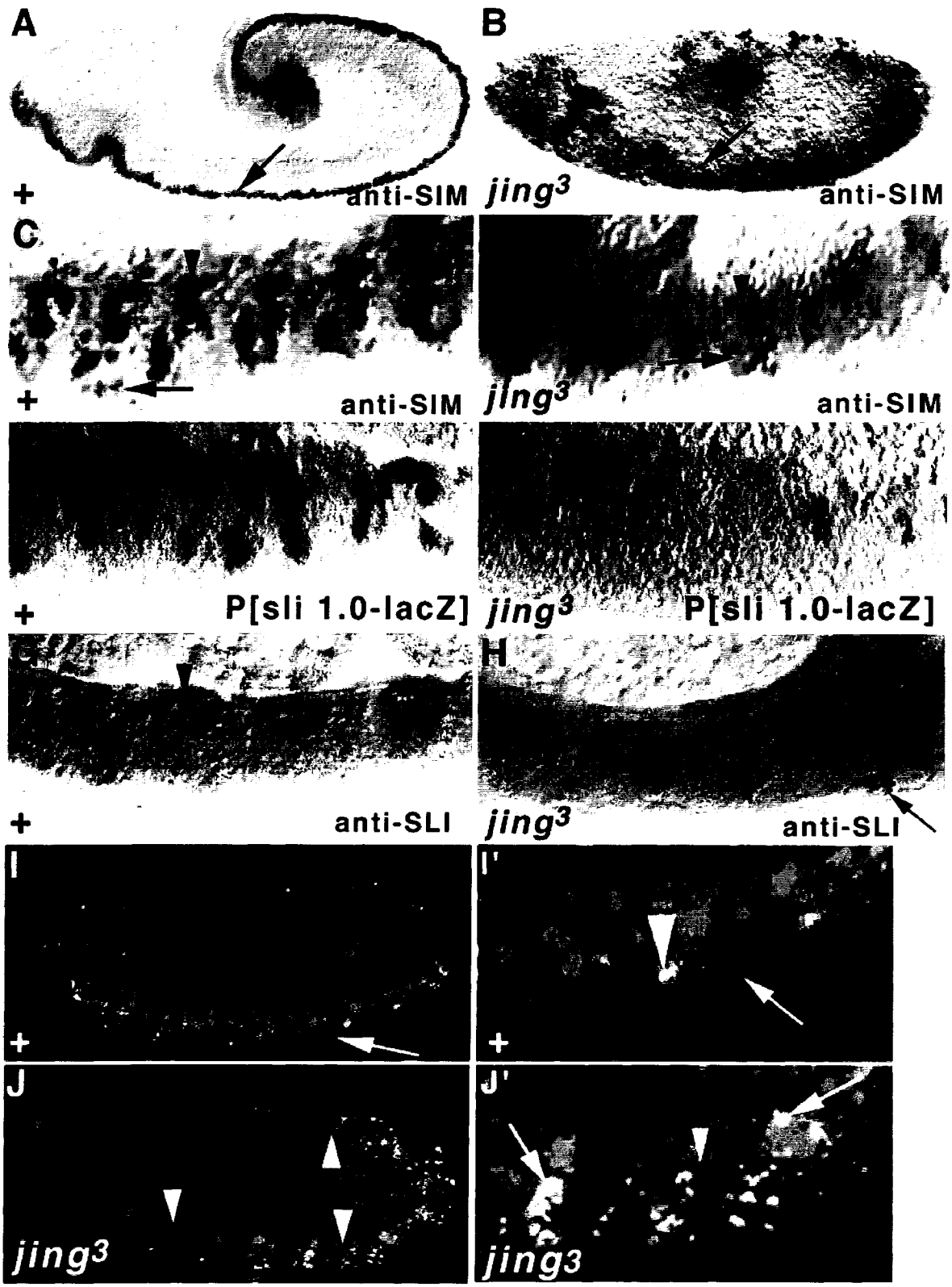

A
+
anti-SIM
B
jing3
anti-SIM
C
+
anti-SIM
jing3
anti-SIM
+
P[sli 1.0-lacZ]
jing3
P[sli 1.0-lacZ]
+
anti-SLI
H
jing3
anti-SLI
I
+
I'
+
J
jing3
J'
jing3

Fig. 7. *jing* mutations disrupt CNS midline glial differentiation. (A-D) Embryos stained with anti-SIM; (E,F) ß-gal expression in embryos carrying *sli* reporter P[*sli* 1.0 HV-*lacZ*]; (G,H) anti-SLI stain to visualize midline glia; (I-J') Stage 12 embryos double labeled with anti-SLI (green) and TUNEL (red), and confocal images of 1 μm serial sections. Sagittal views with anterior towards the left. (A,B) *sim* expression is reduced (arrow) in stage 9 $jing^3$ homozygotes (B) compared with wild-type (A). (C,D) Reduced SIM immunoreactivity and small size of midline neurons (arrows) and glia (arrowheads) in stage 15 $jing^3$ homozygotes (D) compared with wild-type (C). (E) Wild-type stage 11 expression of P[*sli* 1.0 HV-*lacZ*] in six midline glia. (F) P[*sli* 1.0 HV-*lacZ*] expression in average of 3.2 midline glia/segment in stage 11 $jing^3$ homozygotes. (G) Wild-type SLI-positive glia during stage 15. (H) Reduced SLI immunoreactivity (arrowhead) and detection in macrophages outside the nerve cord (arrow) in stage 15 $jing^3$ homozygotes. (I) Stage 12 wild-type *sli-lacZ* expression (green). TUNEL-positive profiles (red) are present only outside the CNS (arrow). (I') Close-up view of I. SLI- and TUNEL-positive glia (arrowhead). (J) Loss in SLI immunoreactivity (arrowheads) in stage 12 $jing^3$ homozygotes. Note increase in TUNEL labeling compared with wild type. (J') Close-up view of J (arrows, dead glia). TUNEL-positive profiles in the nerve cord (arrowhead).

Similar results were obtained using anti-Wrapper as a marker of glial identity (data not shown).

To address whether midline glia enter apoptotic pathways, *jing* mutant embryos were double-labeled with anti-SLI and TUNEL, and the occurrence of apoptotic glia monitored from stages 12 to 15 (Gavrieli et al., 1992). On average, there are one or two apoptotic midline glia within an entire nerve cord of a stage 12 wild-type embryo (Fig. 7I,I', arrowhead; n=11 embryos). By contrast, every nerve cord segment in $jing^3$ mutant embryos contains apoptotic glia in addition to the presence of more TUNEL-positive profiles in the CNS (Fig. 7J,J'; n=13 embryos). The increased occurrence of apoptotic glia correlates with reductions in SLI immunoreactivity in the midline of $jing^3$ mutant embryos (Fig. 7J, arrowheads) and establishes that *jing* function is required for midline glial survival.

Enhancer traps and antibodies were used to follow the development of individual motoneurons (VUMs, 22C10) and interneurons, such as the midline precursors (MP1, dMP2, vMP2; P223, anti-ODD and 22C10) and the median neuroblast (MNB; anti-Engrailed) in wild-type and homozygous $jing^3$ mutant embryos (Fujita et al., 1982; Hummel et al., 2000; Schmid et al., 1999; Skeath and Doe, 1998; Sonnenfeld and Jacobs, 1994; Spana et al., 1995). *jing* loss-of-function mutations are associated with reductions in the expression of all neuronal markers tested. There are absences of immunoreactivity in the VUMs, MNB and MP1 neuronal lineages in some VNC segments in $jing^3$ mutant embryos (Fig. 8B,D,F,H) compared with wild type (Fig. 8A,C,E,G). There is a loss of ODD immunoreactivity as early as stage 10 in MP neurons in homozygous $jing^3$ mutants

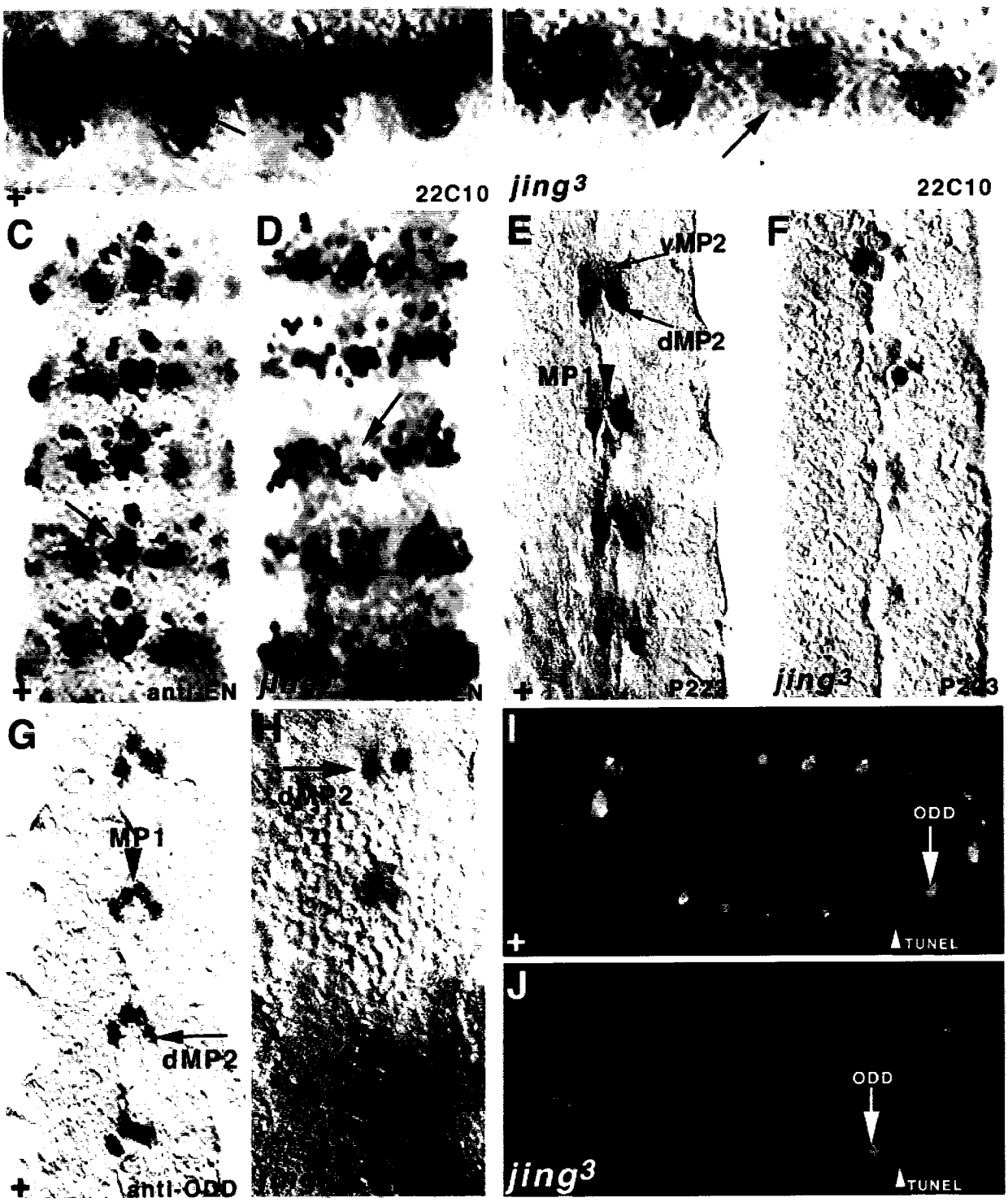

+
22C10
jing3
22C10
C
D
E
vMP2
dMP2
MP1
F
+
jing3
G
MP1
dMP2
+
anti-ODD
H
dMP2
I
ODD
+
TUNEL
J
ODD
jing3
TUNEL

Fig. 8. *jing* mutations disrupt midline neuronal differentiation. Wild-type (A,C,E,G,I) and *jing*^{3} mutant embryos (B,D,F,H,J) stained with neuronal specific antibodies. Sagittal views of whole-mount stage 14 (A,B) and stage 10 embryos (I,J). (C-H) Frontal views of dissected stage 15 nerve cords with anterior up. (A) mAb 22C10 stains the VUM neuron cell bodies and axons projecting dorsally (arrow) in each nerve cord segment. (B) Absence of VUM cell bodies and axons in some *jing*^{3} segments (arrow). (C) Six wild-type Engrailed (EN)-positive neurons (detecting the VUMs and MNB). (D) Absence of EN-positive neurons in the CNS midline of some *jing*^{3} segments (arrow) and reductions in others. Neuroectodermal EN-positive neurons are not reduced from wild-type. (E,F) Wild-type P223 enhancer trap expression in the MP1, vMP2 and dMP2 neurons. (F) Downregulation of P223 expression in MP lineages of *jing*^{3} homozygotes. (G) Wild-type Odd-skipped (ODD) expression in MP1 and dMP2 neurons. (H) Reduced ODD expression in *jing*^{3} MP1 and dMP2 neurons. (I) Stage 10 embryo double-labeled with anti-ODD and TUNEL. Apoptotic MP lineages are not detectable. (J) Stage 10 *jing*^{3} homozygous mutant embryo double-labeled with anti-ODD and TUNEL. Note that ODD immunoreactivity is significantly reduced compared with wild type. TUNEL-positive MP neurons are not present in this embryo.

compared to wild-type (Fig. 8I,J). Similar reductions in the number of immunoreactive vMP2 and dMP2 are observed by 22C10 staining of stage10 homozygous *jing*^{3} mutant embryos (Hummel et al., 2000) (data not shown).

Within a particular VNC segment in *jing*^{3} mutants, there is a loss of Engrailed (EN)-positive neurons while the number of EN-expressing neuroectodermal cells remains equal to that in wild-type embryos (Fig. 8D, arrow). In addition, *jing* mutant embryos displaying reduced 22C10 staining of the VUMs in the CNS midline do not show any visible defects in peripheral nervous system development (data not shown). These results strongly suggest that the primary site of *jing* CNS function is at the midline.

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.

jing tracheal phenotype

To determine the role of *jing* during tracheal development, a phenotypic analysis of homozygous *jing* mutant embryos was performed using antibodies to TRH as a marker of cell identity and to EN for identifying the anterior border of the trachea (Glazer and Shilo, 2001; Isaac and Andrew, 1996; Wilk et al., 1996). Initial defects in tracheal morphogenesis occur during tracheal placode stages in embryos homozygous mutant for all *jing* alleles (Fig. 9B). This correlates with the nuclear localization of JING in tracheal placode cells (Fig. 4). The number of TRH-positive precursors in stage 10 homozygous *jing*^{3} mutant embryos is approximately 22% of the expected number of wild-type cells

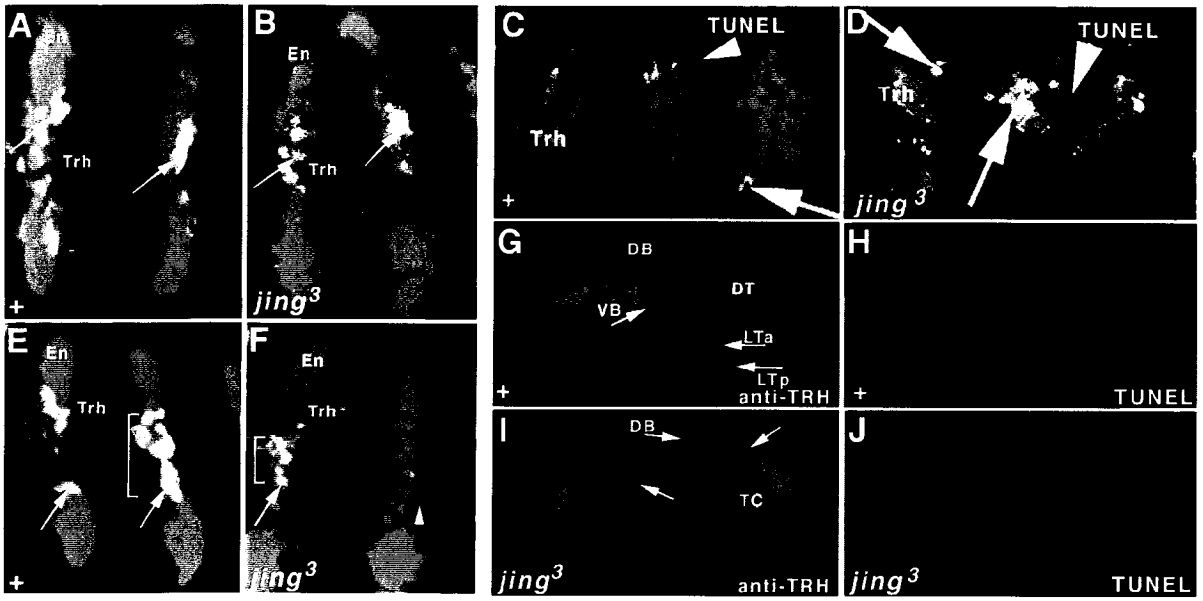


Fig. 9. *jing* tracheal phenotype. (A) Wild-type stage 10 embryo. Tracheal nuclei are visualized with anti-Trachealess (TRH, red) antibodies and anti-Engrailed (EN, green) references the anterior tracheal border. A subset of tracheal cells express EN (arrows, yellow cells). (B) In *jing*^{3} stage 10 homozygous mutant embryos, the number of TRH-positive precursors is reduced. However, the segmental EN-positive stripes and positioning of the placodes appear normal. (C) Wild-type stage 11 embryo stained with anti-TRH (green) and TUNEL (red) showing a rare apoptotic tracheal cell (arrow). (D) Stage 11 *jing*^{3} mutant embryo stained with anti-TRH (green) and TUNEL (red). Many apoptotic tracheal cells are present (arrows) and more apoptotic cells surround the pits than in wild-type embryos (arrowhead). (E) Wild-type embryo at stage 12 shows migration of primary branches across EN-positive stripes. A domain of EN-positive tracheal cells encompasses a region of the segmental stripe (bracket). (F) The domain of EN-positive migrating tracheal cells is smaller (bracket) in some stage 12 *jing*^{3} mutant segments and absent in others (arrowhead). (G,H) Wild-type stage 15 embryo stained with both anti-TRH (green, G) and TUNEL (red, H). (I,J) *jing*^{3} stage 15 homozygous mutant embryo stained with anti-TRH (green, I) and TUNEL (red, J). (I) In *jing*^{3} mutant embryos the EGFR-dependent branches are most severely affected. The visceral branch does not form and regions of the dorsal branch are absent (arrows). The number of cells in the DPP-dependent branches (DB and TC) is reduced compared with wild type. (J) By contrast, the overall apoptotic pattern in *jing*^{3} mutant embryos is not significantly elevated from wild type. DB, dorsal branch; DT, dorsal trunk; VB, visceral branch; TC, transverse connective; LTa, lateral trunk anterior; LTp, lateral trunk posterior.

(Fig. 9A,B). The relatively normal pattern of ectodermal segmentation in *jing*^{3} mutant embryos, as revealed by EN staining, suggests that the improper differentiation of tracheal cells in these mutants is not likely to result from indirect effects of ectodermal patterning (Fig. 9B,F). These results also reveal that the positioning of tracheal placodes in *jing*^{3} mutants is not altered from that of wild-type embryos.

To determine the fate of tracheal lineages we analyzed the pattern of cell death by double labeling wild-type and *jing*^{3} mutant stage 11 embryos with TUNEL and anti-TRH (Fig. 9C,D) (Gavrieli et al., 1992). Cell death is not common in the tracheal pits of wild-type stage 11 embryos (Fig. 9C). On average, there is a maximum of three TUNEL- and TRH-positive cells within an entire stage 11 wild-type embryo (n=24). By contrast, there is an average of 20 TUNEL- and TRH-positive precursors in stage 11 *jing*^{3} mutant embryos (Fig. 9D; n=34). There is also an increase in the number of apoptotic profiles surrounding the tracheal pits in *jing*^{3} compared with wild-type embryos (Fig. 9C,D, arrowhead). Cell death is observed by TUNEL labeling throughout embryogenesis in all tracheal branches in homozygous *jing*^{3} mutant embryos, suggesting that the requirement for *jing* function is not branch specific (data not shown).

In *jing*^{3} homozygous mutant embryos, tracheal cells invaginate but the tracheal branches do not migrate properly anteriorly across EN-positive stripes as they do in wild-type embryos (Fig. 9E,F). In addition, fewer TRH-positive cells express EN in homozygous *jing*^{3} mutant embryos compared with wild-type at stage 12 (Fig. 9E,F). 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 (Fig. 9I; data not

shown). In addition, the visceral branch does not form in *jing*^{3} mutant embryos (Fig. 9I). Therefore, the EGFR-dependent visceral and dorsal trunk branches appear more severely affected than the *Dpp*-dependent dorsal and ganglionic branches, as well as the transverse connectives in *jing*^{3}mutant embryos (Fig. 9I). Despite the death of tracheal cells in *jing* mutant embryos, the overall embryonic pattern of cell death is not significantly altered by the end of embryogenesis from that of wild-type embryos (Fig. 9H,J). Therefore, the tracheal defects in *jing* mutants are not likely to result from widespread defects in embryonic differentiation.

Discussion

In this study, we provide evidence that proper functioning of the *jing* zinc-finger transcription factor is required in the CNS midline and trachea for patterning of CNS axons and tracheal tubules, respectively. The *jing* expression pattern, embryonic lethality, and loss- and gain-of-function phenotypes are consistent with an embryonic role for this locus. Given the recent characterization of *jing* during the migration of border cells in *Drosophila* ovaries, these studies highlight the important role of this gene during cellular differentiation (Lui and Montell, 2001).

Loss-of-function *jing* alleles result in aberrant expression of all CNS midline and tracheal markers tested. Loss of midline *en* expression in segments that have no detectable changes in lateral *en* expression shows that *jing* mutants have defects specific to the midline. The loss of CNS midline and tracheal cells in homozygous *jing* mutant embryos is at least partially mediated by cell death. Therefore, during embryogenesis *jing* is required for terminal differentiation and viability of CNS midline and tracheal cells.

Role of *jing* in the CNS midline

The results presented here show that CNS midline neurons and glia do not differentiate properly in homozygous *jing* mutant embryos. Several lines of evidence support this. The expression of cell-type-specific markers of midline neuronal and glial identity is altered in *jing* mutants compared with that in wild-type embryos. For example, expression of the *sli-lacZ* 1.0 HV reporter initiates in six midline glia in each wild-type nerve cord segment during stage 11 (Wharton and Crews, 1993). By contrast, *sli-lacZ* 1.0 HV reporter expression in *jing* mutants initiates in only an average of three midline glia per nerve cord segment by stage 11. In addition, there are reductions in the number of SIM-positive midline cells and ODD-positive/22C10-positive MP neurons by stage 9 in $jing^3$ homozygous mutant embryos, respectively. Therefore, early midline glial and neuronal differentiation is aberrant in homozygous *jing* mutant embryos. By the end of embryogenesis, many neuronal and glial cell type markers are barely detectable in homozygous *jing* mutant ventral nerve cords.

The loss of *sim*, *sli*, *odd* and 22C10/*futsch* expression in *jing* mutants may reflect improper activation/regulation of gene expression or may be secondary to cell loss. To address this issue, we analyzed the pattern of cell death in the CNS midline of *jing* mutant embryos. Apoptosis occurs in the midline glial lineage in wild-type embryos and begins during stage 12 to refine the number of cells from six to an average of three per nerve cord segment by the end of embryogenesis (Sonnenfeld and Jacobs, 1995; Zhou et al., 1995). In homozygous *jing* mutants, however, there are more apoptotic glia during stage

12 than in wild-type embryos and this correlates with the loss of SLI-positive glia. It is, therefore, likely that the loss in CNS midline gene expression in *jing* mutants results from a loss of cells. In summary, the loss in expression of cell identity markers and inappropriate cell death lead us to conclude that midline neurons and glia do not differentiate properly in *jing* mutant embryos.

The arthropod ventral nerve cord is characterized by the ladder-like pattern of the major CNS axon tracts. The nerve cord is segmental and each neuromere is connected by longitudinal axons, which are separated by anterior and posterior commissures. Disruption of this pattern by *jing* gain-of-function specifically in the CNS midline reveals the requirement for proper *jing* function within these cells for axon patterning. In addition, homozygous mutant *jing* embryos display reductions in CNS midline cells while neuroectodermal and peripheral nervous system development is unperturbed. Together, these results show that *jing* mutations have strong effects on the CNS midline and that *jing* dosage is crucial for their development.

Genetic analysis of axon patterning in the *Drosophila* CNS has revealed the important rôle of neuron-glial function in this process (Klämbt et al., 1991; Hummel et al., 1999b). Mutations leading to reductions in midline neuron numbers correlate with a reduction in the number of commissural tracts, whereas mutations leading to reductions in midline glia numbers show fused commissure phenotypes (Hummel et al., 1999a). These observations are consistent with the hypothesis that midline neurons (such as the VUMs) are required to attract commissural growth cones initially to the CNS midline, whereas midline glia are required subsequently for the organization of commissural axons (Hummel et al.,

1999b). Based on these observations, we propose that defects in the differentiation of midline neuronal precursors, such as the VUMs, in *jing* loss-of-function mutants inhibit the attraction of commissural growth cones to the CNS midline during stage 12. As the attraction of commissural axons to the CNS midline precedes the separation of anterior from posterior commissures, the defects in midline neuronal differentiation and the associated lack of growth cones in the midline of *jing* mutants probably mask subsequent defects in glial-associated functions (Klämbt et al., 1991). During axon patterning, the MP1 interneurons participate in the formation of specific longitudinal pathways (Lin et al., 1995; Hidalgo and Brand, 1997). Therefore, the defects in MP1 neuronal differentiation in *jing* mutants may account for the inhibition in the formation of the longitudinal connectives.

Signals generated by CNS midline cells control the commissural axon pattern by either guiding growth cones toward the midline or preventing them from crossing the midline (Battye et al., 1999; Harris et al., 1996; Kidd et al., 1999; Tessier-Lavigne and Goodman, 1996). Defects in glial-associated functions occur in the CNS of homozygous *jing* mutant embryos. Reduced glial numbers and SLI production in *jing* mutants are consistent with the reduction in midline repulsion of longitudinal pathways as visualized by Fasciclin 2 staining (Fig. 6H). The remaining SLI protein product in stage 12 *jing* mutant nerve cords, however, is apparently sufficient to prevent a total collapse of the longitudinal connectives, as observed in homozygous *sim* and *sli* mutations (Nambu et al., 1990).

Role of *jing* in the trachea

This work has identified multiple roles for *jing* in tracheal morphogenesis. The earliest function of *jing* is to allocate the correct number of cells to the tracheal placodes. Several lines of evidence support this. The number of tracheal placode cells is significantly reduced from wild-type in homozygous *jing* mutant embryos. In addition, tracheal precursors die in *jing* mutant embryos, suggesting that *jing* is essential for their differentiation. As JING localizes to the nuclei of tracheal placode cells and contains potential DNA-binding and transactivation domains, it is possible that it regulates genes essential for the differentiation and survival of tracheal precursors (Mitchell and Tijian, 1989).

Although loss of *jing* function affects cellular differentiation in all tracheal lineages, it appears to have more severe effects on dorsal trunk and visceral branch development. The dorsal trunk and visceral branches derive from the same position in the tracheal placode and are induced by Epidermal Growth Factor Receptor (EGFR) (Wappner et al., 1997). EGFR is activated in the central portion of the tracheal placodes by the restricted expression of *rhomboid* (*rho*) (Bier et al., 1990; Llimargas and Casanova, 1997; Sturtevant et al., 1996; Wappner et al., 1997). The defects in dorsal trunk and visceral branch formation in homozygous *jing* mutant embryos are similar to those in embryos homozygous mutant for *Egfr* signaling (Llimargas and Casanova, 1997; Wappner et al., 1997). Given that mutations in *Egfr* pathway genes do not affect tracheal placode cell numbers, we propose that *jing* may function prior to EGFR signaling.

Several lines of evidence suggest that *jing* functions specifically in tracheal cells. First, we detect JING protein within nuclei of tracheal precursors and differentiated lineages. Second, defective placodes in *jing* mutants are observed in hemisegments with normal *en* expression patterns indicating that defects in the metamerization process do not cause the *jing* tracheal phenotype. However, we cannot rule out the possibility that Hedgehog signaling in segmental ectodermal stripes is affected by *jing* mutations. A requirement for *hh* in determining proper tracheal placode numbers in some hemisegments has recently been shown (Glazer and Shilo, 2001). Third, the most severe defects in tracheal patterning in *jing* mutant embryos occur in the dorsal trunk and visceral branch, suggesting that there is some specificity to *jing* tracheal function. Last, overexpression of *jing* specifically in the trachea results in defects in tracheal patterning that resemble *jing* loss-of-function phenotypes.

Does *jing* function in *sim*- and *trh*-dependent pathways?

Based on genetic and phenotypic analyses, we propose a role for *jing* downstream of *sim* and *trh* during CNS midline and tracheal development, respectively. First, *jing* expression is not observed prior to that of either *sim* or *trh* in the CNS midline and trachea, respectively. *jing* expression is detected in the CNS midline during stage 9, which is after the initiation of *sim* expression and establishment of midline fates (Crews, 1998). JING protein is present in tracheal precursor nuclei, coincident with TRH during stage 10. Second, the CNS axon and tracheal phenotypes of homozygous *jing* mutations are less severe than those of homozygous *sim* and *trh* mutations, respectively. However, we

cannot rule out that maternal JING may rescue the effects of zygotic *jing* mutations or that *jing* functions in a combinatorial fashion and therefore may not display severe phenotypes (Ma et al., 2000). Third, *jing* can be activated by ectopic expression of *sim*, suggesting that *sim* may regulate *jing*. The presence of three E-box ACGTG core sites in the 5' regulatory region of *jing* suggest that this regulation may be direct. Fourth, the *sim* and *trh* embryonic phenotypes are epistatic to that of *jing*, as shown by double mutant analysis. Finally, *jing* mutations genetically interact with mutations in bHLH-PAS target genes such as *sli* and *btl* (Ma et al., 2000; Oshiro and Saigo, 1997). The ventral displacement of midline cells in *jing* and *sli* double heterozygotes strongly suggests that *jing* is required for proper *sli* regulation.

jing and CCAAT-binding proteins

In *Drosophila* ovaries, *jing* is an essential downstream target of the vertebrate homolog of the basic region/leucine zipper transcription factor CCAAT enhancer-binding protein (C/EBP), which is required for border cell migration (Liu and Montell, 2001). The JING protein is most similar to the mouse protein AEBP2, which was identified by its binding to a regulatory sequence in the adipocyte *aP2* gene. Given that C/EBP also binds this sequence, it is proposed that JING and C/EBP coordinate cell differentiation in a co-operative manner (He et al., 1999; Liu and Montell, 2001).

An interesting parallel between the ovarian and embryonic pathways involving *jing* is the activation of *btl*. *btl* is expressed in embryonic tracheal and midline glial cells, as well as border cells of the ovary, and is essential for their migration (Glazer and Shilo, 1991;

Klämbt et al., 1992; Murphy et al., 1995). *btl* is a direct target of C/EBP and TRH::TGO heterodimers in vitro (Murphy et al., 1995; Oshiro and Saigo, 1997). Therefore, the strong dominant interactions between *jing* and *btl* in the embryonic trachea coupled with the role of *jing* in border cell migration implies an important link between the maternal and embryonic pathways involving *jing*. C/EBP is expressed in the embryonic trachea after *btl* expression begins (Rorth et al., 1992). Therefore, C/EBP probably does not regulate *jing* or *btl* in the trachea, as it does in border cells. Furthermore, C/EBP expression is not sufficient to cause ectopic *btl* expression and therefore, it is proposed that this transcription factor carries out the gene expression program initiated by other factors (Murphy et al., 1995). In this way, C/EBP can function in very different pathways, including fat metabolism in adipocytes, long-term memory in Aplysia neurons and cell migration in the ovarian border cells (Murphy et al., 1995). The transcriptional capabilities of *jing* have not yet been tested and therefore it is not known whether *jing* co-operates with TGO::TRH or TGO::SIM heterodimers in activation of *btl* or other targets.

Model of JING function

We propose that bHLH-PAS heterodimers may activate *jing* transcription by binding any or all of the three CNS midline elements (CMEs) present in the 5' regulatory region of *jing* (Fig. 10). The initiation or maintenance of *jing* transcription may also require the function of additional transcription factors such as VVL in the CNS midline and trachea or Fish-hook in the CNS midline (Ma et al., 2000; Llimargas and Casanova, 1997; Boube et al., 2000; Zelzer and Shilo, 2000). The presence of a Fish-hook DNA-binding site

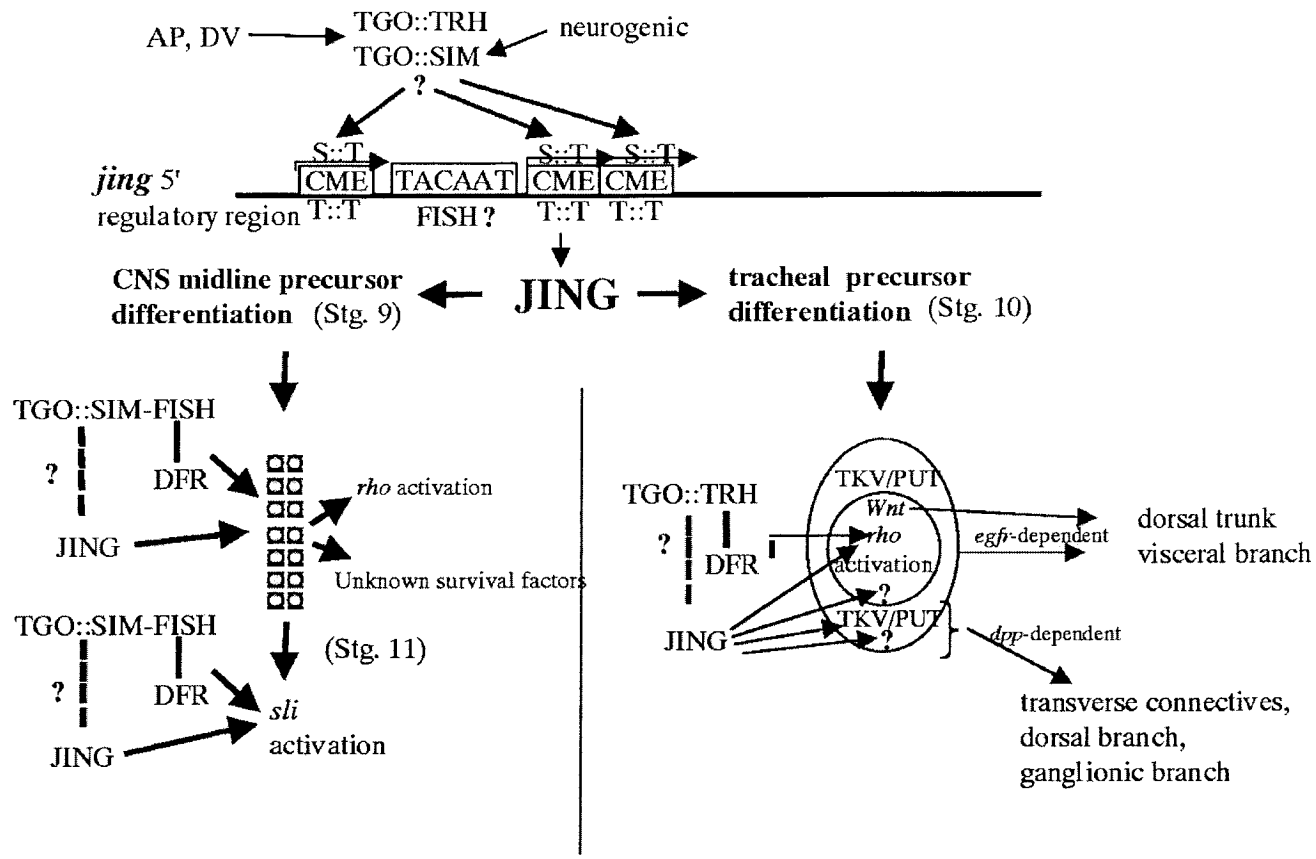


Fig. 10. Model for the role of the JING Zn-finger transcription factor in CNS midline and tracheal cell differentiation. Heterodimers of TGO:SIM and TGO:TRH may bind to the CMEs in the 5' regulatory region of *jing*. In the CNS midline, the SOX HMG protein Dichaete (FISH in the figure) may also bind its target site, TACAAT, to promote *jing* transcription. JING enters the nucleus of CNS midline and tracheal cells where it may perform a regulatory role. This regulatory role is required for the differentiation and survival of CNS midline and tracheal precursors, possibly through the control of EGFR signaling and/or other uncharacterized survival factors. Alternatively, *jing* may function to maintain the transcriptional output initiated by bHLH-PAS, POU and SOX regulatory molecules, and failure to do so results in cell death. JING function is required throughout CNS midline and tracheal development for the formation of a functional central nervous system and tracheal tubule network. Question marks denote that direct interactions have not been demonstrated.

(TACAAT) adjacent to the CMEs in the *jing* 5' regulatory region suggests this possibility (Fig. 4A, Fig. 10).

Genetic and phenotypic evidence suggests that *jing* functions in a different manner from either *vvl* or *fish*. *vvl* and *fish* have combinatorial regulatory roles in CNS midline or tracheal pathways, and therefore *vvl* and *fish*-null embryos do not show strong phenotypes (Boube et al., 2000; Ma et al., 2000; Zelzer and Shilo, 2000). By contrast, the defects in cell numbers and increased cell death during stage 10 and 11 in *jing* mutants precede and are different from the defects in homozygous *vvl* and *dfr-fish* double mutants in the CNS midline or from *dfr* homozygotes in the trachea (Boube et al., 2000; Ma et al., 2000; Zelzer and Shilo, 2000). Therefore, activation of *jing* transcription in the CNS midline or trachea cannot be controlled exclusively by Fish-hook or VVL.

jing encodes a putative DNA-binding protein with putative transcriptional regulatory domains, and its product can be seen in the nuclei of CNS midline and tracheal cells. Based on *jing* expression patterns and phenotypes, we propose that *jing* participates in the activation of genes downstream of both SIM::TGO and TRH::TGO in the CNS midline and trachea, respectively. Whether *jing* targets are also regulated by bHLH-PAS, POU or SOX combinatorial transcriptional activities remains to be determined. Nevertheless, JING function is required to promote the differentiation of CNS midline and tracheal lineages, which, in the absence of *jing* function, do not differentiate and instead undergo apoptosis.

How does *jing* promote cellular differentiation? We propose that *jing* regulates the transcription of important survival factors including those of the EGFR pathway. For

example, the *rho* gene product regulates the processing of the EGF receptor ligand Spitz and is expressed at stage 9/10 in the CNS midline and in the center region of the tracheal placodes (Bier et al., 1990; Schweitzer et al., 1995; Golembo et al., 1996; Wappner et al., 1997). Proper function of EGFR pathway genes is required for the survival of cells most highly affected by *jing* mutations, including the CNS midline glia, the tracheal dorsal trunk and visceral branches (Scholz et al., 1997; Sonnenfeld and Jacobs, 1994; Wappner et al., 1997). Furthermore, the *rho* regulatory region is controlled by TGO::TRH:DFR interactions and also contains multiple CAAT sites similar to those bound by AEBP2, the protein most related to JING (He et al., 1999; Lui and Montell, 2001; Zelzer and Shilo, 2000). This raises the possibility that *jing* may be involved in a combinatorial fashion in the regulation of bHLH-PAS target genes. However, this hypothesis does not account for the role of *jing* in EGFR-independent process such as survival of CNS midline neurons and *dpp*-dependent tracheal branches. One can then argue that *jing* function in the CNS midline and trachea is generic, and that JING carries out the gene expression programs initiated by bHLH-PAS, POU domain and SOX transcription factors. If the transcriptional programs are not maintained by *jing*, cells enter default apoptotic pathways. Alternatively, JING may be responsible for directly activating unknown survival factors in midline neurons and *dpp*-dependent tracheal branches.

Acknowledgments

We thank H. Lipshitz and Angelo Karaiskakis for help with P element-mediated transformation. We are grateful to the Ottawa General Hospital Research Institute for use

of their confocal microscope. Thanks to N. Sant, C. Stefanski and C.-A. Chenard for help isolating *jing* mutations. We thank S. Crews, M. Krasnow and S. Hayashi for reagents and flies. We thank C. S. Goodman, S. Artavanis-Tsakonis and J. Skeath for antibodies. BP102, 4D9 anti-ENGRAILED/INVECTED and mAb 22C10 antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work was supported by a Canadian Institutes for Health Research (CIHR) operating grant to M. J. S.

Chapter Three

Sedaghat and Sonnenfeld (2002). The *jing* gene is required for embryonic brain development in *Drosophila.*

The following outlines the experimental contributions of the authors of this manuscript. Immunostaining, light and laser confocal microscopy and image analysis, TUNEL labeling and statistical analysis was done by Y. Sedaghat. First draft of the manuscript was written by Y. Sedaghat under the guidance of Dr. M. Sonnenfeld. Dr. M. Sonnenfeld revised and completed the manuscript.

The *jing* gene is required for embryonic brain development in *Drosophila*

Yalda Sedaghat[1] and Margaret Sonnenfeld[1]*

Faculty of Medicine, Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, K1H 8M5, Canada

Dev Genes Evol (2002) 212:277-287

Abstract

Loss- and gain-of-function studies have demonstrated a crucial role for the *jing* zinc finger transcription factor in neuronal and glial differentiation and survival in the embryonic central nervous system midline of *Drosophila*. Here, we have studied the role of *jing* during embryonic brain development. Proper *jing* function is required for the formation of the primary brain axon scaffold. In homozygous $jing^3$ mutant embryos the preoral commissure is not pioneered and never forms. Other axon pathways are pioneered but subsequently do not form properly, including the postoral tritocerebral commissure, the circumesophageal connectives and the pathways that connect the brain with the ventral nerve cord. To understand the cellular basis of the axon phenotype the *jing* expression pattern in the brain was characterized using a *jing-lacZ* enhancer trap. *jing-lacZ* enhancer trap expression occurs in glia and neurons in the brain midline and lateral clusters as determined by co-localization of the *lacZ* gene product with Repo and Castor, respectively. In addition, the *jing-lacZ* enhancer trap and the basic helix-loop-helix-PAS gene, *single-minded* (*sim*), are expressed in the only glial midline cluster present in stage-14 wild-type embryos. *jing* function is required for the differentiation of Repo-, Castor- and Sim-positive cells in the embryonic brain as each of these populations contain a reduced number of cells in homozygous $jing^3$ mutant embryos. We further find that *jing* is required for neuronal and glial survival as *repo-* and *castor*-expressing cells undergo cell death in homozygous $jing^3$ mutant embryos, as revealed by double labeling with Tunel. Expression of *jing* in *sim*-expressing cells in the brain disrupts the entire axon scaffold but most significantly results in loss of the preoral and postoral tritocerebral

commissures. In addition, circumesophageal connectives are repelled after expression of two copies of UAS-*jing* in *sim*-expressing cells, suggesting the activation of axon repellent molecules. Over-expression of *sim* in the brain is also associated with loss of preoral and postoral tritocerebral commissures. Therefore, the proper dosage of *jing* and *sim* in the brain is critical for the formation of the primary axon scaffold. These results show that an important role for *jing* in the developing brain is the regulation of neuronal and glial differentiation and survival.

Keywords. *jing* - Zinc finger transcription factor - *Drosophila* - Brain development – Apoptosis

Introduction

jing is a zinc finger transcription factor recently characterized for its roles in the *Drosophila* embryonic central nervous system (CNS) midline and trachea as well as in the ovary (Lui and Montell, 2001; Sedaghat et al., 2002). In the CNS midline and trachea, *jing* function is required for proper cellular differentiation (Sedaghat et al., 2002). Loss- and gain-of-function *jing* is associated with defects in CNS axon and tracheal tubule patterning. In *jing* homozygous mutant embryos, reductions in neuronal and glial gene expression and inappropriate apoptosis in the CNS midline and trachea establish that *jing* is essential for the proper differentiation and survival of these lineages.

In *Drosophila* ovaries, *jing* is an essential downstream target of the vertebrate homolog of the basic region/leucine zipper transcription factor CCAAT enhancer-binding protein (C/EBP) required for border cell migration (Liu and Montell, 2001). The *jing* protein is most similar to the mouse protein, AEBP2, which was identified by its binding to a

regulatory sequence in the *adipocyte* (*a*P2) gene. Given that C/EBP also binds this sequence it is proposed that *jing* and C/EBP coordinate cell differentiation in a co-operative manner (He et al., 1999; Liu and Montell, 2001).

Analysis of the cellular and molecular processes regulating axon scaffold formation in the brain of *Drosophila* embryos reveals that preoral commissures are formed by axons that project across a bridge connecting the right and left brain hemispheres (Therianos et al., 1995). The process that gives rise to the initial set of axon projections has been analyzed in the embryonic brain of the grasshopper, *Schistocerca gregaria* (Boyan et al., 1995). At the beginning of axon scaffold formation the axons of pioneer neurons migrate between glial borders in each brain hemisphere. In *Drosophila* the first axons that project across the brain midline originate from neurons present in the medial edge of each hemisphere and send their axons toward the midline (Therianos et al., 1995). In both *Schistocerca* and *Drosophila* embryos, all the pioneering brain neurons express the cell adhesion molecule Fasciclin I during the initial outgrowth (Boyan et al., 1995; Therianos et al., 1995).

Descending circumesophageal connectives are prefigured by a group of glial cells along which pioneering axons extend. Therefore, the mechanisms of axon scaffold formation and its final structure in the embryonic brain appear similar to that of the ventral nerve cord (VNC) in which commissures follow glial scaffolds, span the midline and connect longitudinal connectives in the bilaterally symmetrical halves of the CNS (Jacobs and Goodman, 1989; Klämbt et al., 1991).

Many of the molecular and genetic processes regulating axon scaffold formation in the VNC have been characterized (Klämbt et al., 1991; Hummel et al., 1999a, b). It is not

known whether many of the genes that regulate cellular differentiation in the VNC also perform a similar function in the brain, however, some of the genes involved in axon scaffold formation in the VNC have been analyzed in the brain. In the VNC of embryos homozygous mutant in the *commissureless* gene, commissures are absent while longitudinal connectives are unaffected (Seeger et al., 1993). In the brain of *commissureless* mutants, however, commissures are pioneered but subsequent projection of commissural axons across the midline does not take place. Therefore, comparative genetic analysis demonstrates that there are differences in the mechanisms of commissure formation in the brain compared to that in the VNC. The *single-minded* (*sim*) gene encodes a basic helix-loop-helix-PAS transcription factor expressed in CNS midline precursors and differentiated neurons and glia in the VNC (Crews et al., 1988). *sim* function regulates the expression of many midline genes that are required for precursors to differentiate into sublineages (Nambu et al., 1990; Crews, 1998). In the VNC of homozygous *sim* mutants, commissures do not form and longitudinal connectives fuse into one tract at the midline (Thomas et al., 1988). In contrast, in the brain of *sim* mutants the preoral commissures form but circumesophageal longitudinal connectives and postoral commissures do not (Therianos et al., 1995). Furthermore, *sim* enhancer expression in the brain and VNC is different. While *sim-lacZ* expression could not be detected in the midline of the embryonic brain, it is exclusively expressed in the midline of the VNC (Nambu et al., 1991; Therianos et al., 1995). Therefore, *sim* phenotypes and expression patterns in the brain and VNC are different. *sim* may be involved in transcriptional regulation in the brain but previous results suggest that it is not likely involved in specification of the brain midline (Therianos et al., 1995).

Given the previously established role of *jing* in VNC axon scaffold formation, we wanted to determine if *jing* is also required for formation of the brain axon scaffold (Sedaghat et al., 2002). Here, we show that *jing* function is required for proper formation of all major axon pathways in the brain suggesting that it may have similar roles in the VNC and brain (Sedaghat et al., 2002). Analysis of *jing-lacZ* enhancer trap expression in the brain reveals that it is expressed in many cells including Repo-positive glia and Castor-positive neurons. The functional role of *jing* was examined by loss-of-function and targeted expression experiments. Quantitative analysis of cell numbers and apoptosis in Repo and Castor lineages in *jing* mutants reveals a role for *jing* in cellular differentiation and survival during embryonic brain development. Targeted expression, by the Gal4/UAS system (Brand and Perrimon, 1993), of *jing* to *sim*-expressing cells interferes with axon scaffold formation and resembles the *jing* loss-of-function phenotype. Over-expression of *sim* in the brain also results in an axon phenotype resembling that of *jing* loss-of-function. Based on these data, we propose that *jing* regulates the expression of essential genes required for the differentiation and survival of neurons and glia during embryonic brain development.

Materials and methods

Fly strains

The wild-type strain was w^{118}. The *jing-lacZ* enhancer trap strain ($jing^{01094}$) was obtained from the Indiana *Drosophila* stock center (Bloomington, Indiana). Heterozygous embryos carrying $jing^{01094}$-*lacZ* were used to study *jing* expression during embryonic brain development. The $jing^{3}$ EMS-induced allele has been previously described (Sedaghat et

al., 2002). For over-expression experiments transgenic strains p[*sim*-Gal4]), p[UAS-*sim*] (gifts of S. Crews) and p[UAS-*jing*] were used (Sedaghat et al., 2002).

Antibodies

Primary antibodies were: mouse BP102 diluted 1:20 [Developmental Studies Hybridoma Bank (DSHB)]; mouse anti-Fasciclin II diluted 1:10 (gift of C.S. Goodman); rat anti-Sim diluted 1:100 (gift of S. Crews); rabbit and mouse anti-β-galactosidase diluted 1:100 (Promega); rabbit anti-Repo diluted 1:300 (a gift from A. Travers); rabbit anti-Castor diluted 1:300 (a gift from W. Odenwald); mouse anti-En diluted 1:3 (DSHB); rabbit anti-HRP diluted 1:250 (Jackson). Secondary antibodies were HRP-conjugated goat anti-mouse (Jackson), IgG biotinylated anti-rat (Vector), and HRP-conjugated goat anti-rabbit (Jackson). For immunofluorescence confocal microscopy, FITC- and Rhodamine-conjugated secondary antibodies (Jackson) were used. Fluorescein-labeled embryos were mounted in vectashield H-1,000.

Immunohistochemsitry, light and laser confocal microscopy

Antibody staining procedures are based on a standard technique as described by Patel (*1994*). Embryo staging was according to Campos-Ortega and Hartenstein(1985). The absence of β-Gal staining was used to identify homozygous $jing^3$ mutant embryos in $jing^3$/*wg-LacZ* strains. For quantification of Sim-positive cells, the number of immunoreactive cells were scored at 40x magnification in each brain lobe (frontal view) of wild-type and homozygous $jing^3$ mutant embryos. A Zeiss Axioskop was used for light microscopy and a Zeiss Axiovert 100 TV was used for laser confocal microscopy.

Optical sections were at 1 μm with a line average of 8. Images were processed using Adobe Photoshop.

TUNEL labeling

To examine cell death, an in situ cell death detection kit was used (TMR red, Roche) according to Booth et al. (2000). In this experiment, in situ tailing of DNA fee ends by terminal deoxynucleotidyl transferase (TdT) was carried out (Gavrieli et al., 1992) and apoptotic nuclei were visualized on a Zeiss Axiovert 100 TV confocal microscope. TUNEL-labeled embryos were double-labeled with anti-Repo, -Castor or -Sim to detect cell-type-specific cell death. The number of glia and neurons was determined using a reticule mounted on the confocal microscope in 20 sagittal wild-type and *jing*^{3} mutant brain lobes. Quantification of apoptotic nuclei was done in Adobe Photoshop after merging double-labeled images. All images were processed with Adobe Photoshop software and quantifications of cell numbers and cell death were plotted using Sigma Plot software.

Results

***jing* function is required to establish the primary axon scaffold in the brain**

To determine whether *jing* plays a role in establishing the primary brain axon scaffold we analyzed homozygous mutant *jing*^{3} embryos stained with BP102 (Fig. 1). The *jing*^{3} allele is a strong hypomorph containing a stop codon in the middle of the second zinc finger and therefore likely truncates the protein product (Sedaghat et al., 2002).

Axonogensis in the wild-type brain begins during stage 12/0 first with formation of the circumesophageal connectives and then with formation of the first preoral brain commissure (Fig. 1A; Therianos et al., 1995). In homozygous *jing*^{3} mutant embryos the initial formation of the circumesophageal connectives appears normal during stage 12 as both descending and ascending axons meet and fasciculate to form a connective (Fig. 1B). However, the first axonal projections to cross the midline in wild-type embryos are absent in stage 12 homozygous *jing*^{3} mutant embryos (Fig. 1B). The postoral tritocerebral commissure is pioneered before the preoral brain commissure in wild-type embryos and forms anterior to the subesophageal ganglion (Fig. 1A; Therianos et al., 1995). In stage 12 homozygous *jing*^{3} mutant embryos the postoral tritocerebral commissure is pioneered as axons extend around the subesophageal ganglion (Fig. 1B).

By stage 15, the wild-type primary axon scaffold has an orthogonal structure consisting of a preoral commissure, circumesophageal connectives (longitudinal fascicle) and postoral tritocerebral commissure (Fig. 1C; Therianos et al., 1995). In the brain of homozygous stage-15 *jing*^{3} mutant embryos, formation of the preoral commissure has still not occurred (Fig. 1D). Therefore, either the follower axons require the pioneering axons for their guidance or the follower neurons are not differentiating properly in *jing* mutants. In addition, the postoral tritocerebral commissure and circumesophageal connectives are significantly reduced in size in homozygous *jing*^{3} mutants compared to wild type (Fig. 1D). The longitudinal connectives do not extend properly and sometimes

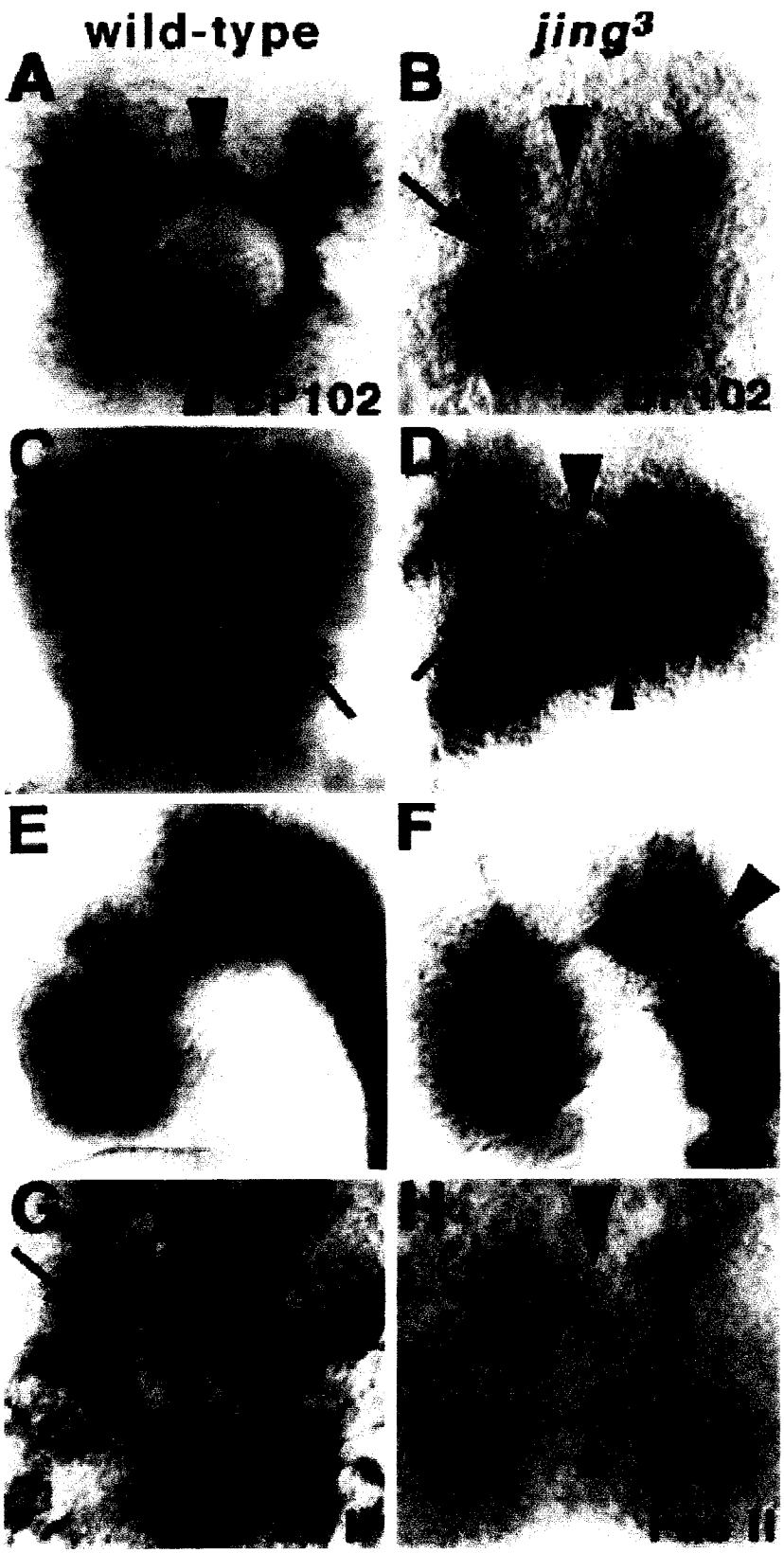

wild-type
jing3
A
B
C
D
E
F
G
H

Fig. 1. Axon phenotype of homozygous *jing*^{3} mutants. Frontal (**A-D, G, H**) and sagittal views (**E, F**) of embryonic brains stained with BP102 (**A-F**) and with anti-Fasciclin II (**G, H**) with anterior oriented *up*. **A** In wild-type stage-12/0 embryos, BP102 stains the preoral commissures (*large arrowhead*), the circumesophageal longitudinal connectives (*arrow*) and the postoral tritocerebral commissures (*small arrowhead*). **B** In homozygous stage-12/0 *jing*^{3} mutant brains the preoral commissure is not pioneered (*large arrowhead*). **C** Wild-type orthogonal structure of a stage-15 brain. **D** Aberrant structure of a stage-15 *jing*^{3} mutant brain. The preoral commissure never forms (*large arrowhead*). The postoral (*small arrowhead*) and circumesophageal connectives (*arrow*) are reduced compared to the wild type. **E** In wild-type embryos, the brain and ventral nerve cord are connected by BP102-positive axons (*arrowhead*). **F** In homozygous *jing*^{3} embryos the connection between the brain and VNC is absent (*arrowhead*). **G** In wild-type stage-15 embryos, Fasciclin II is expressed on axon fascicles in circumesophageal (*arrow*) and commissural (*arrowhead*) brain pathways. **H** In homozygous stage-15 *jing*^{3} embryos stained with anti-Fasciclin II, commissural pathways are absent (*large arrowhead*) and circumesophageal connectives are extremely reduced (*arrow*). The postoral tritocerebral commissure appears thin (*small arrowhead*).

appear bunched together (Fig. 1D). Lastly, the connection between the brain and nerve cord is absent in homozygous stage-15 *jing*^{3} mutant embryos (Fig. 1F) compared to wild type (Fig. 1E).

In the wild-type stage-15 embryonic brain three axon fascicles express Fasciclin II (Fas II) in the circumesophageal connectives and in the preoral commissure (Fig. 1G; Therianos et al., 1995). In the tritocerebral commissure there is one Fas II-positive fascicle. In stage-15 homozygous *jing*^{3} mutant embryos, the Fas II-positive fascicles of the preoral commissure do not form, consistent with the absence of BP102 stain (Fig. 1H). The Fas II-positive fascicles of the circumesophageal connectives are extremely reduced compared to wild type. The tritocerebral Fas II commissure is present in homozygous *jing*^{3} mutants but also appears reduced compared to wild type (Fig. 1H). In summary, the *jing* gene product is necessary for the pioneering and subsequent development of the preoral brain commissure and is required for proper development of the entire axon scaffold.

jing-lacZ expression in the embryonic brain

In order to understand *jing* function in the embryonic brain, expression of the *jing-lacZ* enhancer trap was analyzed. *jing-lacZ* expression is similar to that of endogenous *jing* in the embryonic nerve cord and trachea and therefore is an accurate reflection of *jing* expression (Sedaghat et al., 2002). Embryos heterozygous for *jing-lacZ* were stained with anti-β-galactosidase (anti-β-Gal) and analyzed by confocal microscopy.

To determine whether *jing* is expressed in glia, embryos containing *jing-lacZ* were double-labeled with anti-β-Gal and anti-Repo. *repo* encodes a homeodomain protein

found in glioblasts at stage 9 and is expressed in all differentiated glia in the central and peripheral nervous system, excluding the midline glia of the VNC (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995). *jing-lacZ* expression is first detected during stage 11 in 35% of Repo-positive glia in both posterior and anterior regions of the brain (Fig. 2A). *jing-lacZ* is expressed in the longitudinal glia that connect the brain with the VNC during stages 11 and 15 (Fig. 2A, B, arrowhead; Therianos et al., 1995). By stage 15, approximately 50% of glia in anterior and posterior domains of the brain express *jing-lacZ* (Fig. 2B). A subset of the *jing-lacZ*-expressing midline cells are glia as they also stain with anti-Repo (Fig. 2E, arrowhead). This group of cells consists of four glia that occupy a posterior position in the midline. Lateral *jing-lacZ*-expressing glia follow the circumesophageal longitudinal axon tracts (Fig. 2E, arrow).

To determine whether *jing* is expressed in brain neurons, heterozygous embryos containing *jing-lacZ* were double-labeled with anti-β-Gal and anti-Castor (Cas). *cas* encodes a zinc finger transcription factor expressed in many CNS neurons (Cui and Doe, 1992; Mellerick et al., 1992). Both *jing-lacZ* and Cas show nuclear localization and therefore allow for an accurate co-localization of the gene products. A recent study shows that *Drosophila* neuroblasts in the ventral nerve cord sequentially express the transcription factors *hb, Kr, pdm and cas* (Isshiki et al., 2001). Accurate temporal regulation of Hb, Kr and Cas is critical for proper cellular development in the CNS. Therefore, the presence of Cas within a cell is a good indicator of the status of differentiation. Staining with an anti-Cas antibody shows that the Cas protein is present throughout the developing brain (Fig. 2C, D). *jing-lacZ* expression is first detected during stage 11 in approximately 47% of Cas-positive neurons mostly in the anterior of the brain

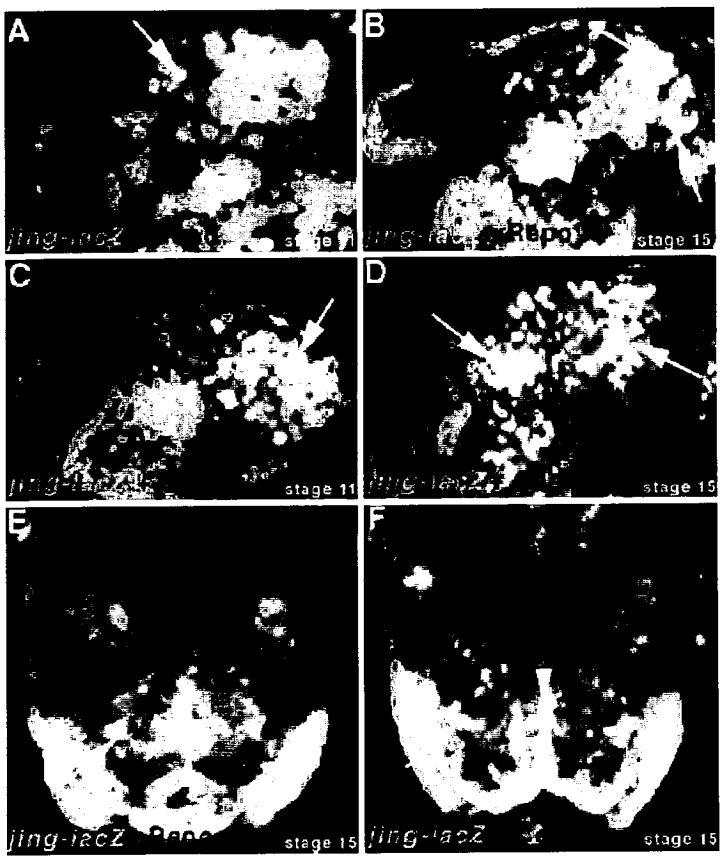


Fig. 2. *jing-lacZ* expression in the embryonic brain. Laser confocal micrographs of stage-11 (**A**, **C**) and -15 (**B**, **D-F**) heterozygous embryos containing *jing-lacZ*. Embryos were double labeled with anti-ß-gal and the glial-specific anti-Repo antibody (**A**, **B**, **E**) or the neural-specific anti-Castor antibody (**C**, **D**, **F**). Images are reconstructions of optical sections shown in sagittal view (**A-D**) and horizontal view (**E**, **F**). (**A**) During stage 11, *jing-lacZ* (*green*) is expressed in a subset of Repo-positive glia (*red*) in the posterior of the brain (*arrows*, *yellow*) and in the longitudinal glia connecting the brain with the ventral nerve cord (*arrowhead*). (**B**) During stage 15, *jing-lacZ* (*green*) is expressed in both anterior and posterior glial domains (*arrows*) and in the longitudinal glia connecting the brain with the ventral nerve cord (*arrowhead*). (**C**) During stage 11, *jing-lacZ* (*green*) is expressed in a subset of anterior Castor-positive neurons (*arrow*). (**D**) During stage 15, *jing-lacZ* (*green*) is expressed in both anterior and posterior Castor-positive neural domains (*arrows*). (**E**) There is a cluster of *jing-lacZ* and Repo-positive cells in the midline of the brain (*arrowhead*) and along circumesophageal longitudinal tracts (*arrow*). (**F**) There is a cluster of *jing-lacZ* and Castor-positive cells in the midline of the brain (*arrowhead*) as well as laterally (*arrow*).

(Fig. 2C). During later embryonic stages, *jing-lacZ* is activated in most Cas-positive neurons in both anterior and posterior regions of the brain (Fig. 2D). In the brain midline, a subset of *jing-lacZ*-expressing cells stain with anti-Cas, revealing their neuronal identity (Fig. 2F). This group of neurons consists of approximately six cells in a more anterior position in the midline than the *jing-lacZ*-expressing glia.

jing function is required for neuronal and glial differentiation and survival

To determine the role of *jing* during brain development the differentiation of glia and neurons was analyzed in homozygous $jing^3$ mutant brains. Glia prefigure the primary preoral commissure and circumesophageal connectives in the wild-type embryonic brain (Therianos et al., 1995). Given the distinct defect in preoral commissural formation in $jing^3$ mutants it was important to determine whether this was due to abnormal glial differentiation.

To assess glial fates, the number of glia and the number of apoptotic glia were counted in homozygous $jing^3$ mutant and wild-type embryos. Apoptotic glia were identified by double-labeling with TUNEL and anti-Repo. In stage-11 wild-type brains few apoptotic glia are present (Fig. 3A). By stage 15, only 4.3% of glia in wild-type embryonic brains are apoptotic (Fig. 3B, E). In stage-11 homozygous $jing^3$ brains, the number of apoptotic glia is not significantly altered compared to wild type (Fig. 3C). Therefore, onset of the developmental apoptotic program does not require *jing* function. In contrast, by stage 15, approximately 18.9% of all glia were TUNEL-positive in homozygous $jing^3$ mutant brains (Fig. 3D, E). Our analysis also reveals an average 33% reduction in the number of brain glia in homozygous $jing^3$ mutant brains compared to wild type. Together, these

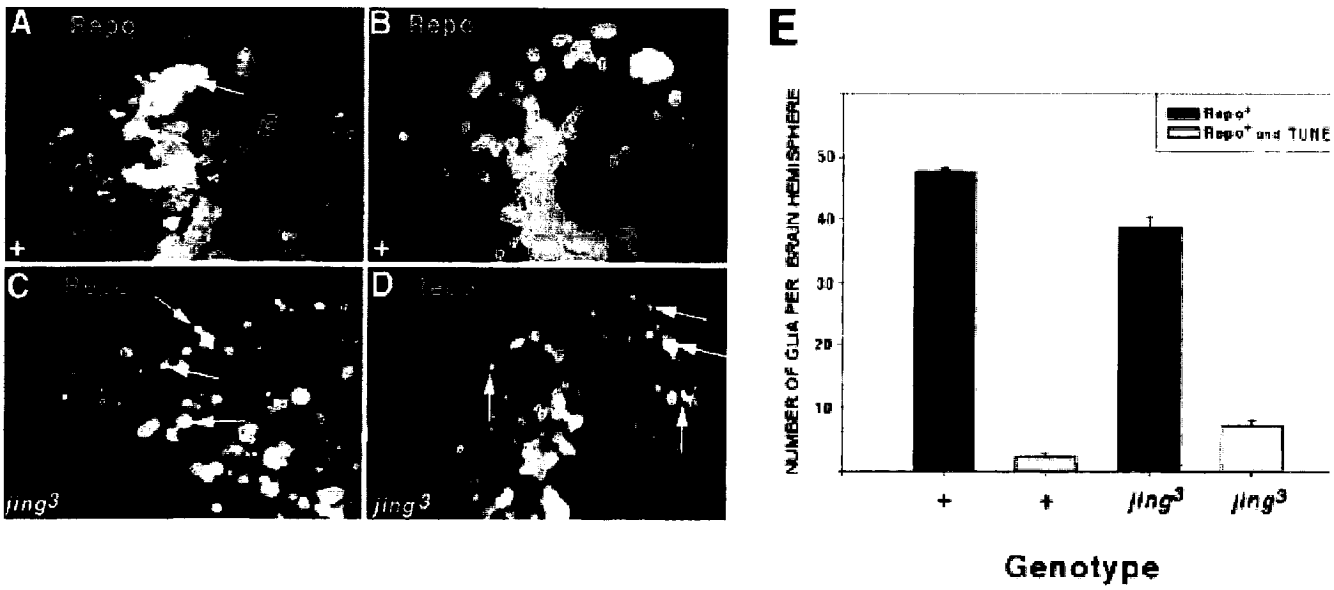


Fig. 3. Pattern of glial cell death in wild-type and homozygous *jing*^{3} embryonic brains. Wild-type (**A**, **B**) and homozygous *jing*^{3} (**C**, **D**) brains at stage 11 (**A, C**) and 15 (**B, D**) were stained with anti-Repo and the cell death marker Tunel (*red*). Laser confocal micrographs show reconstructions of brains in sagittal view. (**A, B**) TUNEL- and Repo-positive cells are present (*yellow, arrows*) in wild-type stage-11 brains but are uncommon in stage-15 brains (*arrows*). **C**) The pattern of glial cell death in homozygous *jing*^{3} stage-11 brains is not altered from that of wild-type but the reduction in glia (*green*) is apparent (*arrows*). (**D**) The pattern of glial and general cell death is significantly altered in stage-15 *jing*^{3} brains compared to the wild type. There are many TUNEL- and Repo-positive cells (*yellow, arrows*). There is also an abundance of TUNEL-positive profiles throughout the brain. The reduction in glia (*green*) is apparent compared to the wild type. **(E)** Quantitative analysis of glial numbers and cell death in wild-type and homozygous *jing*^{3} brains during stage 15. Note the decrease in glial numbers and increase in apoptotic glia in homozygous *jing*^{3} brains relative to the wild type. n =20 in all samples.

results suggest that the decrease in glial numbers in mature *jing* mutant brains is due to improper differentiation and to cell death.

To assess neuronal fates, wild-type and homozygous $jing^3$ mutant embryos were stained with anti-Cas and the number of cells quantified. Our results show that subsets of Cas-positive cells in the embryonic brain are dependent upon *jing* function, as mature stage-15 homozygous $jing^3$ mutant brains show an approximate 26% reduction in the number of Cas-positive neurons from that in wild-type stage-15 brains (Fig. 4B, D, E). There are more TUNEL- and Cas-positive neurons in stage-11 homozygous $jing^3$ mutant brains compared to that in wild type (Fig. 4A, C). By stage 15, 37.2% of Cas-positive neurons are TUNEL-positive in $jing^3$ mutant brains compared to 5.4% in wild-type brains (Fig. 4E). Therefore, these results suggest that the decrease in the number of neurons in *jing* mutant brains is due to a lack of differentiation and cell death.

jing is required for the correct organization and differentiation of Sim cell clusters

We chose to analyze the fate of Sim-positive cells in the brains of *jing* mutants because *jing* is required for the differentiation and survival of Sim-positive cells in the VNC midline (Sedaghat et al., 2002). In addition, a role for *sim* in axon scaffold formation in the brain has been previously described (Therianos et al., 1995). In the brain, mutations in *sim* are associated with absent or aberrant projection of longitudinal pathways and an absence of the postoral commissures (Therianos et al., 1995). Previous analysis using a *sim-lacZ* reporter indicated that *sim* is expressed in the lower part of the brain and that some *sim*-expressing cells were glia (Therianos et al., 1995). These results suggested a key role for *sim* in the development of the circumesophageal connectives.

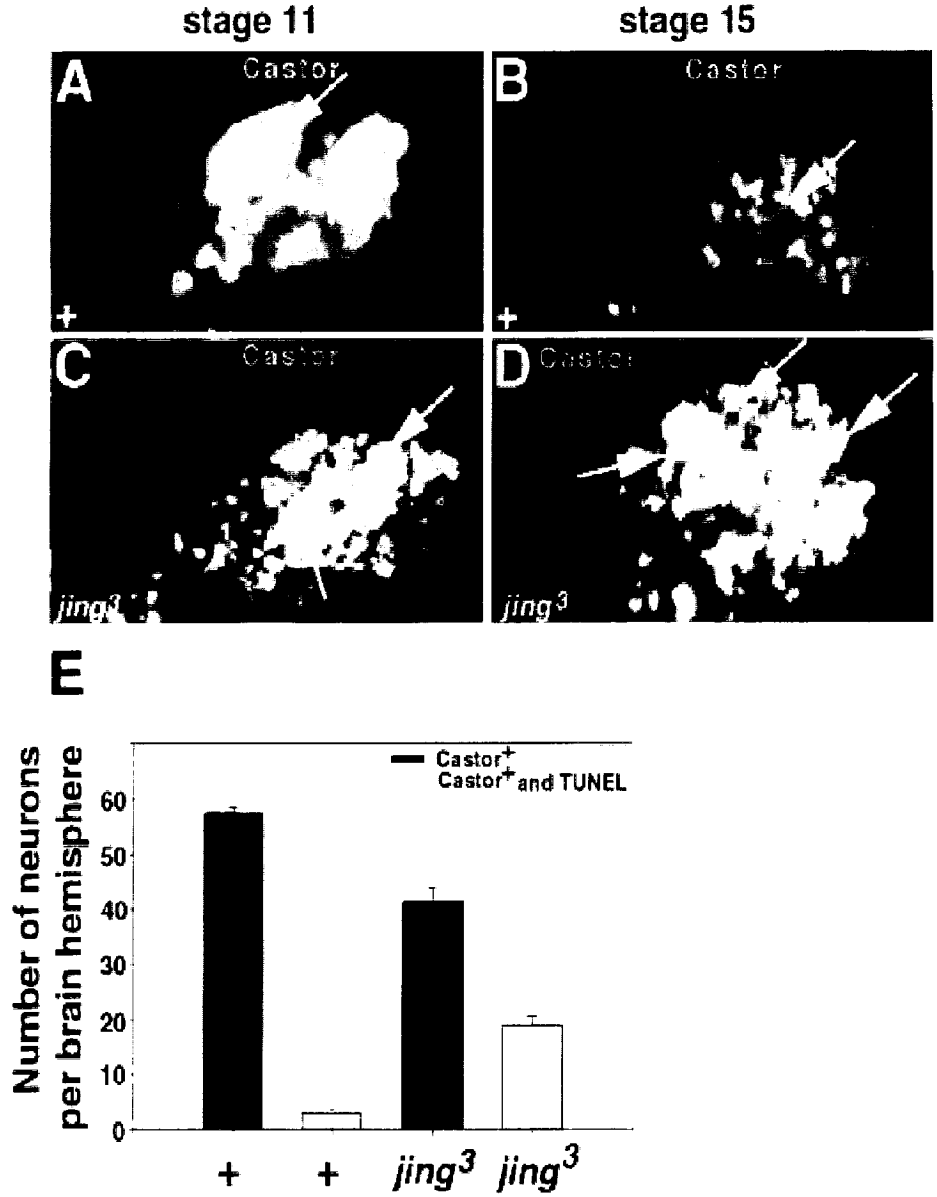


Fig. 4. Pattern of neuronal cell death in wild-type and homozygous *jing*^{3} mutant brains. Confocal micrographs of embryos double labeled with Tunel (*red*) and anti-Castor (*green*; **A-D**). Laser confocal micrographs show reconstructions of brains in sagittal view. (**A, B**) In wild-type embryos a small number of Castor-positive neurons in the brain undergo apoptosis during stage 11 (**A**) and 15 (**B**; *arrows*). (**C, D**) In homozygous *jing*^{3} mutant brains there are many Castor-positive apoptotic neurons during stage 11 (**C**) and 15 (**D**; *arrows*). (**E**) Quantitative analysis of neuronal numbers and cell death in Castor-positive populations in wild-type and *jing*^{3} brains during stage 15. Note decrease in Castor-positive neurons and increase in apoptotic Castor-positive neurons in homozygous *jing*^{3} mutant brains relative to the wild type. n =20 in all samples.

We have analyzed the distribution of Sim protein in the embryonic brain using an anti-Sim antibody. Sim protein is first detected in the brain during stage 12 in one three-cell cluster in the posterior of each hemisphere (data not shown). In the mature stage-15 brain, Sim is present in the nuclei of cells in five distinct clusters in each hemisphere (Fig. 5A). Clusters 2, 3 and 4 are in close proximity to where the circumesophageal connectives form. There is one large cluster of Sim-positive cells located in the midline of the brain at a position where glia are located (Fig. 5A, arrowhead; compare to Fig. 2E).

To determine whether *jing* is required for the development of *sim*-expressing cells homozygous $jing^3$ mutant embryos were stained with anti-Sim. The pattern of Sim immunoreactivity is altered in homozygous $jing^3$ mutant brains (Fig. 5B). In wild-type stage-15 brains, Sim cluster 1 contains an average of 6.5 cells (n =26 hemispheres) whereas Sim cluster 1 contains only an average of 2.5 cells in similarly staged homozygous $jing^3$ mutant brains. Sim clusters 3, 4 and 5 also contain a significantly reduced number of cells in stage-15 homozygous $jing^3$ mutant brains compared to wild type. The total number of Sim-positive cells in wild-type and homozygous $jing^3$ mutant brains was quantified during stage 15 (Fig. 5C). This analysis reveals an approximate 50% reduction in the total number of Sim-positive cells in homozygous $jing^3$ mutant brains compared to wild type. In summary, these results show that *jing* is required for the proper differentiation of Sim-positive cells during embryonic brain development.

Expression phenotypes of *jing* and *sim* in the embryonic brain

To assess *jing* function in a specific cell type in the embryonic brain, transgenic flies were used that express *jing* (p[UAS-*jing*]) under the control of the *sim* promoter (p[*sim*-

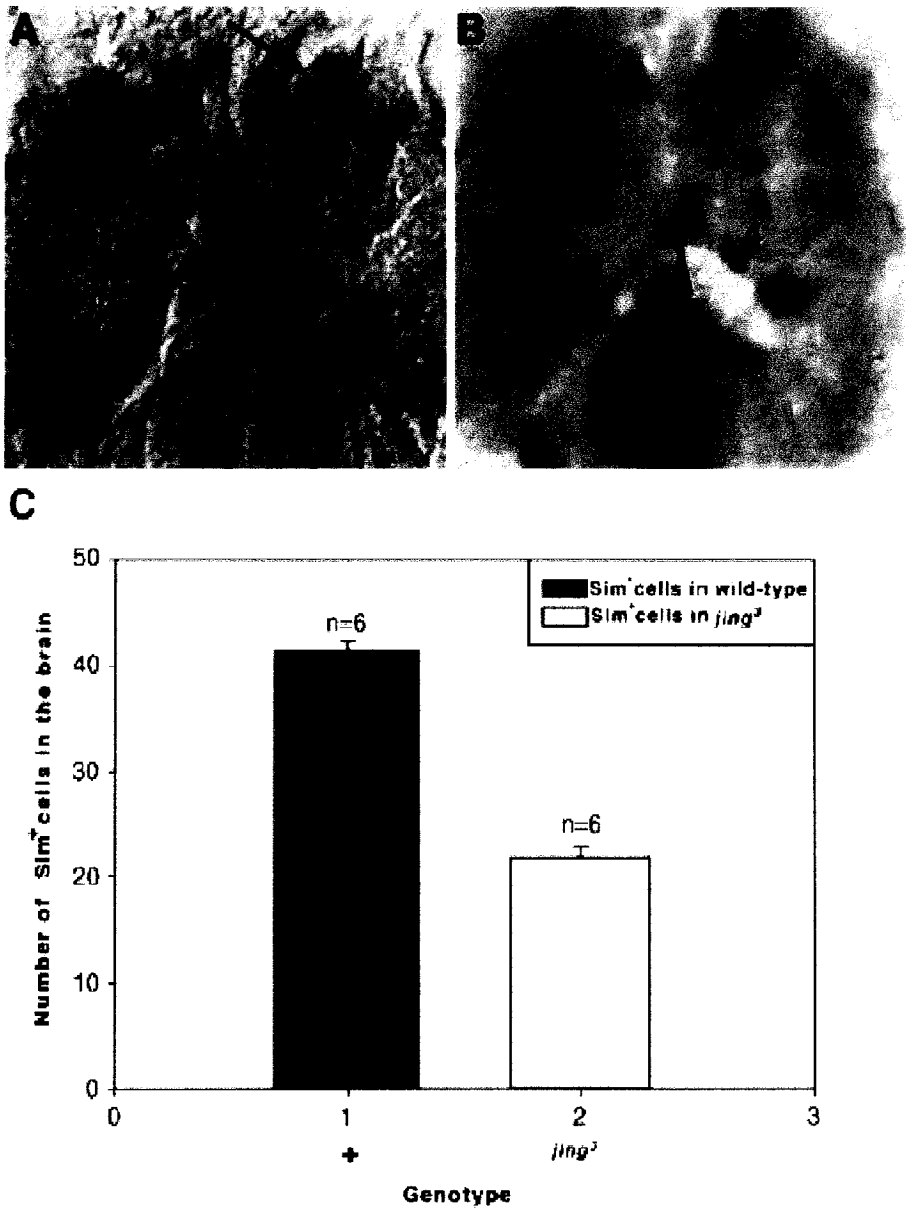


Fig. 5. *sim* expression pattern in wild-type and homozygous *jing*^{3} brains. Sim immunoreactivity is shown at stage 15 in frontal view with anterior oriented *up*. Wild-type (**A**) and *jing*^{3} (**B**) homozygous mutant brains. (**A**) Sim is present in the nuclei of cells in five clusters (*1-5*) in each brain hemisphere. Sim is also present in a cluster of glia in the midline (*arrowhead*). (**B**) In homozygous *jing*^{3} mutant brains Sim immunoreactivity is weaker relative to the wild type and some Sim-positive cells are absent. The midline cluster (*arrowhead*) contains a reduced number of cells as do lateral clusters 1, 2 and 5 (*arrow*). Cluster 4 is absent in the left hemisphere. (**C**) Quantification of Sim-positive cells during stage 15 in wild-type and *jing*^{3} homozygous mutant brains. The number of Sim-positive cells is reduced by 50% in *jing*^{3} homozygous mutant brains.

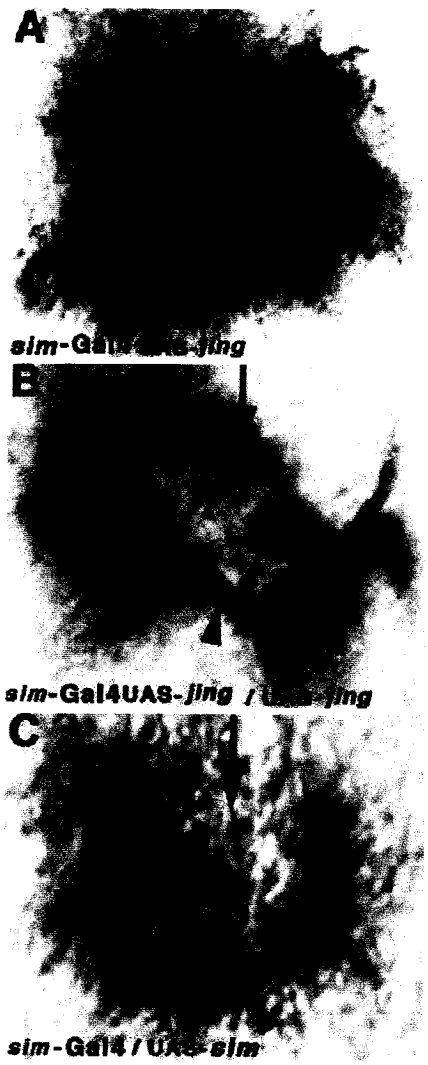


Fig. 6. Over-expression of *sim* and expression of *jing* in Sim-positive cells in the brain. UAS-*jing* was expressed in the brain in one copy (**A**) or two copies (**B**) using *sim*-Gal4 as a driver. (**C**) Over-expression of *sim* in the brain using *sim*-Gal4 and UAS-*sim*. CNS axons were visualized by BP102 staining and HRP immunocytochemistry. (**A, B**) Malformations occur in the preoral (*arrows*) and postoral (*small arrowheads*) commissures after expression of *jing* in *sim*-expressing brain cells. Expression of one copy of UAS-*jing* results in gaps in the circumesophageal connectives (**A**, *large arrowhead*). After expression of two copies of UAS-*jing* the circumesophageal connectives extend away from the brain midline. (**C**) Over-expression of one copy of UAS-*sim* in the brain is associated with loss of the preoral commissure (*arrows*) and reduced postoral (*small arrowhead*) commissures. Circumesophageal connectives are present but are thinner than wild-type (*large arrowhead*; compare to Fig. 1A).

Gal4]) using the Gal4/UAS system of Brand and Perrimon (1993). Flies containing p[UAS-*jing*] were crossed to those containing p[*sim*-Gal4] and their progeny were stained with BP102 to visualize the brain axon scaffold. Expression of *jing* in *sim* cells results in an axon scaffold phenotype similar to that in *jing* loss-of-function embryos (Fig. 6A, B). Expression of one copy of p[UAS-*jing*] is sufficient to inhibit preoral and postoral tritocerebral commissural formation (Fig. 6A). In addition, the circumesophageal longitudinal connectives sometimes do not extend properly and have large gaps (Fig. 6A). Expression of two copies of p[UAS-*jing*] driven by p[*sim*-Gal4] is also associated with commissural loss (Fig. 6B). In addition, the circumesophageal longitudinal connectives project away from the brain midline suggesting the activation of an axon repulsive molecule by *jing*.

To determine the effect of *sim* over-expression on the development of the primary brain axon scaffold, flies containing p[*sim*-Gal4] were crossed to those containing p[UAS-*sim*] and their progeny were stained with BP102. Over-expression of *sim* in the brain results in loss of preoral and postoral commissural formation (Fig. 6C). The circumesophageal longitudinal connectives form but are thinner than those in wild-type embryos. In summary, these results show that proper *jing* and *sim* dosage is required in the cells that pattern the brain axon scaffold.

Discussion

The results presented in this study allow for a comparative analysis of the role of the *jing* zinc finger transcription factor in the embryonic CNS. There are both similarities and differences in the fate of neurons and glia in the VNC and brain of homozygous *jing*^{3}

mutants. However, in both cases, *jing* is essential for the terminal differentiation and survival of neurons and glia suggesting that it has a conserved biological function.

In the brain, proper formation of the commissures, longitudinal connectives and the connection between the brain and VNC depends on functional *jing* gene product. In loss-of-function *jing* mutants there is an absence of the preoral and postoral commissures and the axon connection between the brain and VNC as well as a thinning of the circumesophageal connectives. Therefore, *jing* function is required to establish the brain axon scaffold.

The preoral brain commissure develops from neural extensions that originate from the medial edges of each hemisphere and project towards the midline (Therianos et al., 1995). Along with these axonal projections are neuronal cell bodies and glial cells that reach the midline of the brain and establish an interhemispheric cell bridge (Therianos et al., 1995). A row of glial cells extends across the midline and is associated with the preoral commissure (Fig. 2E; Therianos et al., 1995). *jing-lacZ* is expressed in the row of glia that are associated with the preoral commissure (Fig. 2E). Loss of the preoral commissure in homozygous $jing^{3}$ mutants may therefore result from improper differentiation of the glia and/or the neurons that make up the commissure.

The role of longitudinal glial cells in the formation of longitudinal axon tracts has been described in the VNC (Jacobs and Goodman, 1989; Jacobs, 1993). In the wild-type brain, a row of longitudinal glial cells prefigures the circumesophageal connectives (Therianos et al., 1995). In homozygous $jing^{3}$ mutant brains the circumesophageal longitudinal connectives are pioneered but do not form properly. *jing-lacZ* is expressed in the longitudinal Repo-positive glia that follow the circumesophageal longitudinal

connectives and therefore it is possible that improper circumesophageal longitudinal formation in *jing* mutants results from defective longitudinal glial differentiation. Similarly, the absence of the axonal connection between the brain and VNC correlates with expression of *jing-lacZ* in the glia in this region. In addition, there is a severe reduction in the number of longitudinal glia at the connection between the brain and VNC in *jing* mutant embryos and many of the longitudinal glia in homozygous $jing^3$ mutants undergo inappropriate apoptosis. These results support the notion that glia have a conserved role in prefiguring axon pathways in invertebrate and mammalian brains (Jacobs and Goodman, 1989; Chitnis and Kuwada, 1990; Klämbt et al., 1991; Easter et al., 1993; Jacobs, 1993; Silver, 1993; Steindler et al., 1993; Boyan et al., 1995).

To determine the role of *jing* in the brain, we analyzed glial and neuronal fates in homozygous $jing^3$ mutant embryos. Brain glia do not appear to require *jing* function for their specification as reductions in glial cell numbers or increased glial apoptosis are not observed in homozygous $jing^3$ mutants until after stage 12. Therefore, terminal glial differentiation requires *jing* function as loss-of-function *jing* is associated with increased apoptosis in glial lineages. In contrast, brain neurons require *jing* function for their survival during early (stage 11) and late stages of differentiation. While apoptosis is barely detectable in Cas-positive neurons in wild-type stage-11 embryos it is significantly more prevalent in similarly staged homozygous $jing^3$ mutant embryos (Fig. 4). Many apoptotic Cas-positive neurons are present until the end of embryogenesis in homozygous $jing^3$ mutant embryos compared to that in wild-type embryos. Therefore, *jing* plays important and different roles in glial and neuronal differentiation in the embryonic brain.

single-minded (*sim*) encodes a basic helix-loop-helix-PAS (bHLH-PAS) transcription factor that functions as a heterodimer with another bHLH-PAS transcription factor known as Tango (Tgo) to control downstream gene transcription in the embryonic VNC midline (Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997). Together, this transcriptional heterodimer acts as a lineage control switch (Crews, 1998). Because *jing* controls the differentiation and survival of Sim-positive neurons and glia in the VNC we wanted to determine if it plays a similar role in the brain. We first characterized the pattern of Sim protein localization in the embryonic brain. During stage 12, Sim is present in only one cluster in the posterior of each brain hemisphere which is after the specification of neurons and glia (Therianos et al., 1995). This suggests that *sim* is not likely involved in cellular specification in the brain as it is in the VNC (Nambu et al., 1991). This analysis shows that Sim is found in five discrete clusters in each stage-15 brain hemisphere suggesting that its expression is precisely regulated (Fig. 5A). Interestingly, Sim is also found in one midline cluster situated in the posterior region of the stage-15 brain in a location where Repo-positive glia are also found.

The function of *sim* in the embryonic brain appears to be different from its function in the VNC (Therianos et al., 1995). In the VNC of homozygous *sim* mutants, commissures do not form and longitudinal axons run as a single tract down the midline. This is due to a loss of the midline cells and their associated repellent molecules such as Slit (Nambu et al., 1991; Battye et al., 1999; Kidd et al., 1999). In contrast, the preoral commissure of the brain does not require *sim* function for its formation while the circumesophageal connectives and postoral commissure are dependent on *sim* function (Therianos et al., 1995). The axon phenotypes of *jing* and *sim* loss-of-function in the brain overlap and *jing*

function is required for proper differentiation of Sim-positive cells. Since *jing* expression precedes that of *sim* in both neurons and glia in the brain *jing* is not likely a downstream component of bHLH-PAS pathways in the brain as it is in the VNC (Sedaghat et al., 2002). *jing* and *sim* are both expressed in a midline glial cluster whose role in axon formation is not yet known.

Targeted gene expression studies were performed to address whether the dosage of *jing* and *sim* is important for formation of the brain axon scaffold. Our results show that *jing* and *sim* are expressed in the only group of Repo-positive glia present in the wild-type brain midline, while their co-localization in each hemisphere remains to be determined. Expression of one copy of UAS-*jing* in *sim*-expressing cells results in loss of the preoral and postoral commissures and aberrant circumesophageal formation. This phenotype resembles the *jing* loss-of-function phenotype and suggests that *jing* function was altered in the *sim*-expressing cells. Furthermore, this functional alteration suggests that *jing* dosage is critical in the cells that pattern the brain axon scaffold. Expression of two copies of UAS-*jing* in *sim*-expressing cells results in the repulsion of circumesophageal connectives from the midline suggesting the activation of axon repellent molecules. Over-expression of *sim* in the brain results in the same phenotype as targeted *jing* expression. It remains to be determined whether *jing* is expressed in Sim-positive cells in the brain. However, these results show that an alteration of normal *jing* dosage in Sim-positive cells in the brain interferes with axonogenesis in a manner similar to that of *sim* over-expression. It is therefore possible that similar molecules are activated and/or repressed by expression of *sim* and *jing* in Sim-positive cells.

It is interesting that in the brain midline, the *jing* enhancer is activated in glia and Cas-expressing neurons as well as additional unidentified neurons. *jing* therefore has a more extensive midline expression than that of *sim* revealing that regulation of *jing* expression in the midline of the brain and VNC is different. In the VNC, *sim* is required for *jing* expression and can ectopically activate the *jing-lacZ* enhancer in *Drosophila* embryos (Sedaghat et al., 2002). Further differences exist in gene regulation between the brain and VNC midline. For example, Repo and Cas are present in the brain midline but are excluded from the VNC midline, while both proteins are found in the VNC neuroectoderm (Cui and Doe, 1992; Mellerick et al., 1992). Given the importance of *jing* function in cellular differentiation it will be important to determine its mechanism(s) of action.

Acknowledgements. We thank Ward Odenwald for anti-Castor antibody, Andrew Travers for anti-Repo antibody, Corey Goodman for anti-Fas II and Steve Crews for *sim*-Gal4 and UAS-*sim*. This work was supported by a Canadian Institutes for Health Research (CIHR) operating grant to M.J.S. and by an Ontario Graduate Scholarship to Y. S.

Chapter Four

Sedaghat et al. (2005). *Drosophila jing* is a target of TRACHEALESS::TANGO heterodimers and and it is involved in the regulation of *breathless FGFR* expression in the trachea.

.

The following outlines the experimental contributions of the authors of this manuscript. Cell culture, transcription assay and RNA extraction for microarray analysis was established, optimized and performed by Y. Sedaghat. All Confocal microscopy, and light microscopy of embryos stained with α-Crb were performed and analyzed by Y. Sedaghat. Preparation of eye samples for scanning electron microscopy, required fly crossing, screening the flies for rough eye phenotype, statistical analysis of rough eye penetrance, light microscopy of rough eye samples; screen, cross and isolation of three transgenic lines (*1.5jing-lacZ, A1-LacZ* and *A3-lacZ*) were performed by Y. Sedaghat. Y. Sedaghat wrote sections of the manuscript related to the work she had performed, under Dr. M. Sonnenfeld's supervision.

N. Barazesh helped with maintaining some of the fly lines, in situ hybridization and α-Crb and α-2A12 antibody staining. T. Morozova helped with antibody staining and in situ hybridization, which were analyzed by Dr. M. Sonnenfeld. Dr. M. Sonnenfeld revised and completed the manuscript.

Drosophila jing is a target of TRACHEALESS::TANGO heterodimers and it is involved in the regulation of _breathless FGFR_ expression in the trachea

Yalda Sedaghat, Nasrin Barazesh, Tatiana Morozova and Margaret Sonnenfeld*

Key Words: jing, Drosophila, trachea, bHLH-PAS, *breathless*, *branchless*, ectodermal gene expression, adherens junction

Abstract

Drosophila tracheal development is dependent on the cooperative activity of TRACHEALESS (TRH) and TANGO (TGO), two members of the basic helix-loop-helix-PAS transcription factor family. TRH and TGO activate the expression of the *breathless* (*btl*) *fibroblast growth factor receptor* (*FGFR*), which is necessary for primary branch outgrowth in the trachea. We previously demonstrated that proper function of the *jing* zinc finger gene product is required for tracheal development and show here that JING is a key cofactor in the initiation of tracheal-specific gene expression. We show that TRH and TGO activate *jing* transcription in S2 cells. We establish the in vivo relevance of this by showing that a 1.5Kb genomic fragment from the *jing* promoter has tracheal-specific enhancer activity in a pattern resembling *btl* expression. JING cooperates with TRH and TGO to activate *btl* expression as shown by the activation of *btl* by *jing* in vivo, synergistic genetic interactions between *jing* and *trh* and reductions in *btl* mRNA expression in homozygous *jing* mutants. Gene chip data, in vivo expression analysis and genetic experiments support a role for *jing* in regulating *btl* expression and that of downstream genes including *Delta* and *DE-Cadherin*. The requirement for *jing* in establishing fusion cell fates is consistent with *btl* pathway regulation in vivo. *jing* expression may be under multiple control mechanisms to control and restrict *btl* expression in the tracheal placodes and primary branches, including auto-inhibition and inhibition by the POU domain transcription factor DRIFTER and the ETS transcription factor AOP/YAN. Together, these results reveal that *jing* is required for the balance between repression and activation that regulates TRH- and TGO-mediated *btl*

transcription and subsequent growth factor levels to control signaling during the primary and secondary branching processes.

Introduction

The formation of the *Drosophila* respiratory organ (trachea) follows well-conserved processes of branching morphogenesis and tubule growth. Some of these processes include cell migration, cell guidance, cytoskeletal rearrangement accompanying cell shape changes, cellular fusion and intracellular lumen formation (Manning and Krasnow, 1993; Samakovlis et al., 1996a, b; Metzger and Krasnow, 1999; Uv et al., 2003; Affolter et al., 2003). The fully formed tracheal network is a composite of seamless tubes that carries air to target tissues and in which gas exchange occurs. Cells in each tracheal placode arise from the ectoderm and become subdivided into distinct domains by the actions of multiple pathways involving Decapentaplegic (DPP) (Vincent et al., 1997), Epidermal growth factor receptor (EGFR) (Llimargas and Casanova, 1997; Wappner et al., 1997; Llimargas and Casanova, 1999), Wingless (WG)/WNT (Chihara and Hayashi, 2000; Llimargas, 2000) and Hedgehog (Glazer and Shilo, 2001).

The *trachealess* (*trh*) and *tango* (*tgo*) bHLH-PAS transcription factors activate a number of target genes encoding molecules essential for the invagination and branching processes in the trachea (Wilk et al., 1996; Isaac and Andrew, 1996; Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997; Zelzer et al., 1997; Zelzer and Shilo, 2000). TRH makes cells competent to EGF signaling and promotes invagination by activating the expression

of *rhomboid* and other unidentified factors (Zelzer and Shilo, 2000; Llimargas and Casanova, 1999; Isaac and Andrew, 1996; Wilk et al., 1996).

The localized expression of the FGF-like molecule BRANCHLESS (BNL) in ectodermal cells adjacent to the tracheal primordium is critical for the primary branching process (Sutherland et al., 1996). In embryos mutant in *bnl* and its receptor *breathless* (*btl*), tracheal cells invaginate but do not branch. TRH::TGO heterodimers are required for *btl* expression in the tracheal placodes and have been shown to bind and activate the *btl* promoter by gel shift analysis and transcription assays (Ohshiro and Saigo, 1997; Jin et al., 2001). Later in development, the expression of *btl* becomes restricted to secondary-branch-forming cells suggesting that there must be some means of negative regulation given that TRH::TGO are continuously and ubiquitously present in the trachea (Ohshiro and Saigo, 1997; Ohshiro et al., 2002; Ward et al., 1998). This role is carried out by the ETS protein ANTERIOR OPEN (AOP)/YAN in the transverse connectives (Ohshiro et al., 2002). Late *btl* expression has also been shown to be regulated by the POU domain transcription factor DRIFTER/VENTRAL VEINLESS (DFR/VVL) (Isaac and Andrew, 1996; Llimargas and Casanova, 1997; Boube et al., 2000). However, since *btl* expression initiates properly in the tracheal placodes of *dfr/vvl* mutant embryos it has been suggested that an additional transcription factor substitutes for the role of DFR/VVL in activating early *btl* expression (Boube et al., 2000).

We previously showed strong genetic interactions between *trh*, *btl* and the *jing* C_2H_2-type zinc finger gene during tracheal development suggesting that these genes are functionally related (Sedaghat et al., 2002). *jing* was originally identified in two genetic screens for regulators of border cell migration in the ovary and for midline cell function

during embryogenesis (Liu and Montell, 2001; Sedaghat et al., 2002). JING protein co-localizes with TRH from the beginnings of tracheal formation (Sedaghat et al., 2002). The JING protein is present in greater amounts in tracheal nuclei in the dorsal branch, visceral branch, lateral trunk anterior/posterior and ganglionic branches and in very low amounts in the dorsal trunk and transverse connectives. The expression pattern therefore suggests a regulatory role for JING throughout tubulogenesis in a pattern similar to that of *btl* (Sedaghat et al., 2002; Ohshiro et al., 2002). *jing* regulates tyrosine kinase signaling through the *epidermal growth factor receptor* (*Egfr*) and *btl* in the tracheal pits and midline glia (Sonnenfeld et al., 2004). However, the exact relationship with TRH::TGO, the mechanism of *jing* function and its downstream targets are not known.

The tracheal system is derived from ten pairs of ectodermal invaginations that each extends six tubular branches, which subsequently branch again to generate terminal and fusion branches (Samakovlis et al., 1996a). The secondary-branch forming cells that were initially equivalent get subdivided into terminal and fusion cells by the NOTCH/DELTA signalling pathway (Ikeya and Hayashi, 1999; Llimargas, 1999). Fusion branches migrate to find branches from adjacent segments with which they fuse to form a seamless tubular network. Specialized cells at the branch tips, known as fusion cells, express the transcription factor *escargot* (*esg*) and connect branches to form a continuous tubular network in a process known as anastomosis (Samakovlis et al., 1996a, 1996b; Tanaka-Matakatsu et al., 1996; Whiteley et al., 1992; Fuse et al., 1994).

The deposition of *D*E-CADHERIN where two fusion partners contact each other is the first molecular event of the fusion process (Tanaka-Matakatsu et al., 1996; Oda et al., 1994; Uemura et al., 1996; Tepass et al., 1996; Nüsslein-Volhard et al., 1984). The

cytoplasmic domain of *D*E-CADHERIN is associated with ARMADILLO and α-CATENIN which are linked to the actin network (Tanaka-Matakatsu et al., 1996; Lee and Kolodziej, 2002). *crumbs* (*crb*) encodes an EGF repeat family transmembrane protein present on the apical surface of tracheal cells facing the lumen and is required for formation of zonula adherens between cells that contain *D*E-CADHERIN (Tepass, 1996; Tepass et al., 1990). CRB is present at the contact interface of fusion cells when the ring of *D*E-CADHERIN is expanding and it is suggested that CRB contributes to apical-basal polarity (Wodarz et al., 1995). Additional genes involved in *D*E-CADHERIN-mediated adhesion include *grainyhead* (*grh*), *discs-large1* (*dlg1*) and *α-catenin*. *grh* is a transcription factor that is controlled by BNL signalling and controls apical membrane growth during the primary branching process (Hemphala et al., 2003).

How the various signalling pathways integrate information to control cellular invagination, migration and adhesion in the *Drosophila* trachea is under intensive investigation (Manning and Krasnow, 1993; Samakovlis et al., 1996a, b; Metzger and Krasnow, 1999; Uv et al., 2003; Affolter et al., 2003). We established that TRH::TGO heterodimers activate *jing* transcription. *jing*1.5-*lacZ* element is strongly activated during the branching process in cells at the tips of the ganglionic, dorsal and lateral branches which are known to express *btl*. Transcription assays and in vivo expression analyses reveal that JING enhances and is required for TRH::TGO-mediated *btl* activation in the tracheal placodes. DFR/VVL and JING both inhibited expression of *jing*1.5-*lacZ* and showed synergy in its inhibition. *jing* affects gene expression in the *D*E-*cadherin* and *bnl/btl* pathways and shows dominant genetic interactions with *D*E-*cadherin* and *bnl* in the trachea. JING function is required in this pathway for fusion cell fates. These results,

together, demonstrate that JING controls *btl* expression levels in combination with TRH::TGO and support previous models proposing that *btl* expression is controlled by a combination of positive and negative regulatory molecules that are spatially and temporally under strict control.

Materials and methods

Cell line and culture

Drosophila Schneider 2 (S2) cells [American Type Culture Collection (ATCC) Manassas, VA USA] were maintained in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% heat-inactivated foetal calf serum, 100 µg/ml penicillin and 100 U/ml streptomycin. Cells were kept at 25°C and transient transfections were carried out when the cells were at a confluency of 60-75%.

Fly strains

The control strain was w^{118}. For directed-expression experiments, transgenic strains P[*GMR-Gal4*] (Hay et al., 1994) and P[*btl-Gal4*] (Shiga et al., 1996) were used. *Gal4* responsive target genes were P[*UAS-tgo*], P[*UAS-trh*] and P[*UAS-jing*] (Sedaghat et al., 2002). The P[5′ *jing*1.5-*lacZ*] strains carry 1.553 Kb of the *jing* promoter and are discussed below. The enhancer trap line, *escargot*^{G66B} (*eSg*^{G66B}), expresses ß–

GALACTOSIDASE in the cytoplasm in a pattern similar to that of *esg* (Whiteley et al., 1992) and was obtained from S. Hayashi (Ikeya and Hayashi, 1999). *D*E-c*adherin* alleles, $shg^{G\text{-}3173}$ *and* shg^{R696}, were provided by U. Tepass (Tepass et al., 1996). The *branchless*^{17074} (bnl^{17074}), *anterior open*^{1} (aop^{1}) and *anterior open*^{03953a} (aop^{03953a}) alleles (Nüsslein-Volhard et al., 1984) were obtained from the *Drosophila* Bloomington stock centre (Indiana). Homozygous mutant embryos were identified using a *TM3* [*Ubx-lacZ*] balancer or *Cyo* [*wg-lacZ*] balancer. Ectopic expression was achieved using a P[*paired-Gal4*] driver.

RNA extraction and microarray analysis

These procedures were carried out according to previous protocols (Sonnenfeld et al., 2004)

Microscopy and eye imaging

For confocal microscopy, antibody staining was visualized using FITC- and rhodamine-conjugated secondary antibodies (Jackson). Fluorescently-labeled embryos were mounted in 4% n-propyl gallate to inhibit photobleaching and analyzed on a Zeiss Axiovert 100 TV confocal microscope. Optical sections of 1 μm were recorded in line average mode and parameters were set by the TRH fluorescence.

The adult fly heads were dissected, washed in PBS and fixed in fixative solution (2% glutaraldehyde in 0.1M phosphate buffer) overnight. After removing the fixative

residues by washing the fixed heads in PBS, they were dried by an ethanol dehydration series. Samples were kept in 100% ethanol. The ommatidial arrangement was analyzed in images generated by scanning electron microscopy by the Bioimaging Centre at Mount Sinai Hospital (Toronto, Canada). At least three images for each sample were analyzed. Pigmentation was analyzed by light microscopy using Nomarski optics on a Zeiss Axioskop microscope. Images were captured on a Nikon DXM1200 digital camera and processed using Adobe Photoshop software.

Antibodies, immunohistochemistry and in situ hybridization

Antibody staining was based on a standard technique as described by Patel (1994). Embryo staging was according to Campos-Ortega and Hartenstein (1985; 1997). To elucidate the effects of *jing* in the formation of tracheal branches, a 1:200 dilution of rat anti-TRH was used (gift of Dr. S. Crews). The enhancer activity of multiple strains carrying P[*jing*1.5-*lacZ*] was detected by staining the embryos for *lacZ* expression with a monoclonal antibody against *E.coli* β-GALACTOSIDASE (1:200, Jackson). Anti-CRUMBS (ĆRB) and 2A12 were used to assess primary and secondary tracheal branching and were obtained from the Developmental Studies Hybridoma Bank. Secondary antibodies were HRP-conjugated goat anti-mouse (Jackson), HRP-conjugated goat anti-rat (Jackson) and HRP-conjugated goat anti-rabbit. Stained embryos were dehydrated through an ethanol series and mounted in methyl salicylate. A Zeiss Axioskop with Nomarski optics was used for light microscopy and images were processed using Adobe Photoshop software.

Whole-mount in situ hybridization was carried out using digoxigenin-labeled riboprobes prepared from *bnl* and *btl* cDNAs according to Janody et al., (2000) (Ohshiro et al., 2002). Embryos were collected from (1) w^{118}, (2) $jing^{3}$, (3) $jing^{3}$/Df(2R)ST1, (4) P[*btl*-Gal4]; P[UAS-*jing*]/+; P[UAS-*jing*] and (5) P[*prd*-Gal4]/P[UAS-*jing*].

Construction of plasmids

The pGL3 basic promoter was a gift from Dr. P. Albert (OGHRI, Ottawa). The pPac3 basic vector, pPac3-*trh*, pPac3-*tgo*, pGL3-*btl* (P[B123]) and pPac3-*βgal* were provided by Dr. Woodgett (University of Toronto) (Ohshiro and Saigo, 1997). The 1.553 Kb-5'*jing* was obtained by PCR using *Drosophila* w^{118} genomic DNA as template, sequenced and cloned into the *Kpn* 1 and *Xho*1 sites of pGL3 for transcription assays and into the *Kpn* 1 and *Nhe* 1 sites of the pCaSper vector for in vivo expression. Deletion constructs of the 1.553 Kb-5' *jing* regulatory region including Δ*jing*-mut5 which has the TACAAT site removed, Δ*jing*-B3 missing the first CME and TACAAT, Δ*jing*-A1 missing the second and third CME and Δ*jing*-A3 lacking the first CME, were made by site-directed mutagenesis (Stratagene). These constructs were cloned into the pGL3 vector, which has the luciferase reporter gene for transcription assays, and into pCaSper. The pCaSper constructs were injected into embryos and incorporated into the genome by P-element mediated transformation (Spradling and Rubin, 1982) by Genetic Services (Cambridge, MA). At least three independent transgenic lines were monitored for *lacZ* expression of each construct. To express *jing* in S2 cells, the coding sequence previously

subcloned into pUAST was excised and subcloned into the pPac3 vector (Sedaghat et al., 2002).

Transfection and luciferase assays

Schneider 2 (S2) cells were transiently transfected using the calcium phosphate method. A combination of pGL3 and pPac3 (3 μg) vectors containing the sequence of interest in the presence of the pPac3-βGAL (2 μg) was added to 1 ml mixture of 1:1 ratio of 0.25 M $CaCl_2$ and Na_2HPO_4, HEPES buffer (280 mM NaCl, 10 mM KCl, 12 mM Dextrose and 50 mM HEPES). S2 cells at a confluency of 60-70% in 100X20 mm dishes were transfected with this mixture. Transfection was stopped 7 hours later by washing the cells with 1X HBBS buffer (10X HBBS: 1.18 M NaCl, 46 mM KCl, 100 mM Dextrose and 200 mM HEPES), fresh media was added to the cells and they were incubated for 40 hours at 25°C. Transfected cells were harvested after being washed with 1X PBS in Reporter Lysis Buffer (Promega), frozen at -80°C for one hour and centrifuged at 13,000 rpm for 2 min. The supernatant was used to measure the luciferase (Promega) and β-GAL (MUG substrate, Sigma) activities. Luciferase activities normalized to β-GAL activities were calculated by determining the Luciferase/β-GAL ratios and averaging the values from at least four independent triplicate experiments, from which means and standard deviations were calculated.

Results

jing interacts synergistically with *trh*

Over-expression of *jing* in the *Drosophila* eye results in a disruption of ommatidial formation leading to a rough eye appearance (Sonnenfeld et al., 2004). This suggests that ommatidial development is sensitive to the dosage of *jing*. Therefore, to obtain evidence for a functional interaction between *jing* and *trachealess* (*trh*) (Isaac and Andrew, 1996; Wilk et al., 1996), we first looked for genetic interactions in the eye using the *GAL4/UAS* binary system (Brand and Perrimon, 1993). The *glass* multiple promoter fused to *GAL4* (P[*GMR-GAL4*]) was used as a driver to specifically express these genes in developing eye imaginal discs (Hay et al., 1994). Adult eyes were then analyzed by scanning and light microscopy for ommatidial, bristle and pigmentation development.

Ectopic *trh* or *jing* expression was not sufficient to disturb eye development (Fig. 1G) (Sonnenfeld et al. 2004). However, co-expression of *trh* and *tgo*, as a positive control, resulted in alterations in the organization of ommatidia and bristles and in reduced pigmentation, which were manifested by rough eyes with a 52% (+/-4.4) penetrance (Fig. 1B, 1E, 1G). In comparison, co-expression of *jing* and *trh* resulted in a rough eye with a penetrance of 61% (+/- 1.5)(Fig. 1C, 1G). There also were drastic reductions in the eye pigmentation of flies co-expressing *jing* and *trh* (Fig.1F) suggesting that the pigment cells were affected in a similar manner as *trh* and *tgo* co-expression. These results show that ectopic expression of *jing* and *trh* synergistically alters eye

development suggesting that they functionally interact and may influence gene expression together.

TRH and TGO activate *jing* transcription in S2 cells

Previous analysis of genomic sequence in the *jing* regulatory region revealed the presence of three bHLH-PAS consensus sites (CME, ACGTG) (Fig. 2, in red) (Sedaghat et al., 2002). To determine whether there were additional regulatory sequences responsible for *jing* expression further examination was made of the cis regulatory sequences upstream of the *jing* open reading frame. Sequence analysis of a 1.5Kb fragment revealed the presence of a SOX consensus site (TACAAT) (Fig. 2, underlined) (Sedaghat et al., 2002; Wharton et al., 1994; Ma et al., 2000).

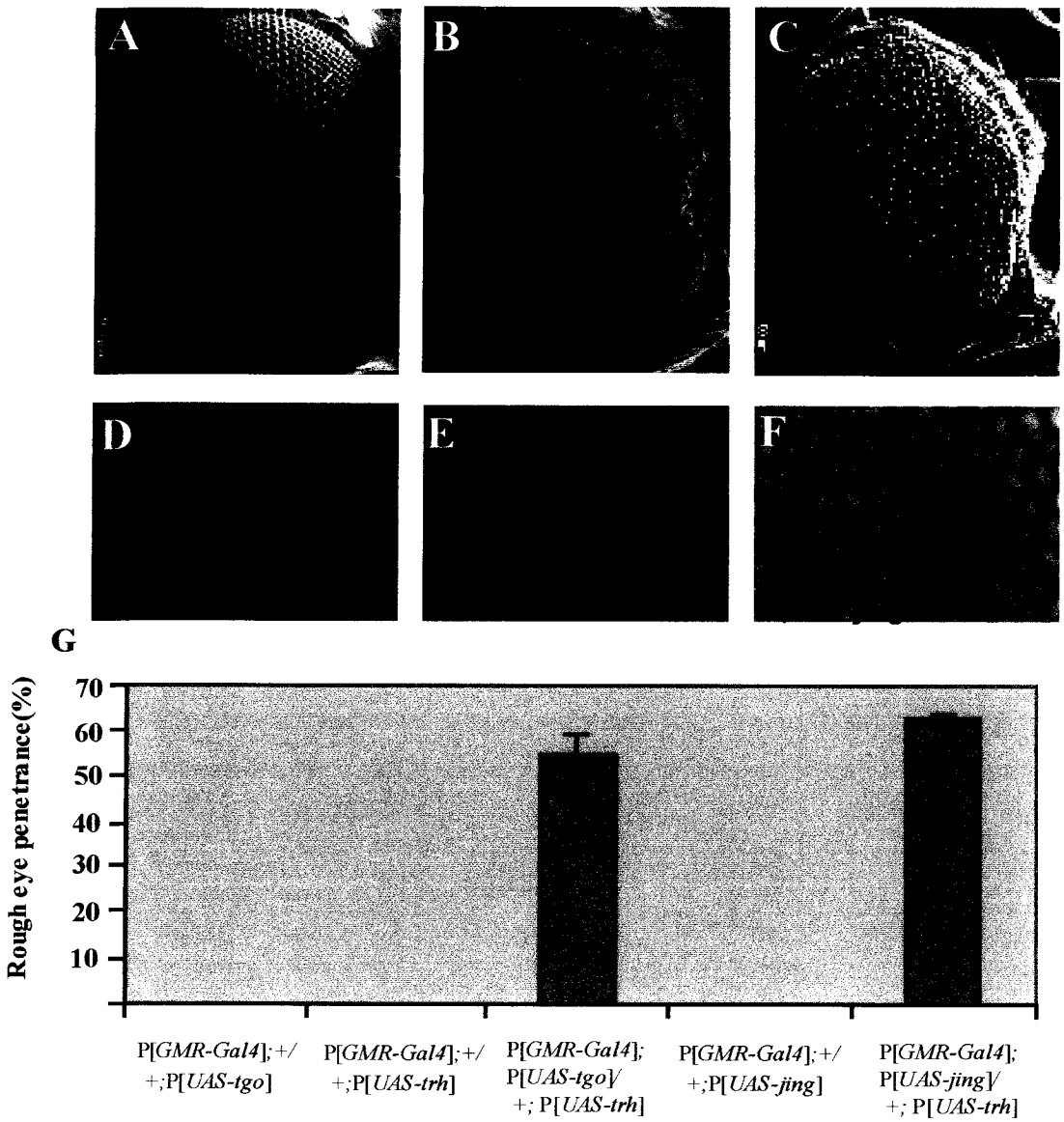


Fig 1. Genetic interactions between *jing* and *trh* detected via gain-of-function assays. Scanning electron micrographs (A-C) and light level images (D-F) of the following genotypes are shown: (A, D) P[*GMR-GAL4*]/+; (B, E) P[*GMR-GAL4*]; P[*UAS-tgo*]/+; P[*UAS-trh*]; (C, F) P[*GMR-GAL4*]; P[*UAS-jing*]/+; P[*UAS-trh*]. Rough eyes consisted of disruptions in ommatidial arrangment and reductions in pigmentation. (G) Quantification of rough eyes.

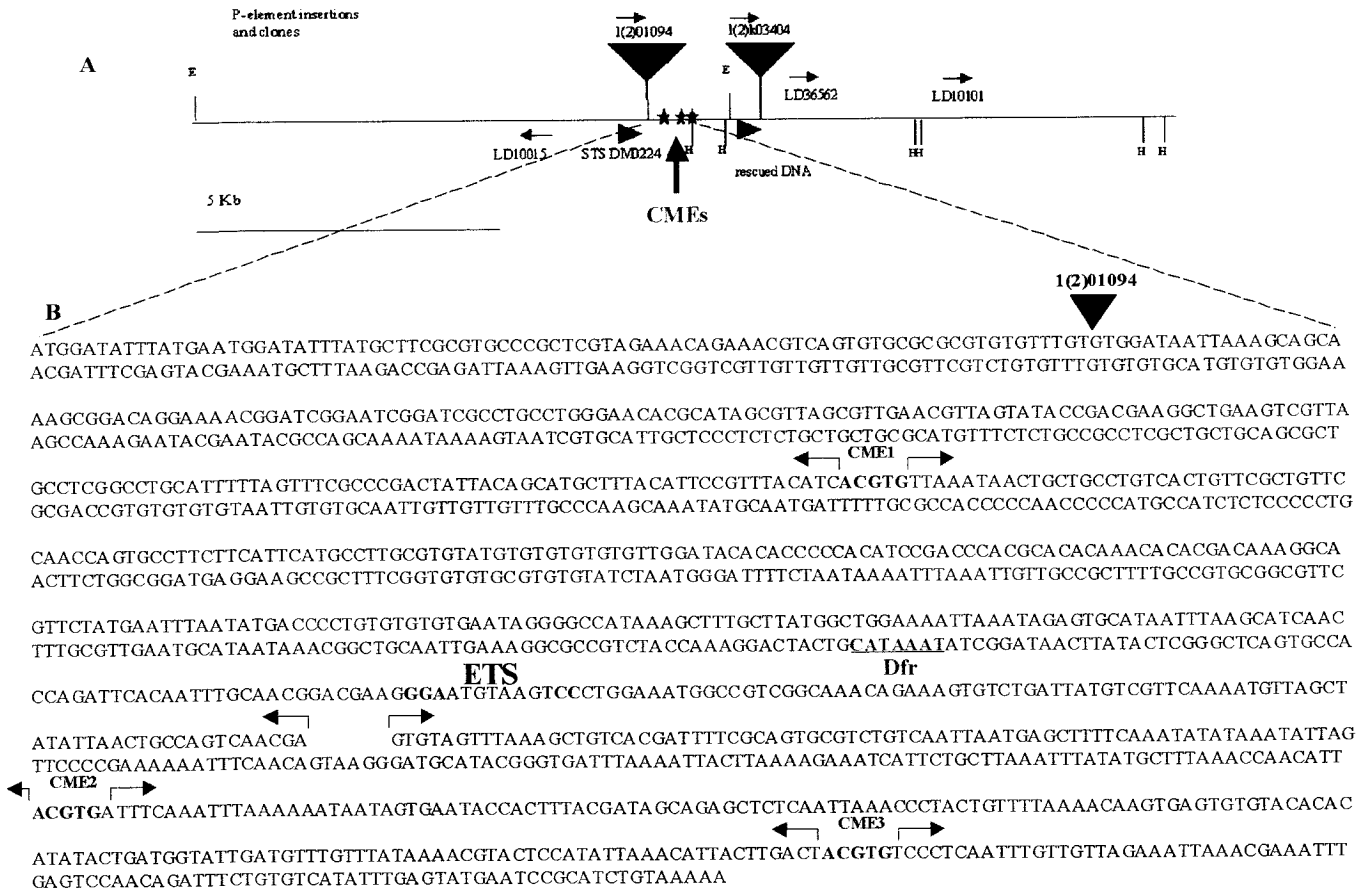


Fig 2. Nucleotide sequence analysis of the *jing* cis-regulatory region. (A) Schematic of *jing* P-element enhancer trap insertions (adapted from Sedaghat et al. 2002). (B) 1.553Kb sequence from the 5'*jing* regulatory region includes three bHLH-PAS consensus sites (CME, ACGTG in red), a SOX consensus binding site (TACAAT), a DRIFTER/VENTRAL VEINLESS (Dfr) binding site (CATAAAT) and an ETS transcription factor binding site (GGA TCC). The filled triangle in the sequence represents the P-*lacW* insertion site in the $jing^{01094}$ fly strain. The blue arrows show the deleted areas.

In addition, we identified consensus binding sites for the POU domain transcription factor DRIFTER(DFR)/VENTRAL VEINLESS (VVL) (CATAAT) and ETS proteins (inverted repeat GGA TCC) in the *jing* regulatory sequence (Fig. 2) (Anderson et al., 1996; de Celis et al., 1995; Ohshiro et al., 2002).

To determine whether TRH and TGO were able to regulate transcription of *jing* through an interaction with the bHLH-PAS CME sites, we monitored the transcriptional activity of a *jing* promoter fusion with the luciferase gene (5'*jing*-1.5-*luc*) in tissue culture cells. Co-expression of *trh* and *tgo* in S2 cells resulted in a synergistic activation of the *jing* regulatory sequence. The effect of TRH::TGO on the expression of 5'*jing*-1.5-*luc* was 17 fold higher than the activity in the absence of TRH and TGO or in the presence of either TRH or TGO alone (Fig. 3; Fig. 6).

To determine whether the CMEs and SOX consensus sequence (TACAAT motif) in the 5'*jing*-1.5 promoter were required for transcriptional regulation of *jing*, four different deletion constructs were made using site-directed mutagenesis (Fig. 3). Deleting the SOX consensus sequence (Δ*jing*-mut5) did not significantly alter TGO::TRH-mediated activation of *jing*. In contrast, removing CME1 (Δ*jing*-A3; Δ*jing*-B3) or CME2 and CME3 (Δ*jing*-A1) abolished *jing* reporter expression (Fig. 3). Therefore, the three bHLH-PAS consensus CME sites are required for TGO::TRH heterodimers to regulate the transcription of *jing* in insect cells.

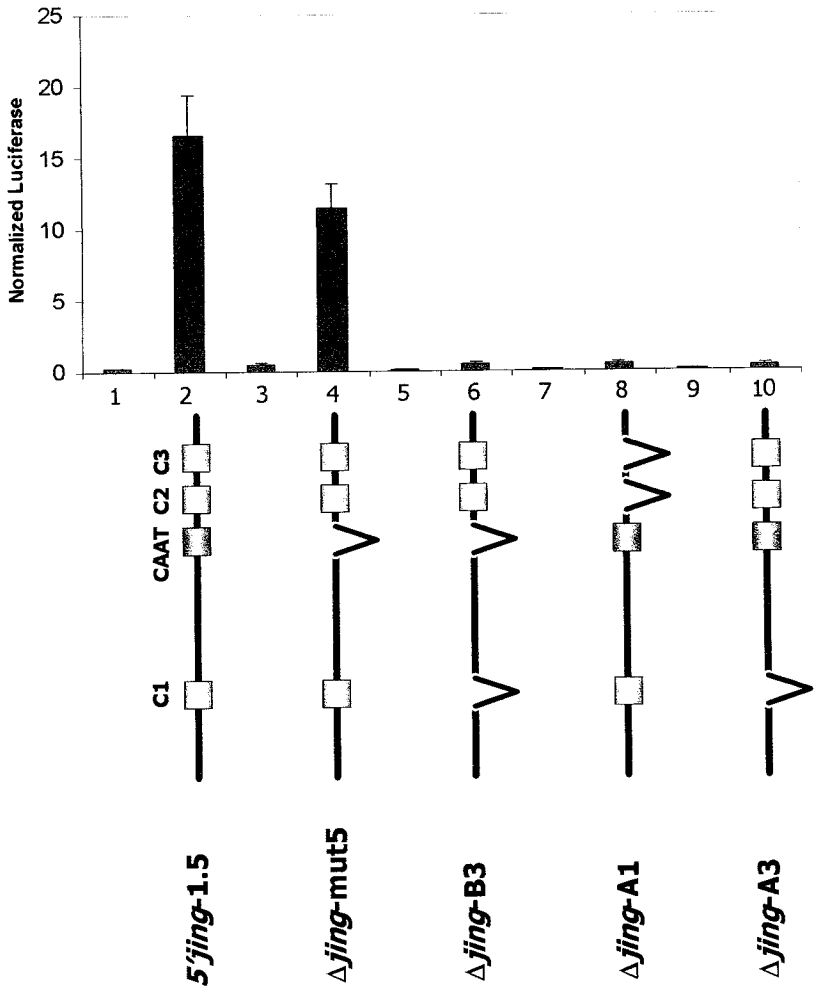


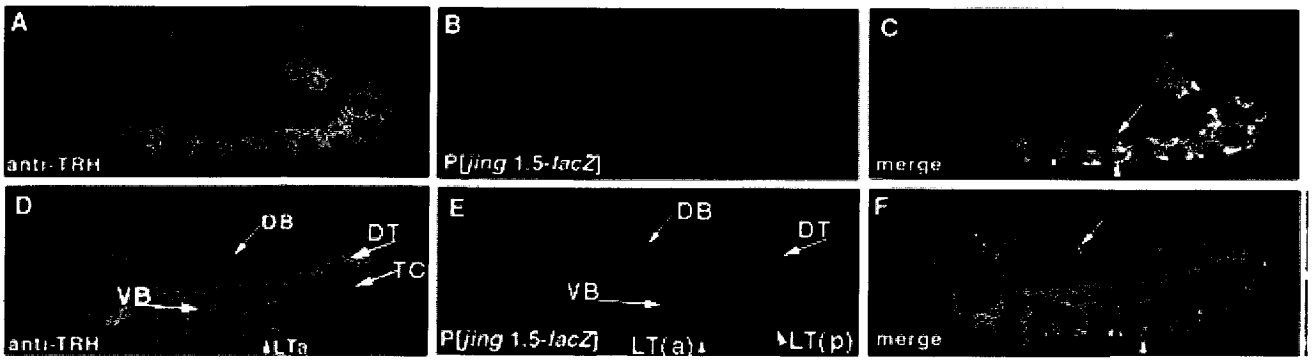
Fig 3. Activation of the *jing* promoter by TRH::TGO. Schematic diagram of different *jing* promoter constructs next to reporter gene assays of 5'*jing*1.5-*luc* promoter luciferase activity. Various *jing* promoter constructs were co-transfected with *trh* and *tgo* into S2 cells with a constitutively expressed control construct (ß-GALACTOSIDASE reporter) for normalization. Gene expression was driven by the *Drosophila Act5C* promoter. The bars show the average normalized luciferase activity (n≥3) and standard deviation. (1) 5'*jing*1.5-*luc*; (2) 5'*jing*1.5-*luc* + TGO + TRH; (3) 5'*jing*-mut5-*luc*; (4) 5'*jing*-mut5-*luc* + TGO + TRH; (5) 5'*jing*-B3-*luc*; (6) 5'*jing*-B3-*luc* + TGO + TRH; (7) 5'*jing*A1-*luc*; (8) 5'*jing*A1-*luc* + TGO + TRH; (9) 5'*jing*A3-*luc*; (10) 5'*jing*A3-*luc* + TGO + TRH. C1:CME1; C2: CME2; C3: CME3; CAAT: SOX site.

The *jing* promoter is activated at the tips of primary tracheal branches

To confirm that the 1.5Kb sequence of the *jing* gene has tracheal-specific enhancer activity and to evaluate the potential contribution of tracheal regulatory genes to this activity, we generated transgenic flies that carried a *jing1.5-lacZ* reporter. Embryos from multiple transgenic lines were stained with monoclonal anti-ß-GAL to identify the pattern of activation during embryogenesis. *jing1.5-lacZ* was activated during stage 12 in the trachea as determined by double labelling with anti-ß-GAL and anti-TRH and examination by confocal microscopy (Fig. 4B, C). The *jing* reporter was most strongly activated in cells at the tip of the primary branches where MAPK activity is present (Fig. 4C, arrow, arrowhead) (Gabay et al., 1997). During stage 13, the reporter was most strongly activated in secondary branch forming cells in the dorsal and ganglionic branches (DB, GB) and in the lateral trunk anterior (LTa) and lateral trunk posterior (LTp) (Fig. 4E, 4F-F"). Expression in the dorsal trunk (DT), visceral branch (VB) and transverse connectives (TC) was very weak to non-existent (Fig. 4E) compared with TRH localization in these branches (Fig. 4D).

To determine the in vivo significance of the CMEs in the *jing* regulatory region, transgenic flies were generated that carried the Δ*jing*-A1 and Δ*jing*-A3 deletion constructs (see Fig. 3). Neither enhancer construct was activated in the trachea establishing the importance of the CMEs for *jing* activation in vivo (Fig. 4G). These observations indicate that the 1.5Kb sequence of *jing* has tracheal system-specific enhancer activity and that the integrity of the bHLH-PAS binding sites is essential for this activity.

We next wanted to determine the in vivo significance of the ETS binding site in the *jing* 1.5Kb regulatory sequence. Previous studies showed that the ETS proteins AOP/YAN and POINTED (PNT) compete for binding with each other to the inverted repeat sequence, GGA TCC, and result in the repression or activation of the *btl* promoter, respectively (Ohshiro et al., 2002). In wild-type stage 12 embryos, the *jing*1.5-*lacZ* reporter was not activated in the CNS midline but in similarly staged embryos homozygous mutant for *aop/yan*, *jing*1.5- *lacZ* was activated in the CNS midline (Fig. 5A, 5B, arrow). In wild-type stage 14 embryos, *jing*1.5-*lacZ* reporter expression was restricted to the dorsal branch and lateral trunk cells in the trachea (Fig. 5C, arrows). Therefore, TRH::TGO are not sufficient for activation of *jing*1.5-*lacZ* in the dorsal trunk, visceral branch and transverse connectives since there are no additional CME binding sites in sequences 5' or 3' to the *jing* open reading frame (data not shown). However, *jing*1.5-*lacZ* was activated in the visceral branch (Fig. 5D, arrow) and dorsal trunk (Fig. 5D, arrowhead) of embryos homozygous mutant for two different *aop/yan* alleles (Fig. 5 and data not shown). Given the endogenous expression pattern of *jing* in the CNS midline as well as in the tracheal visceral branch and dorsal trunk (Sedaghat et al., 2002), and the absence of that in embryos carrying *jing*1.5-*lacZ*, these results suggest that additional factors are required to overcome *jing* repression in these regions during wild-type development. The loss in AOP-mediated repression suggests that there may then be sufficient levels of MAPK-activated PNT to induce *jing*1.5-*lacZ* expression.



G

	trachea	midline
5' *jing*-1.5	+	-
Δ*jing*-A1	-	-
Δ*jing*-A3	-	-

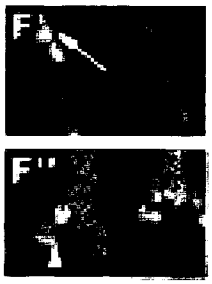


Fig. 4. Expression of the *jing*1.5-*lacZ* reporter in *Drosophila* embryos. Analysis of *lacZ* activation in embryos carrying *jing*1.5-*lacZ*. Confocal microscopic images of embryos double-labeled with anti-ß-GAL to identify *jing* promoter activation and anti-TRH to identify tracheal cells (A-F''). (A-C) *jing*1.5-*lacZ* is activated in stage 12/3 embryos at the tips of the primary branches (C, arrow and arrowhead). (D-F'') During stage 13, *jing*1.5-*lacZ* is expressed at the leading edge of cells in the dorsal branch (DB) (E, arrow) and lateral trunk anterior (LTa) (E, F, F'', arrowheads). (F' and F'') Close-up view of the DB (F') and LTa (F'') from the embryo in F. (G) Analysis of *jing* 1.5-*lacZ* deletions missing the CME bHLH-PAS binding sites. Absence of *lacZ* expression indicates a requirement of the CMEs in the *jing* promoter for tracheal activation. DT, dorsal trunk; VB, visceral branch; LTa, lateral trunk anterior.

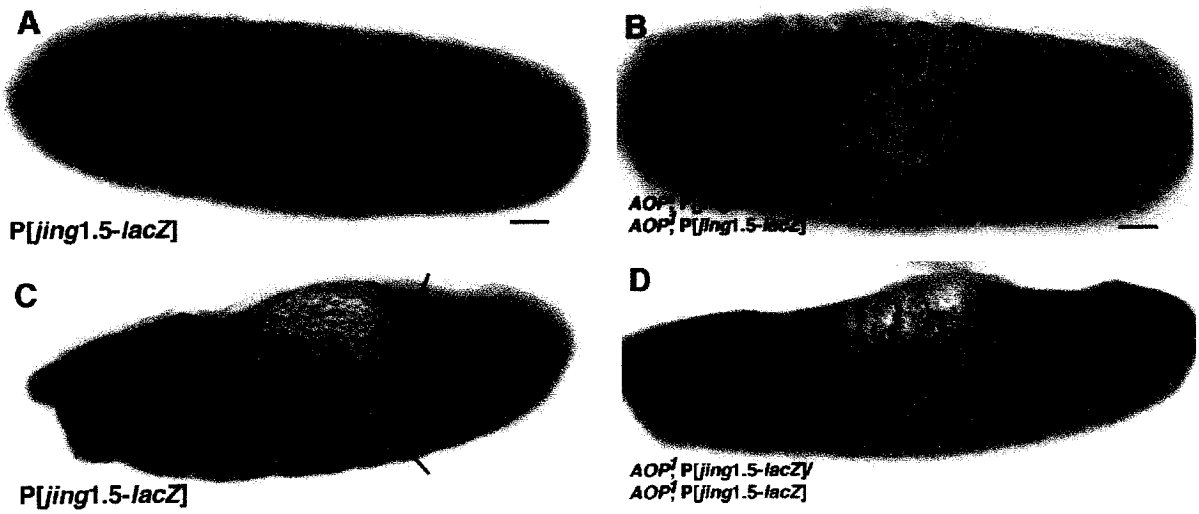


Fig 5. AOP/YAN represses *jing*1.5-*lacZ* in the trachea and CNS midline. (A) During stage 12, *jing*1.5-*lacZ* expression is not detected in CNS midline cells. (B) In stage 12 embryos homozygous for AOP^{1}, *jing*1.5-*lacZ* expression is activated in CNS midline cells (arrow). (C) In stage 14 embryos, *jing*1.5-*lacZ* expression is restricted to the dorsal and lateral branches (arrows). (D) In stage 14 embryos homozygous for AOP^{1}, *jing*1.5-*lacZ* expression is now activated in the visceral branch (arrow) and dorsal trunk (arrowhead). Scale bars: 50 μm.

JING affects on *btl* expression in S2 cells and in vivo

To determine whether JING could activate *btl* expression, the transcriptional activity of *btl* was monitored in S2 cells using a previously characterized *btl* P[B123]-*luc* reporter (Ohshiro and Saigo, 1997; Jin et al., 2001). TRH, TGO or JING were unable to independently activate the *btl* reporter while TRH and TGO activated it by 27-fold, consistent with previous results (Jin et al., 2001). However, addition of JING with TRH and TGO consistently increased the expression of the *btl* reporter to 38-fold above control levels showing that *jing* acts cooperatively. Surprisingly, JING inhibited transcription of the 5'*jing*-1.5-*luc* promoter by 15 fold in the presence of TRH and TGO, (Fig 6). Deletion of the SOX consensus site (TACAAT motif; Δ*jing*-mut5) reduced *jing* auto-inhibition suggesting that this may be a binding site for JING.

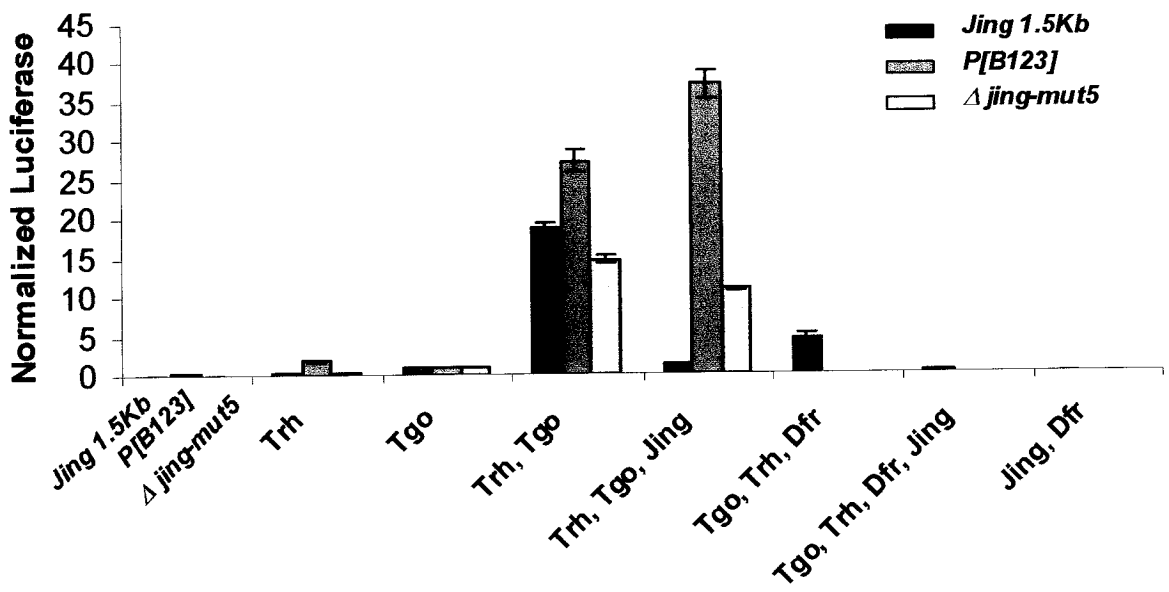


Fig. 6. JING increases TRH::TGO-mediated *btl* expression and inhibits transcription of the *jing* promoter in cultured *Drosophila* S2 cells. S2 cells were co-transfected with the *jing*1.5Kb-*luc* reporter, the *btl* promoter (P[B123]) or the *jing*1.5Kb promoter missing the SOX consensus TACAAT site (mut5) and with various combinations of expression plasmids. Transfection efficiencies were normalized using a β-GAL expression plasmid. Addition of JING to a mixture of TRH::TGO increased the activation of the *btl* P[B123] reporter ($P<0.05$) but inhibited *jing* 1.5Kb-*luc*. JING auto-inhibition was suppressed by deletion of the TACAAT site (mut5). The POU domain transcription factor, DFR/VVL, inhibited TGO::TRH-mediated activation of *jing*1.5Kb-*luc* and *btl*. JING was unable to increase TRH::TGO-mediated activation of the *btl* promoter in the presence of DFR/VVL.

To determine whether *jing* was required for *btl* expression, homozygous *jing*^{3} embryos were hybridized with a digoxigenin-labelled *btl* RNA probe (Fig. 7B, 7D, 7F; data not shown). In wild-type stage 10 embryos, *btl* expression was strong in the tracheal pits (arrow) and CNS midline (arrowhead) (Fig. 7A). However, in *jing* stage 10 and 13 homozygous mutant embryos, *btl* expression was significantly reduced but not completely absent in the CNS midline (Fig. 7B, arrowhead) and trachea (Fig. 7B, 7D and 7F, arrows). *btl* expression was sometimes higher in the last tracheal metamere than that in other metameres in homozygous *jing* mutant embryos (Fig. 7 F, arrowhead).

To determine whether *jing* activates *btl* expression in vivo, *jing* was over-expressed in the trachea using a *btl*-Gal4 driver (Shiga et al., 1996). *btl* expression was expanded around the tracheal pits during stage 10 and 12 in embryos over-expressing *jing* (Fig. 7G and 7I) and compared with wild-type (Fig. 7E and 7H). These results were not observed in embryos over-expressing *trh* in the trachea and *jing* was not able to ectopically induce *btl* expression using a *paired* (*prd*)-Gal4 driver (n=300 embryos) (data not shown). We conclude that *jing* is required for maximal *btl* expression in developing tracheal cells and that *jing* expression is maintained by JING itself, DFR and AOP/YAN.

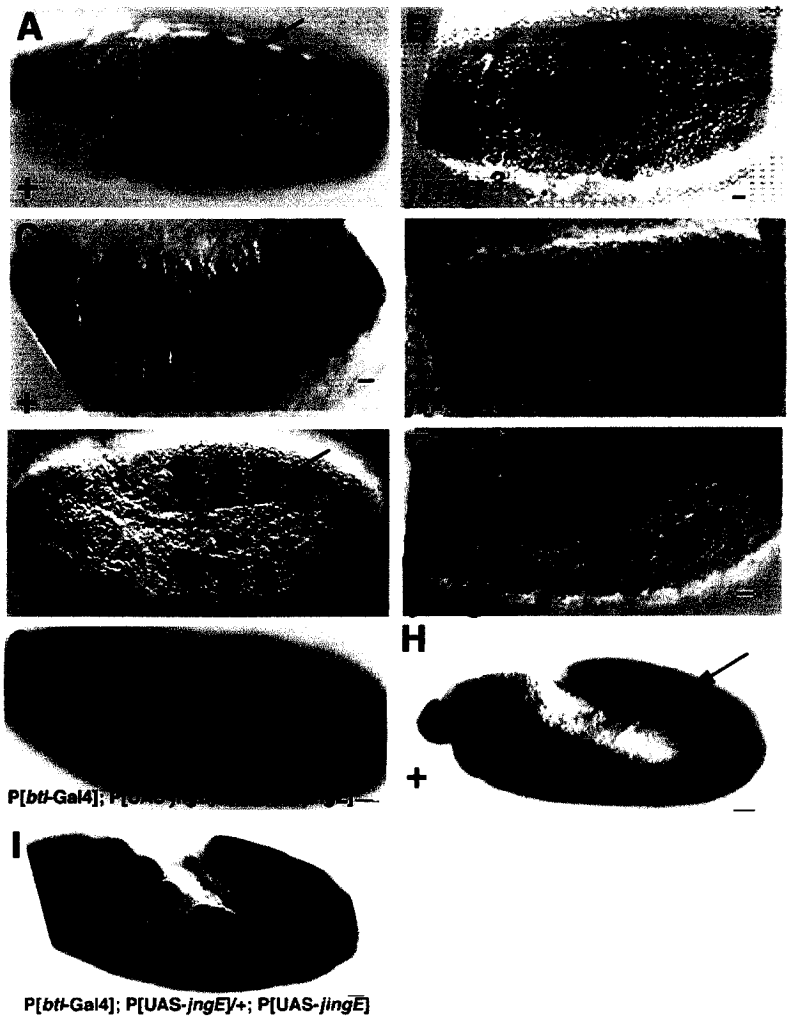


Fig. 7. JING regulates *btl* expression in vivo. Whole-mount wild-type (A, C, E, H) and homozygous *jing*^{3} mutant embryos (B, D, F) or embryos over-expressing *jing* (G, I), were hybridized with a *btl* digoxigenin-labelled RNA probe. Embryos are shown with anterior to the left and in ventral views (A, B) and sagittal views (C- I). *jing* is required for wild-type levels of *btl* expression during stage 10 (B, F) and stage 14 (D). Arrowheads identify the CNS midline and arrows show tracheal cells. Note the relatively stronger *btl* expression in the last tracheal metamere (F, arrowhead). (G, I) Over-expression of *jing* specifically in the trachea as driven by P[*btl-Gal4*]. (G) During stage 10, *btl* expression is expanded around the tracheal pits in embryos over-expressing *jing* (G) as compared to wild-type (E). (H) During stage 12, the beginnings of *btl* expression in tracheal branches can be seen in wild-type embryos (H, arrow). However, in stage 12 embryos over-expressing *jing*, an increase in *btl* expression occurs around the tracheal pits in the absence of primary branching (I, arrow). Scale bars: 50 μm.

jing dominantly interacts with *bnl* and regulates *bnl* pathway gene expression

To determine whether *jing* influences tracheal gene transcription, we analyzed the mRNA levels of genes controlling primary branching, lumen growth and fusion. RNA was prepared from embryos homozygous mutant for *jing* and from those ubiquitously over-expressing *jing* specifically in the trachea as driven by P[*btl-Gal4*] and using the *Gal4/UAS* targeted gene expression system (Brand and Perrimon, 1993; Shiga et al., 1996; Sonnenfeld et al., 2004).

As shown by microarray analysis, the levels of *Delta* (*Dl*) mRNA were reduced by more than 50% in *jing* homozygous mutant embryos compared to the levels of control w^{118} and other tracheal-expressed genes such as *crumbs* (*crb*) (Tepass et al., 1990) and *grainyhead* (*grh*) (Fig. 8A) (Hemphala et al., 2003). When *jing* was over-expressed in the CNS midline and trachea, statistically significant 2-fold increases in *Dl* mRNA levels were observed. Since the BNL-BTL pathway normally stimulates *Dl* expression at the tip of the primary branches (Ikeya and Hayashi, 1999) it is consistent that *Dl* mRNA levels were reduced in *jing* mutant embryos and possible that it is due to the reduction in *bnl* expression.

BNL is a secreted fibroblast growth factor (FGF)-like molecule and is the main inducer of primary branch growth, (Sutherland et al., 1996). During stage 10/11, *bnl* mRNA is expressed in close proximity to the tracheal placodes in wild-type embryos (Fig. 8B) but was reduced in stage 11 embryos homozygous mutant for *jing* (Fig. 8C). In addition, in embryos heterozygous for both *jing* and *bnl* mutations, tracheal branching was reduced in the visceral (Fig. 8E, arrowhead), lateral anterior and posterior (Fig. 8E,

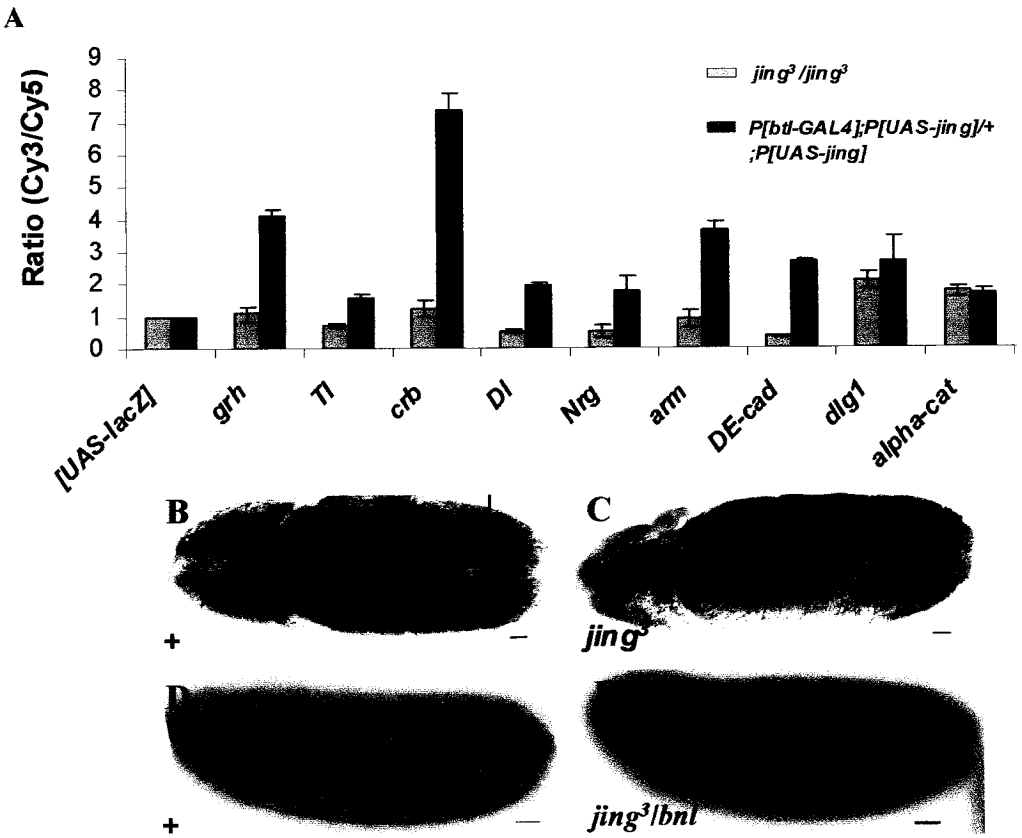


Fig. 8. *jing* dominantly interacts with *bnl* and regulates *bnl/btl* pathway gene expression. (A) Microarray analysis of total RNA extracted from embryos homozygous mutant for *jing*^{3} (light blue bars) and over-expressing *jing* (dark blue bars). Gene expression was driven in CNS midline glia and tracheal cells using P[*btl-Gal4*]. Ratios of experimental (Cy3) to control (Cy5) mRNA fluorescent intensities are shown. Alterations in the expression of *Delta* (*Dl*) and *D*E-*cadherin* (*cad*) were noted in response to both *jing* loss-of-function and gain-of-function (GOF). Large increases in the expression of *crumbs* (*crb*), *armadillo* (*arm*) and *grainyhead* (*grh*) resulted from *jing* GOF. (B and C) in situ hybridization of whole-mount stage 11 embryos with a *bnl* riboprobe. (B) Dorsal surface of a wild-type embryos showing *bnl* expression (arrow). (C) Reductions in *bnl* expression are observed in *jing*^{3}/*jing*^{3} embryos compared with wild-type (B). (D-G) Sagittal (D, E) views of stage 15 embryos stained with monoclonal 2A12. Branching is reduced in embryos double heterozygous for *jing* and *bnl* (E, arrow) compared with wild type (D). Visceral branches do not extend to adjacent metameres (E, arrowhead). Scale bars: 50μm

arrow) as well as the dorsal branches, as determined by staining with the lumen marker, monoclonal 2A12 (Fig. 8E). Together, these data establish that *jing* is required for proper *bnl* pathway gene expression and function.

jing is required for primary branching

Given the regulation of *bnl* and *btl* mRNA expression by *jing*, we next wanted to determine whether *jing* function was needed for the primary branching process. The first branches that form in wild-type embryos in each tracheal epithelial placode are six primary branches known as the dorsal trunk anterior (Dta) and posterior (Dtp), lateral trunk anterior (LTa) and posterior (LTp), dorsal branch (DB), ganglionic branch (GB) and visceral branch (VB). These branches can be identified by anti-TRH staining of wild-type stage 13 embryos (Fig. 9A). In contrast, after *jing* over-expression specifically in the trachea, the DB, LTa and LTp branches did not form (Fig. 9B). This is similar to the tracheal over-expression phenotypes of *bnl* and *btl* (Ikeya and Hayashi, 1999). The lumen of wild-type tracheal branches can be viewed during embryonic stage 11 using an antibody to CRUMBS (CRB), an EGF repeat family transmembrane protein (Fig. 9C, E) (Tepass et al., 1990). In homozygous and hemizygous *jing* mutant embryos, CRB protein was reduced by stage 11 and did not appear in branches by stage 13 when branching becomes more extensive in wild-type embryos (Fig. 9D, F). Together with previous results, these data demonstrate that *jing* function is required for primary branching in the BNL/BTL pathway.

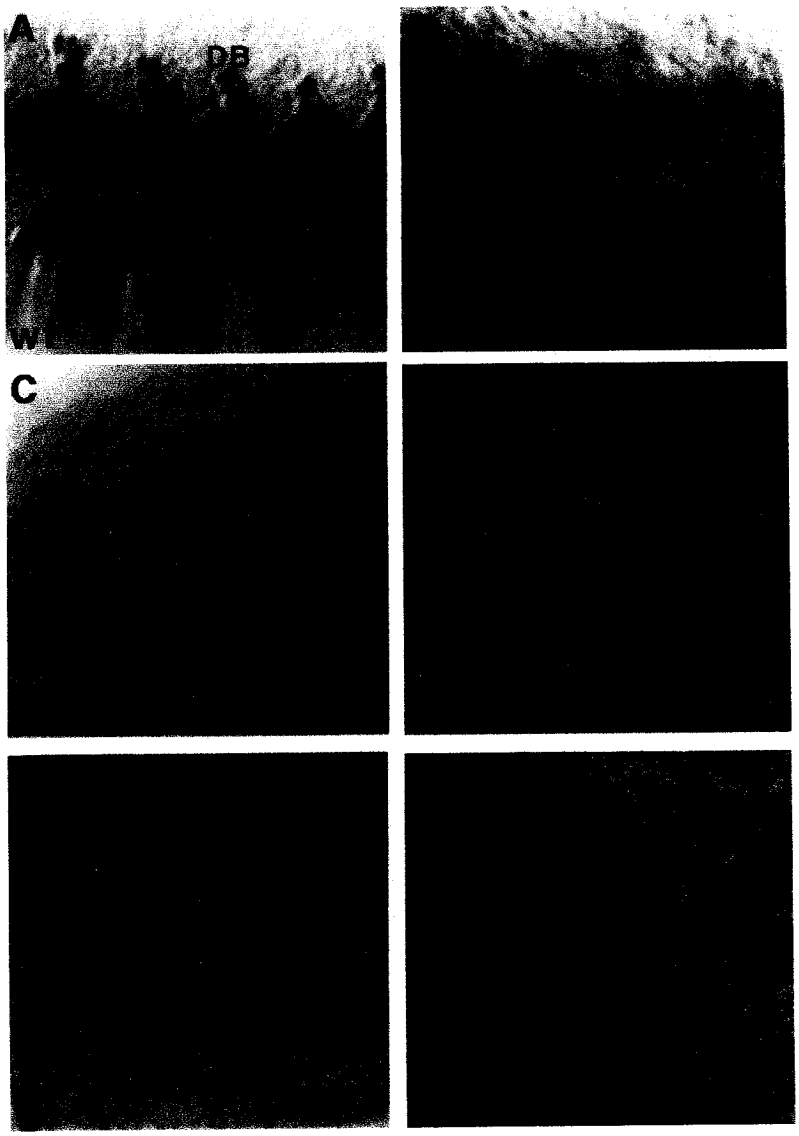


Fig. 9. *jing* is required for primary branching. (A) Tracheal branching in wild-type stage 14 embryos stained with anti-TRH. (B) *jing* over-expression specifically in the trachea interferes with branching of the dorsal branch, dorsal trunk and lateral trunks (LTa and LTp). (C-F) Wild-type and various *jing* mutants were stained with antibodies recognizing CRUMBS (CRB). (C, E) Branching is visible during stage 12 and becomes more extensive during stage 13 in wild-type embryos (arrows). (D, F) Branching is not apparent in homozygous or hemizygous *jing* embryos. Small CRB protein patches are apparent (D, arrow). DT, dorsal trunk; TC, transverse connective; LTa, Lateral trunk anterior; LTp, Lateral trunk posterior. Scale bars: 50μm.

***jing* is required for DE-cadherin pathway gene expression and anastomosis in the dorsal tracheal branch**

In the trachea, two cells at the tips of each branch, one terminal and one fusion cell, form branches consisting of cellular processes that move away from the primary branch. These leading cells are attracted to BNL and produce cytoplasmic protrusions to generate type-I tubes (Uv et al., 2003). Fusion cells undergo branch fusion to connect primary branches and form a continuous tubular network between adjacent metameres (Samakovlis et al., 1996b). The cell adhesion molecule *D*E-CADHERIN is needed for initial fusion cell contact and interacts with ARMADILLO (ARM)(β-CATENIN) and D-α-CATENIN to connect with actin filaments and link to the plasma membrane. (Oda et al., 1994; Uemura et al., 1996; Tepass et al., 1996; Tanaka-Matakatsu et al., 1996). A continuous tubular network is established at the *D*E-CADHERIN ring in a process known as anastomosis which is a good indicator of fusion cell differentiation (Tanaka-Matakatsu et al., 1996).

There were reductions in *DE-cadherin* (*DE-cad)* expression after *jing* loss-of-function and a two-fold increase after tracheal over-expression of *jing* as detected by microarray analysis (Fig.8A). The expression of other genes belonging to the adherens junction complex was also elevated in response to *jing* gain-of-function including *arm* and *discs large* (*dlgl*). The most significant changes after tracheal over-expression were 4-fold and 8-fold increases in the mRNA levels of *grainyhead* (*grh*) and *crumbs* (*crb*), respectively, compared with control levels (Fig. 8A). Therefore, *jing* controls the

expression of genes involved in primary branching, lumen growth and fusion in the developing trachea.

To first assess the state of fusion cell differentiation, we analyzed tubule formation in the dorsal branch of the trachea. In wild-type embryos, anastomosis can be seen at the dorsal midline by stage 16 as visualized with the monoclonal antibody 2A12 and by contact between tubules of contralateral metameres (Fig. 10A, arrowhead) (Samakovlis et al., 1996b). In contrast, in *jing*^{3} hemizygous embryos, anastomosis did not occur due to truncation of the dorsal branches (Fig. 10B, arrow). On average, 99% of the dorsal branches failed to turn at the dorsal midline or fuse with the contralateral branch (n=120 segments) whereas in control embryos this phenotype was not observed (w^{118}, n=90 segments). After over-expression of *jing* in the trachea, similar stalled dorsal branch phenotypes were observed (Fig. 10C, arrowhead). 60% of dorsal branches stalled at the dorsal midline and instead of turning, the branches truncated as circular structures (Fig. 10C, arrowhead) (n=50). Anastomosis of fusion cells in the dorsal branch did not occur in *jing* and *D*E-*cad* (shg^{R696} and shg^{G-317}) double heterozygotes (n= 150 and n=90 segments, respectively) as determined by staining with monoclonal 2A12 (Fig. 10D) and compared with that in wild-type embryos (Fig. 10A, arrowhead). These results suggest that *jing* is required for the fusion of tracheal tubules.

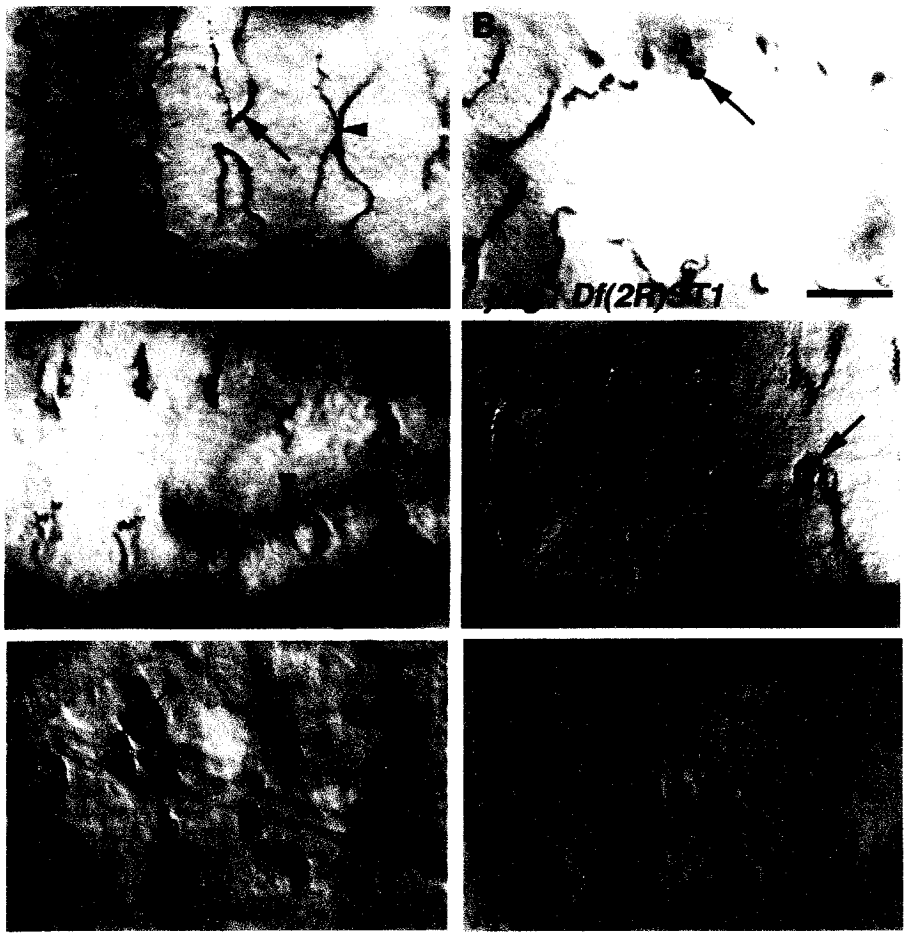


Figure 10. *jing* is required for anastomosis and fusion cell fates in the dorsal branch. (A-D) Dorsal views of stage 16 whole-mount embryos stained with the lumen marker 2A12 to visualize the tubules of the dorsal branches. (A) In wild-type embryos, anastomosis fuses the opposite sides of the tracheal branches (arrowhead). Before anastomosis, branches form a U-shaped structure to migrate to their targets (arrow). (B, C) Tubules truncate (B, arrow) and form circular structures (C, arrowhead) in embryos hemizygous (B) or over-expressing *jing* specifically in the trachea (C). (D) In embryos double heterozygous for *jing* and shg^{R696}, tubules truncate and instead form circular shapes (arrow). (E) In wild-type stage 16 embryos, fusion cells express the enhancer trap $escargot^{G66B}$ (esg^{G66B}) at the dorsal midline (arrow). (F) However, in homozygous $jing^{3}$ mutant embryos, there are 50% reductions in the number of esg^{G66B}-expressing fusion cells in the dorsal branch. Some fusion cells appear smaller than usual (arrow). Scale bars: 50μm.

jing is required for fusion cell fates

NOTCH-DELTA signalling plays an important role in regulating fusion cell fates (Llimargas et al., 1999; Ikeya and Hayashi, 1999; Steneberg et al., 1999). BNL activates the expression of *Delta* to coordinate primary branching with fusion cell determination. Therefore, the defects in anastomosis in *jing* mutant embryos could result from alterations in the differentiation of the fusion cells which express the transcription factor *escargot* (*esg*) and form the anastomoses that connect consecutive hemisegments (Samakovlis et al., 1996; Tanaka-Matakatsu et al., 1996). We therefore, investigated the fate of the fusion cells in homozygous *jing* mutant embryos using the esg^{G66B}-*lacZ* enhancer trap as a fusion cell marker (Tanaka-Matakatsu et al., 1996).

In wild-type embryos, the fusion cells in the dorsal branch of the trachea can be seen at the dorsal midline by stage 16 (Fig. 10E, arrow). In similarly staged embryos homozygous (Fig. 9F) and hemizygous (data not shown) for *jing*, there were 50% reductions in the number of esg^{G66B} -expressing fusion cells in the dorsal branch (n=230 metameres). Fusion cell reductions were not observed in esg^{G66B}-*lacZ* heterozygous embryos. Therefore, *jing* function is required for fusion cell fates and this is consistent with the regulation of *Dl* mRNA expression by *jing* (Fig. 8).

Discussion

The developing trachea must precisely coordinate transcriptional regulation with growth factor signalling in ectodermal cells (Llimargas and Casanova, 1997; Wappner et al.,

1997; Llimargas and Casanova, 1999). This is a valuable model with which to study the mechanisms controlling many aspects of cellular behaviour (Manning and Krasnow, 1993; Samakovlis et al., 1996a, b; Metzger and Krasnow, 1999; Uv et al., 2003; Affolter et al., 2003). However, despite this elegant model system, little is known about branch- and stage-specific transcriptional regulation during tracheal development. Here, we present an analysis of the *Drosophila* protein JING during tracheal development. This, together with previous work establishes that JING has a key role in transcriptional regulation in tyrosine kinase pathways (Sonnenfeld et al., 2004). In particular, JING is required for maximal *btl* FGFR expression and signalling during the initial and later stages of tracheal cell differentiation.

jing functions as a transcriptional co-activator and co-repressor

TRH and TGO are transcription factors belonging to the bHLH-PAS protein family (Isaac and Andrew, 1996; Wilk et al., 1996; Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997; Zelzer et al., 1997). The binding of TRH::TGO heterodimers to consensus DNA binding sites (CMEs) in the *breathless* (*btl*) *FGFR* regulatory region is required for primary branching and tracheal development to proceed (Ohshiro and Saigo, 1997). However, other direct targets of TRH::TGO heterodimers have not been identified. There is evidence for co-activators of *btl* expression in the *Drosophila* trachea, including POINTED and DFR/VVL, but they do not act at the placode stage (Ohshiro et al., 2002). Our present results show that TRH and TGO activate the expression of the *jing* Zn finger

transcription factor in vitro. The JING protein then cooperates with TRH::TGO to increase the levels of *btl* expression.

Transcription assays and in vivo expression analysis show that JING cooperates with TRH::TGO in *btl* activation. Supportive data includes; (1) induction of *btl* expression around the tracheal pits by *jing* over-expression; (2) reduction, but not loss, in *btl* expression in homozygous *jing* mutant embryos; (3) the strong dominant genetic interactions between *jing*, *trh* and *btl* in the trachea (Sedaghat et al., 2002); (4) the synergistic interactions between *trh* and *jing* in the developing eye; (5) the absence of primary branching in *jing* mutants; (6) the reduced *btl*-dependent MAPK activity prior to primary branching observed in *jing* homozygous mutants (Sonnenfeld et al., 2004); and (7) the co-expression of *trh*, *jing* and *btl* in the tracheal placodes and later in secondary branch-forming cells. These results support a role for *jing* as a co-activator of *btl* expression in primary and secondary tracheal branches.

The three CME DNA binding sites in the *jing* promoter are required for activation and binding by TRH::TGO as their deletion or mutation abolishes reporter activation and DNA binding. However, our results suggest that *jing* CMEs are not sufficient to activate early *jing* expression in the tracheal placodes during stage 10. Therefore, *jing* expression in the tracheal placodes requires factors other than or in addition to TRH and TGO since there are no detectable CMEs in the remaining 5′ regulatory sequence of *jing*. These results reveal that additional mechanisms may be required for the stage-specific transcriptional regulation of *jing*.

While JING increased *btl* expression, it strongly repressed TRH::TGO-mediated activation of the *jing*1.5-*luciferase* reporter as shown by transcription assays. This

inhibitory auto-regulation was not due to a non-specific effect as it did not occur in combination with TRH and TGO during *btl* activation. In cultured *Drosophila* cells, the ability of JING to repress the *jing*1.5-*lacZ* reporter gene was dependent on the presence of the SOX consensus site (TACAAT) in the reporter, suggesting that JING represses transcription by irectly binding this motif. In support of this, the mouse protein AEBP2, which has zinc fingers most similar to JING, binds to CCAAT sequences in the adipocyte *Ap2* gene (He et al., 1999). Together, the data suggests that JING negatively auto-regulates and that it may mediate both transcriptional repression and activation.

The purpose of JING negative auto-regulation may be to maintain appropriate protein levels for proper regulation of gene expression in signalling pathways. Consistent with this, a higher dosage of *jing* increases *Egfr* expression in CNS midline glia, altering apoptosis and cell numbers during development (Sonnenfeld et al., 2004). In the trachea, the activation of *jing* by TRH::TGO may occur for the duration of tubulogenesis, given the co-expression of these genes, and necessitating a means of *jing* down-regulation (Sedaghat et al., 2002; Isaac and Andrew, 1996; Wilk et al., 1996; Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997; Zelzer et al., 1997). We show the consequence of *jing* over-expression in the trachea by the increase in *btl* expression. An additional *jing* inhibitory role may also be carried out by the ETS protein, AOP/YAN, which is known to repress *btl* except at the tips of growing branches where MAPK activity is highest (Ohshiro et al., 2002). There is an ETS consensus sequence in the regulatory region of *jing* located between the DFR and SOX binding sites supporting this view. In addition, we find that AOP/YAN represses *jing* reporter expression in the visceral branch, dorsal trunk and CNS midline suggesting that factors in addition to TRH and TGO are required

for endogenous *jing* expression in these regions (Sedaghat et al., 2002). DFR/VVL also has the ability to repress TRH::TGO-mediated activation of the *jing*1.5-*lacZ* reporter gene in *Drosophila* cells. Therefore, there may be multiple checks on *jing* expression in early tracheal cells to properly maintain gene expression in target pathways for the branching process (Fig. 11).

Together, these results suggest that the relative levels of at least JING, TRH, TGO, DFR/VVL and PNT/YAN in a particular tracheal cell dictate the transcriptional response of the *btl* and *jing* promoters.

jing is required for primary and secondary branching

BNL/BTL signaling in the developing trachea controls cell migration during primary branch formation (Reichman-Fried and Shilo, 1995; Lee et al., 1996; Sutherland et al., 1996). This pathway also induces the expression of genes in cells participating in secondary and terminal branch formation. BNL acts as a chemoattractant and can induce new terminal branches during oxygen deprivation (Jarecki et al., 1999). Our in vivo studies suggest that *jing* co-activates *btl* expression at different stages of tracheal development. First, primary branching defects were observed in *jing* mutants after staining with anti-CRUMBs and anti-TRH, which is consistent with *btl* regulation by *jing* (this work; Sedaghat et al., 2002). These defects were also associated with tracheal-specific *jing* expression suggesting an autonomous tracheal function for JING. Second,

defects in secondary branching were observed after *jing* tracheal over-expression and in *jing* and *bnl* or *D*E-*cad* double heterozygous embryos.

During stage 12, the *jing* 1.5Kb enhancer is strongly activated at the tips of growing tracheal branches, including the DB, LTa, LTp and GB, that are active in MAPK and express *btl* (Ohshiro et al., 2002). This expression pattern is similar to the localization of nuclear JING protein in the trachea except that JING is also present in the visceral branch suggesting that this partial promoter is a good representation of *jing* activation except in the visceral branch and tracheal pits (Sedaghat et al., 2002). The expression pattern of *jing* at the tips of growing tracheal branches suggests that *jing* is activated where BTL signaling is most needed for the branching process and may be required for regulation of *btl* during primary, secondary and/or terminal branch formation (Reichman-Fried and Shilo, 1995; Samakovlis et al., 1996; Ohshiro et al., 2002; Gabay et al., 1997).

The BNL/BTL pathway induces NOTCH signaling which activates *Delta* (*Dl*) expression in the tip cells of the primary branches (Ikeya and Hayashi, 1999). A high level of Dl then inhibits autonomous NOTCH signalling and allows activation of *esg* and MAPK, which establishes fusion cell fates and promotes anastomosis. Microarray analysis and/or in situ hybridization show that *jing* is required for *bnl* and *Dl* expression and is therefore consistent with the reductions in fusion cells and the lack of anastomosis in the dorsal branch of the trachea in homozygous *jing* mutants (this work; Tanaka-Matakatsu et al., 1996).

Our model is that TRH and TGO activate *jing* expression by binding to the three CME sites in the *jing* promoter (Fig. 11). The three CME sites in the *jing* 1.5Kb

promoter are not sufficient for strong activation of *jing* in the tracheal placodes and we therefore postulate that additional factors are needed for strong initial *jing* expression in the trachea (denoted by question mark). JING and DFR/VVL negatively regulate *jing* expression in the placodes. JING co-activates *btl* gene expression in the placodes and migrating tracheal tip cells and this maintains *Dl* mRNA expression to establish fusion cell fates and promote subsequent branch formation (Fig. 11).

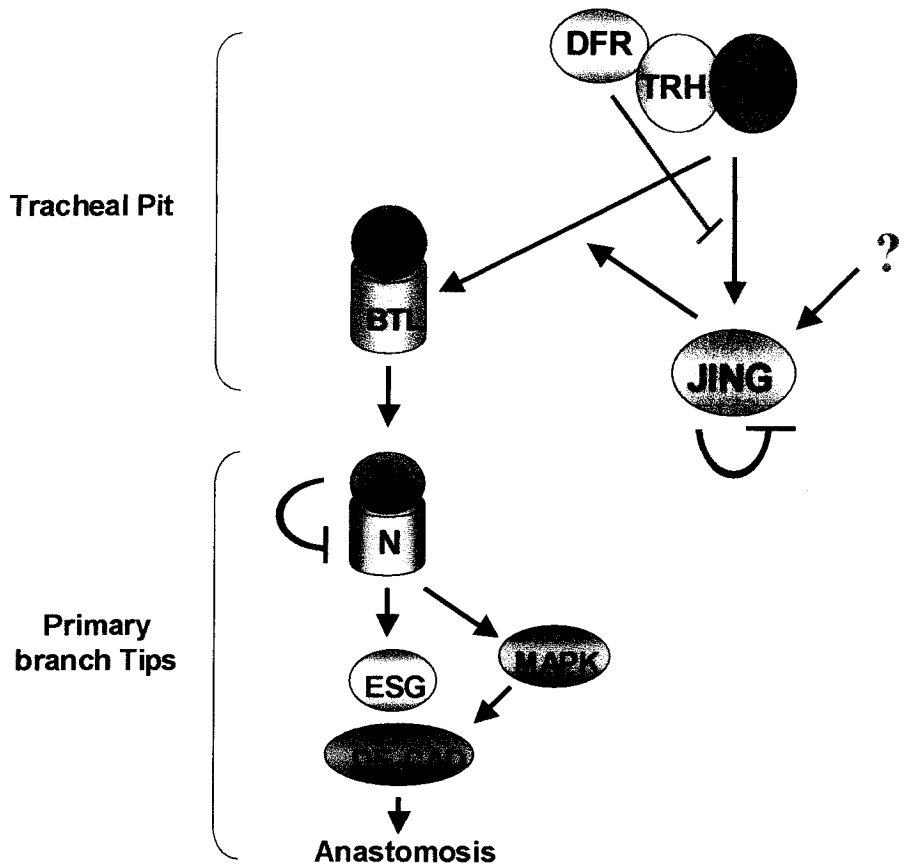


Figure 11. Model of *jing* function in the embryonic trachea. TRH::TGO heterodimers activate the transcription of *jing* through the bHLH-PAS CME binding sites. The question mark indicates that additional unidentified factors are required for *jing* expression in the tracheal placodes. JING then cooperates with TRH::TGO heterodimers to elevate *btl* expression levels in the tracheal placodes. In the absence of *jing* function, *btl* is still activated by TRH::TGO heterodimers but at significantly reduced levels. DFR and JING synergistically negatively regulate *jing* transcription. JING negative auto-regulation requires TACAAT sites in the *jing* promoter. Later, JING regulates *btl* during primary branch formation thereby maintaining the DL/N cascade, *esg* and MAPK+ levels. Fusion cells fates and subsequent *DE-cadherin* expression require the autonomous inactivation of Notch by Delta (Tanaka-Matakatsu et al., 1996; Ikeya and Hayashi, 1999), the latter of which is down-regulated in *jing* mutants.

Studies of tracheal branching processes have been successful in identifying how various mechanisms of transcriptional control integrate with signalling molecules. In this paper, we showed that the JING zinc finger transcription factor, co-activated gene expression in the FGF tyrosine kinase signalling pathway in the trachea and established that JING is a major factor necessary for epithelial tubule formation. The need to regulate protein levels can occur by a number of processes including protein degradation, ratios of negative/positive co-regulators, or negative and positive transcriptional auto-regulation. These studies show that the latter two processes are key mechanisms controlling tracheal tubulogenesis during *Drosophila* embryogenesis.

Acknowledgements

We thank J. Woodgett for *trh*, *tgo* and *btl* constructs for transcription assays. We thank S. Hayashi for esg^{G66B}, *bnl* and *btl* cDNAs and S. Crews for P[UAS-*trh*]. We thank Doug Holmyard at the Bioimaging Centre at Mount Sinai Hospital for performing SEM. We thank D. Godt and U. Tepass for *DE-cadherin* mutants. We are grateful to Roger Jacobs and David Lohnes for critical reading of the manuscript. This work was supported through a grant from the Canadian Institutes of Health Research to M.J. S and an Ontario Graduate Scholarship to Y.S.

Chapter Five

General Conclusions and Discussion

General Conclusion and Discussion

One of the basic goals of modern biology is to elucidate the molecular mechanisms of development in higher organisms. Genetic approaches have been extremely advantageous to understand these mechanisms. These achievements have been possible because of the widespread use of model systems, such as the fruit fly, *Drosophila melanogaster*. Molecular genetic studies of embryogenesis in *Drosophila* have allowed us to identify and isolate numerous genes involved in the development of different aspects of this organism. Many of these genes share sequence similarities and functional conservation with that of vertebrates. Therefore, studying the developmental processes in this model system will assist us in understanding similar complex pathways in vertebrates.

This thesis describes the molecular genetic analysis of *jing*, a novel gene that was discovered in separate screens for genes involved in border cell migration (Liu and Montell, 1999 and 2001) and CNS patterning related to *sim/tgo* genes (Sedaghat et al., 2002). Molecular characterization of *jing* revealed that its cDNA structure consisted of three zinc finger domains and a putative nuclear localization sequence. *jing* is expressed in CNS midline and tracheal cells during early and late stages of development, and we hypothesized that it plays a regulatory role in these cells. Our studies demonstrate that *jing* is required for the proper expression of a subset of factors that participate in axon guidance decisions involving midline glial cells, commissural, longitudinal connective axon fascicle development, and tracheal formation. We showed that Trh::Tgo complexes regulate *jing* transcription through binding to CMEs and that *jing* function is required for the expression of Trh::Tgo target genes in Btl-Bnl pathway.

A. *jing* genomic structure and embryonic expression pattern

jing has also been known in FlyBase as CG9403, l(2)01094, l(2)22F3 and rhumba. *jing* is located on the second chromosome and has been mapped cytologically to 42C1 (http://www.flybase.net). The analysis of the genomic structure of *jing* revealed that it contains seven exons. Exon 2 possesses repeats of poly-glutamine (Q), poly-serine (S), and alanine-glutamine (AQ), which may be considered as putative transcriptional regulatory motifs, based on functional analysis of other proteins (Sedaghat et al., 2002). Proline-rich and glutamine-rich domains are two classes of transcriptional activation domains (Ptashne and Gann, 1997; Cress and Triezenberg, 1990). It has been shown that activation domains of the glutamine-rich SP1 transcription factor interact with at least one component of the *Drosophila* TFIID complex, which is one of the accessory factors in the general transcription machinery (Gill et al., 1994).

There are three C_2H_2-type zinc fingers present on the fourth and fifth exons of *jing*, which categorize Jing as a putative transcription factor that binds to DNA transcription-control elements to stimulate or repress transcription (Liu and Montell, 2001; Sedaghat et al., 2002). C_2H_2 zinc fingers were initially found in the transcription factor IIIA, which is required for transcription of 5S rRNA genes by RNA polymerase III (Miller et al., 1985). Each repeating unit, which has two cysteine and two histidine residues, binds one zinc ion through the cysteine and histidine side chains. This compact domain can insert its α helix into the major groove of DNA.

The zinc finger motifs in Jing are followed by a putative nuclear localization sequence (NLS) (Sedaghat et al., 2002). To verify the role of the NLS in importing Jing into the nucleus, the following experiments should be performed: (1) Jing-GFP fusions

should be generated to see if this complex goes to the nucleus, (2) the putative NLS should be mutated to see if Jing accumulates in the cytoplasm, and (3) the NLS should be fused to a heterologous protein and nuclear localization of the complex should be identified.

To study the *jing* function in CNS and tracheal development, *jing* mutations were made using EMS (Lewis and Bacher, 1968). The $jing^3$ EMS-induced mutation is a G-to-A change at nucleotide 3806 of cDNA LD36562, which results in the conversion of W^{1200} to a stop codon in the middle of the second zinc finger located within the fourth exon (Sedaghat et al., 2002). The $jing^3$ mutant allele lacks part of the second, the entire third zinc finger motifs, and the putative NLS, thus I predict that the mutant allele is never transported to the nucleus.

It is possible that the disruption of the second zinc finger could remove transcriptional activation, as this region serves a repressive function in AEBP2, the most similar protein to Jing (He et al., 1999; Liu and Montell, 2001). AEBP2 represses the transcription of *aP2* and this repression involves the middle zinc finger, as a point mutation in this region abolishes the repressive effect of AEBP2, but not its ability to bind to DNA (He et al., 1999).

Jing mRNA and protein can be detected at the blastoderm stage of embryogenesis, which suggests there is a maternal component for *jing* expression in the early embryo. *jing* expression is detectable again from stage 9 in the CNS midline, neuroectoderm, and tracheal pits and persists throughout embryogenesis and is localized to the nucleus (Sedaghat et al., 2002; Liu and Montell, 2001). Double staining with markers for midline glia, neurons, and trachea in combination with a *jing-lacZ* enhancer trap confirmed the

expression of *jing* in the midline and tracheal cells. *jing* is also expressed in border cells and nurse cells within the ovary and this expression depends upon *slow border cell* (*slbo)* (Liu and Montell, 2001). Mutation in *jing* promted defects in border cell migration, which initially brought up the name jing for this gene; jing means "still" in Chinese (Liu and Montell, 2001).

A. 1. The most similar mammalian protein to Jing

A blast search using the Jing protein sequence demonstrated that it is related to AEBP2 (mouse adipocyte enhancer binding protein 2), which is a member of the C_2H_2 zinc finger family and binds to a small regulatory region in the *adipocyte P2* gene (He et al., 1999). AEBP2 has three Gli-Krüppel C_2H_2 zinc finger motifs (He at al., 1999).

AEBP2 also contains a single consensus phosphorylation site ((Ser/Thr)-Pro-X-(Lys/Arg)) (SPSK) for Cdc2 kinase, which is a highly conserved cell cycle regulatory kinase. The SPSK is located between the third zinc finger and the NLS (He et al., 1999). It has been suggested that the SPSK site may affect the function of the NLS and regulate import of the AEBP2 protein into the nucleus (He et al., 1999). Jing shows 50% amino acid identity with AEBP2 within the zinc finger regions and 20% in the C-terminal region flanking the zinc fingers. Furthermore, there is a SPTK site between the first stretch of serine and the second glutamine-rich sequences in Jing, which can also be a putative phosphorylation site for Cdc2 kinase. However, unlike AEBP2, this site is closer to the N-terminus and 955 amino acids away from the putative NLS in the Jing protein, therefore phosphorylation of this site might not affect the function of the NLS. Since the SPTK site is flanked by the serine and glutamine- rich regions, it is possible that phosphorylation at this site may have effects on the transcriptional activity of Jing. To

determine that SPTK residues can be phosphorylated by Cdc2, two-dimensional peptide mapping should be used (Futter et al., 2005). To study the role of the SPTK site in Jing's transcriptional activity, a mutated version of Jing with a change of Serine to Alanine nucleotide in the SPTK site should be tested *in vivo* and in cell culture to see if it could affect the transactivation of *btl* promoter (LeHoux et al., 2004).

In *Drosophila*, *jing* is required for the initiation of border cell migration. Slbo encodes the *Drosophila* homologue of the CCAAT enhancer binding protein (C/EBP) (Ro and Roncari, 1991). The *Drosophila* C/EBP protein is expressed in the border cells just prior to their migration, and is required for the initiation of border cell migration. C/EBP regulates the transcription of target genes through binding to a site similar to that of AE-1, AATTGGGCAAT (Ro and Roncari, 1991). Several lines of evidence indicate that *jing* functions in the *slbo* pathway (Liu and Montell, 2001). Jing cooperates with Slbo in activating transcription of downstream target genes. Both *jing* and *slbo* are normally required for expression of a border cell marker, *PZ6356*. Over-expression of *jing* can compensate for reduced levels of Slbo *in vivo* (Liu and Montell, 2001).

AEBP2 was also identified in a screen for proteins that bind to the same enhancer element as C/EBP in the *adipocyte protein 2* gene (*aP2*) (He et al., 1999). AEBP2 binds to the AE-1 site (AXTTXAXAAAT), which serves as a bifunctional element in the regulation of *aP2* gene expression (Ro and Roncari, 1991; He et al., 1995). It is known that the AE-1 site is bound by either C/EBPα, a transcriptional activator, or the transcriptional repressor AEBP1 (Herrera et al., 1989; He et al., 1995).

There is an interesting parallel between ovary and embryonic pathways that involves *jing* and *breathless (btl)*. *btl* is expressed in embryonic tracheal and midline glial cells, as

well as border cells of the ovary, and is essential for their migration (Glazer and Shilo, 1991; Klämbt et al., 1992; Murphy et al., 1995). *btl* is a direct target of C/EBP and of TRH::TGO heterodimers (Murphy et al., 1995; Oshiro and Saigo, 1997). C/EBP regulates *btl* expression in the ovaries, but probably not in the trachea since C/EBP is expressed in the embryonic trachea after *btl* expression begins (Rorth et al., 1992). However, the strong dominant interactions between *jing* and *btl* in the embryonic trachea coupled with the role of *jing* in border cell migration suggest that Jing might be able to bind to the AE-1 site and regulate the transcription of *btl*. This suggests that JING and C/EBP coordinate cell differentiation in a co-operative manner (He et al., 1999; Liu and Montell, 2001).

AEBP2 is ubiquitously expressed in the adult mouse and in the embryonic brain, liver, and placenta, suggesting that this gene is involved in development and in tissue homeostasis in the adult (He at al., 1999). AEBP2 is involved in the regulation of adipocyte differentiation, a process that bears little obvious resemblance of the requirement for *jing* function in border cell migration in the ovaries, CNS midline survival, and differentiation and tracheal formation. One similarity between border cells and adipocytes is that both cell types coordinate their differentiation with the nutritional status of the organism (Liu and Montell, 2001).

In addition to AEBP2, Jing exhibits some similarity to the Gli family of zinc finger transcription factors. This homology is limited to 25% amino acid identity within the zinc finger motifs and no homology outside of these regions (Liu and Montell, 2001). In vertebrates, there are three Gli genes, Gli1, Gli2, and Gli3, which are expressed in a number of tissues during embryogenesis, including the CNS (Hui et al., 1994; Ruiz I

Altaba, 1998). These proteins are known to be transcriptional mediators of Shh signaling that coordinately pattern the dorsal-ventral axis of the spinal cord (Ruiz I Altaba, 1998). The *Drosophila* Gli homologue is Cubitus interruptus (Ci), which is the key regulator of Hh target genes and functions as both repressor and activator (Orenic, 1987; Methot and Basler, 2001; Aza-Blanc et al., 1997).

B. *jing* and embryonic CNS development in *Drosophila*

jing function is required for the embryonic development of ventral nerve cord and brain in *Drosophila*.

B. 1. Ventral nerve cord (VNC) development

single-minded (sim) and *tango (tgo)* are two members of the bHLH-PAS proteins that are required for the proper formation of the CNS (Nambu et al., 1991; Sonnenfeld et al., 1997). To further characterize the relationship between *jing* and CNS midline development, CNS formation was studied in *jing* loss- and gain-of-function embryos. In $jing^{3}$ homozygous embryos, commissural axons, and longitudinal connectives are disrupted; meanwhile *jing* and *sim* double mutant embryos exhibit a similar phenotype as *sim* homozygotes, indicating that *jing* functions downstream of *sim* (Sedaghat et al., 2002).

Based on our genetic data, Sim and Tgo might heterodimerize to regulate *jing* function through interaction with CMEs (Crews, 1998; Crews and Fan, 1999). To examine this, activation of a luciferase reporter fused to 1.5 kb fragment of the *jing* 5′ flanking region was tested in the presence of Sim and Tgo in transiently transfected S2 cells. Sim and Tgo expression did not increase the activity of the *jing* reporter compared to basal activity in S2 cells (data not shown). We also co-transfected the S2 cells with

cofactors, which have been demonstrated to positively affect on Sim function. The two well-known factors are Fish-hook (Fish), which is a Sox HMG domain (Nambu and Nambu, 1996; Russell et al., 1996) and Drifter (Dfr) is a POU domain protein (Anderson et al., 1995). It has been shown that the combination of Fish, Dfr with Sim::Tgo increases the activity of a *slit-lacZ* reporter gene in S2 cells by 15 fold, while Sim and Tgo are capable of increasing the reporter activity only by one fold (Ma et al., 2000). The 1.5 kb *jing* promoter fragment has DNA binding sites for both Fish and Dfr (Ma et al., 1998; Certel et al., 1996). However, co-expression of these genes had no effect on reporter gene activity (data not shown).

There are possible reasons to consider why we were unable to observe Sim::Tgo-mediated transactivation of the *jing* promoter in our system. First, the transcriptional activity of a gene can be influenced by increasing the copy number of a transcription factor binding site as has been demonstrated for transactivation of *slit* by Sim::Tgo (Sonnenfeld et al., 1997). The regulatory region of *slit* has one CME. Sim and Tgo together yielded only minimal *slit-lacZ* reporter activity in S2 cells. However, Sim::Tgo heterodimers strongly activate expression of 6XCMEs-*lacZ* reporter (Sonnenfeld et al., 1997). Similar findings have been reported for transcriptional function of AEBP2 on the *aP2* promoter (He et al., 1999). AEBP2 regulates *aP2* transcription through interaction with the AE-1 site. To obtain the reporter activity in the presence of this transcription factor, three copies of the AE-1 sequence were inserted upstream of the *aP2* promoter, which resulted in the repression function of AEBP2 on the reporter (He et al., 1999). Therefore, the effect of Sim::Tgo on *jing* transcription should be re-evaluated in S2 cells using a reporter construct that contains multiple copies of the CME sequence.

Second, the location of the binding site for a transcription factor on the promoter of interest could also be critical. For instance, Rhomboid (Rho) functions as a regulator in the Egfr pathway by processing the Spitz protein, an Egfr ligand (Schweitzer et al., 1995; Golembo et al., 1996). *rho* itself is a transcriptional target of Sim and Trh. These bHLH-PAS proteins regulate the transcription of *rho* through binding to CMEs. *rho* promoter contains four CMEs (Zelzer and Shilo, 2000). Only the furthest two CMEs from the start codon are necessary for Sim::Tgo or Trh::Tgo heterodimers to regulate the transcription of the *rho* in CNS midline and trachea, respectively (Zelzer and Shilo, 2000). It seems that the flanking regions of the CMEs also play an important role in transactivation of *rho* promoter. Therefore, this might be the reason that Sim::Tgo were not capable of mediating transactivation through the 1.5 kb *jing* promoter fragment in our system. In addition, there might be other regulatory elements needed for *jing* transcription that are not present in the 1.5 kb fragment of the *jing* 5′ flanking region. Consistent with this possibility is our observation that this 1.5 kb *jing* promoter fragment did not drive *lacZ* expression in the CNS of the transgenic embryos.

B. 1. i. Role of *jing* in CNS midline development

We also observed that the number of the midline neurons and glia were reduced in $jing^3$ homozygous embryos. To determine whether loss of midline cells is due to cell death and/or improper differentiation of the cells at early stages of development, we performed TUNEL labeling and observed an increase in apoptosis in midline cells in $jing^3$ homozygous embryos throughout embryogenesis. Applying stage-specific markers showed loss of these cells at early stages of midline cell differentiation (Sedaghat et al.,

2002). Therefore, we conclude that *jing* function is required for both midline cell survival and differentiation.

jing loss-of-function also affected CNS axon patterning, which raised the question of how the axon patterning is regulated by *jing*. During the formation of axonal tracts in wild type embryos, midline repulsive mechanisms prevent the ipsilateral fascicles from crossing the midline (Hummel et al., 1996; Kidd et al., 1999). In $jing^3$ homozygous embryos, however, ipsilateral axons project contralaterally and cross the midline, suggesting that midline repulsive mechanisms are not intact in $jing^3$ mutants (Sedaghat et al, 2002). Meanwhile, over-expression of one copy of *jing* in the CNS midline results in a similar phenotype as the *jing* mutation, suggesting that CNS development is sensitive to *jing* dosage (Sedaghat et al., 2002). In both insects and vertebrates, chemorepellents and chemoattractants provide guidance cues to axons and regulate their pathfinding along stereotypical pathways toward their synaptic targets (Goodman, 1986; Tessier-Lavigne, 1994). Thus, activities of axon repellents and axon attractants regulate the formation of commissure that cross the midline and connect the two sides of the CNS, and longitudinal connectives, which travel along the nerve cord and connect different segments along the anterior-posterior axis and join the brain to the nerve cord.

Axon repulsion is mediated by Slit (Sli)-Roundabout (Robo) signaling. *Drosophila* has a single Slit receptor and three Robo receptors (Robo, Robo2 and Robo3) (Kidd et al., 1999). Axon attraction is mediated by the Netrin (Net)-Frazzled (Fra) signaling (Harris et al., 1996; Mitchell et al., 1996). It has been shown that growth cones that express *robo* will not enter and cross the midline where *sli* is expressed at high levels. Therefore, in *robo* mutants axons cross and re-cross the midline, whereas in *sli* mutants, axons that

normally do not enter the midline freely do, but they do not leave the midline after entering (Kidd et al., 1999; Rajagopalan et al., 2002). Therefore, the Sli-Robo repellent complex might be affected in *jing*^{3} mutants (Kidd et al., 1999; Bashaw et al., 2000).

To determine the role for *jing* function during axonal guidance, we employed the GAL4/UAS system and established *UAS-jing* transgenic flies (Brand and Perrimon, 1993). Expression of Jing protein was directed in CNS midline neuron and glia of mutant *jing*^{3} embryos using the *sim*-GAL4 driver strains (Scholz et al., 1997). Midline expression of *jing* could partially rescue the *jing*^{3} mutant CNS phenotype indicating that *jing* function is required in midline cells, which express both Slit and Netrin. In *jing*^{3} homozygous embryos at stage 15, 25.7% of segments show commissure crossing the midline, however after over-expressing *jing* in midline cells, this percentage is increased up to 53.8% (n=210) (Data not shown). Therefore, *jing* functions in the CNS midline to control commissural crossing.

In *jing*^{3} homozygous embryos, CNS midline cells die prematurely, as they do in EGFR pathway mutants, suggesting a functional relationship between these genes (Sonnenfeld and Jacobs, 1994; Sonnenfeld et al. 2004). It has been shown that the epidermal growth factor receptor (EGFR)-mediated RAS1 signaling pathway regulates the differentiation and survival of midline glial cells (Sonnenfeld and Jacobs, 1994; Baker, 2001). In *jing*^{3} homozygous embryos, the number of CNS midline cells is reduced during embryogenesis by excessive apoptosis (Sedaghat et al., 2002). In these embryos, RTK activity, which was mediated by the diphosphorylated form of MAPK (ppMAPK), was very low compared with wild-type embryos (Sonnenfeld et al., 2004). This result suggests that MAPK activation is aberrant in the absence of *jing*.

To prove that *jing* function is required for *Egfr* pathway transcription, mRNA levels of *Egfr* pathway genes were examined by microarray analysis in $jing^3$ homozygous embryos and in embryos over-expressing *jing* in the CNS midline and trachea. In $jing^3$ homozygous embryos, *ras1* mRNA levels were reduced by 50%, and Egfr pathway mRNA levels were increased in embryos over-expressing *jing* in midline. Following the microarray data, the expression of *Egfr* was studied by *in situ* hybridization. *Egfr* expression was reduced in $jing^3$ homozygous embryos midline cells and increased in the embryos over-expressing *jing* in the CNS midline compared to controls (Sonnenfeld et al. 2004). Therefore, we propose that *jing* function is essential for *Egfr* pathway gene expression in the CNS midline.

Over-expression of *jing* in the CNS midline led to the appearance of extra glia and lack of commissural fascicle formation (Sedaghat et al., 2002; Sonnenfeld et al., 2004). Over-expression of *Egfr* pathway genes in the CNS midline has a similar effect (Klaes et al., 1994; Sonnenfeld and Jacobs, 1995). Interestingly, there is no evidence of cell death in the glial cells of the embryos over-expressing *jing* compared to the small amount of cell death normally observed in glial cells in the wild type CNS midline (Sonnenfeld et al., 2004; Sonnenfeld and Jacobs, 1995). The absence of commissural fascicles in these embryos could be a result of the lateral migration of the glial cells toward the longitudinal tracts, which is similar to the *commissureless* homozygous phenotype and the effect of ectopically expressing *pointed* in the midline (Klaes et al., 1994; Sonnenfeld and Jacobs, 1995). Therefore, *jing* over-expression can induce extra glial cells, similar to over-expression of pointed. It suppresses normal apoptosis in the midline glial cells. In the absence of the gene *reaper*, previously established to be required for apoptosis (White et

al, 1994), there is also an increase in midline glia number relative to the wild-type midline glia number (White et al, 1994; Sonnenfeld and Jacob, 1995). Further study of *reaper* expression in *jing* gain-of-function embryos might help to understand the role of *jing* in glial survival.

Interestingly, the effect of *jing* over-expression on the induction of extra glia could be suppressed by mutation in genes in *Egfr* pathway such as *star (s)* and *spitz (spi),* which suggests that *jing* is acting upstream of *s* and *spi* (Sonnenfeld et al., 2004; Schweitzer et al., 1995; Tsruya et al., 2002; Urban et al., 2001).

In contrast, over-expression of two other genes of Egfr pathway, *ras* and *ellipse* (Egfr gain-of-function mutation), in the CNS midline could suppress the *jing* loss-of-function phenotype, which appeared as midline cell reduction (Sedaghat et al., 2002; Sonnenfeld et al., 2004). Meanwhile, simultaneous reduction of *jing* and *S/spi* by one copy reduced the number of the CNS midline cells; ectopic apoptosis was also observed in these embryos. These results are consistent with the idea of Ras1/MAPK pathway involvement in midline cell survival downstream of *jing.* Therefore, we suggest that *jing* and *Egfr* pathway gene function is inter-dependent and the proper dosage of these genes is required for midline cell survival.

B. 2. Role of *jing* in the development of the brain axon scaffold

Formation of the axon scaffold in the embryonic brain is slightly different from that in the VNC. In the brain, the axon scaffold forms around the ingrowing forgut, following three structures (Wildemann *et al.,* 1997). First, the connectives grow along longitudinal rows of glial cells at the medial brain margin facing the foregut. Second, the preoral

commissure (supraesophageal commissure), which links the two cephalic hemispheres, appears to be guided by cellular bridges across the midline composed of neuronal and glial somata. Third, the postoral tritocerebral commissure extends across the midline in close contact with the ventral epithelium of the forgut. In *jing*^{3} homozygous embryos at stage 12, longitudinal connectives in the brain are formed improperly. Since the formation of these axonal tracts depends on the longitudinal glia, it is possible that these cells are abnormal in *jing*^{3} homozygous embryos (Therianos et al., 1995). The connection between the longitudinal tracts and the VNC, as well as the preoral commissure are also abnormal in *jing*^{3} homozygous embryos (Sedaghat and Sonnenfeld, 2002).

The defect in the formation of the preoral commissure, the commissure that connect the anterior part of the two brain hemispheres, indicates a role for *jing* function in regulating chemorepellents and chemoattractants that guide the growing axons (Therianos et al., 1995). There is evidence that Robo is involved in commissure formation. In humans there is a disorder called HGPPS where patients exhibit horizontal gaze palsy with progressive scoliosis and hindbrain dysplasia (Jen et al., 2004). In these patients two major nerve tracts, the motor corticospinal tract and the dorsal somatosensory tract, fail to cross the midline in the hindbrain. These patients carry mutations in the *robo3* gene (Jen et al., 2004). A complementary study describes the effects of engineering mice that lack the *robo3* gene. These animal models show similar phenotype to those observed in HGPPS patients (Sabatier et al., 2004). Robo3 releases the block that Robo1 had applied on the Netrin receptor, enabling Netrin to bind to its receptor and act as a chemoattractant for the growing axon tip (Wong and Ren, 2001; Bulow and Hobert, 2004). Therefore, we speculate that *jing* phenotype is caused by disrupted Robo signaling. Loss-of-function of

jing might affect on Robo3 and inhibit it from releasing the effect of Robo1 as a block of the Fra receptor. Therefore, Netrin is not able to bind to Fra and act as a chemoattractant for the growing axon tip (Bulow and Hobert, 2004).

Timothy et al. studied the formation of the boundary between adjacent groups of cells in the *Drosophila* brain. They showed that Slit and the Robo receptors are key factors that prevent mixing between adjacent groups of cells in the fly brain (Timothy et al., 2004). Since the structure of the brain in $jing^3$ homozygous embryos seems to be affected, further analysis of *slit* and *robo* expression in these embryos is required.

B. 2. i. Role of *jing* in the development of neurons and glia in the brain

To analyze the role of *jing* in the brain, we studied neuronal and glial cell fates in $jing^3$ homozygous embryos. This study revealed that *jing* expression is necessary for glial and neuronal cell survival at the late stages of the embryogenesis.

The *sim* expression pattern is also disturbed in the brains of $jing^3$ homozygous embryos compared to wild type (Sedaghat et al., 2002). Considering that *sim* is a master regulator of midline cell differentiation, the observed abnormalities in $jing^3$embryonic brain could be secondary to a failure to specify midline cells (Nambu et al., 1991; Therianos et al., 1995). It has been reported that the absence of *sim* causes a gap in the longitudinal row of glial cells between the brain and the VNC. This gap cannot be crossed over by longitudinal pioneer axons and, therefore results in the absence of the longitudinal connectives. This suggests that *sim* is involved in controlling the development of the longitudinal connective pathways (Therianos et al., 1995; Sedaghat and Sonnenfeld, 2002).

C. *jing* and tracheal development in *Drosophila*

Drosophila tracheal development is dependent on the cooperative activity of Trh and Tgo, two members of the bHLH-PAS proteins, which activate the expression of the *breathless* (*btl*) *fibroblast growth factor receptor* (*FGFR*) (Isaac and Andrew, 1996; Sonnenfeld et al., 1997; Glazer and Shilo, 1991). This process is necessary for primary branch outgrowth in the trachea.

jing expression is observed in the tracheal pits and tracheal branches throughout embryogenesis (Sedaghat et al., 2002; Isaac and Andrew, 1996; Glazer and Shilo, 2001). The number of trh^{+} cells is reduced in $jing^{3}$ homozygous embryos. To determine whether this reduction reflects a requirement for *jing* in cell survival, embryos were double labeled with anti-Trh and TUNEL (Isaac and Andrew, 1996). Increased tracheal cell death was observed in $jing^{3}$ homozygous embryos. In these mutant embryos, parts of the dorsal trunk, dorsal branch, and transverse connectives were missing by stage 15, suggesting the role of *jing* in tracheal formation during embryogenesis, and the defects could be related to the inappropriate apoptosis in the cells (Sedaghat et al., 2002).

In these embryos the RTK activity is very low compared with wild type embryos. MAPK activity in the tracheal pits, at stage 10, is dependent on signaling through the EGFR pathway genes and by stage 11, the FGF receptor, *btl*, is responsible for this activity (Gabay et al., 1997; Reichman-Fried and Shilo, 1995). Considering the reduction in MAPK activity in $jing^{3}$ homozygous embryos, we suggest that *jing* function is required for MAPK activity in both cases.

Microarray analysis of $jing^{3}$ homozygous embryos and embryos that over-expressed *jing* in the tracheal system revealed alterations in *Egfr* pathway genes. Gain-of-function

for *jing* in the trachea was associated with a 2-3 fold increase in the mRNA levels for *Egfr* and *ras*. Whole-mount *in situ* hybridization performed on the *jing* homozygous embryos with an *Egfr* probe showed reduction in the *Egfr* expression in the tracheal pits at stage 12 (Sonnenfeld et al., 2004). Thus, *jing* function is essential for *Egfr* expression in the trachea. Similar to the CNS, there was a genetic interaction between *jing* and Egfr pathway in the trachea. *jing* homozygous embryos, embryos double heterozygous for *jing* and *star (S),* and *S* homozygous embryos showed breaks in the dorsal trunk and transverse connectives and inappropriate apoptosis of trh^{+} cells (Sonnenfeld et al., 2004; Sedaghat et al., 2002).

The observed breaks in the dorsal trunk can be a result of the reduction in *Egfr* expression in $jing^{3}$ homozygous embryos and double heterozygous of *jing* and *S*, although there is a possibility that in $jing^{3}$ homozygous embryos other processes that are required for the proper formation of the trachea are also affected. Therefore, reduction in the *Egfr* expression may not be the only factor involved in the tracheal defects.

One of the essential events during tracheal formation is fusion to form the connected tubule network. Microarray analysis of $jing^{3}$ homozygous embryos and embryos that over-express *jing* in the tracheal system revealed the reduction of *Delta (Dl)* and *DE-Cadherin (DE-cad)* mRNA by almost 50% in $jing^{3}$ homozygous mutant compared to wild type. When *jing* was over-expressed in the trachea, the mRNA levels of *crumbs (crb), grainyhead (grh), armadillo (arm), DE-cad,* and *Dl* were increased compared to wild type. These findings raise the possibility that *jing* function is also required for cell fusion, which will be discussed at the end of this chapter.

Genetic studies of *jing* and *trh* show that they interact synergistically. We constitutively mis-expressed *trh* and *jing* in the eye imaginal disc using the driver line GMR-Gal4 (Freeman, 1996). Neither construct had an effect on the morphology of the eyes of adult flies. However, when *trh* were co-expressed together with *jing,* the development of the eye was perturbed. Therefore, we suggested that Trh and Jing might work together and disturb the development of R7 cells resulting in the disruption of ommatidial and bristle formation. This event results in the appearance of a rough eye phenotype (Sedaghat et al., 2005). Therefore, if the *jing* products act in the same pathway as Trh during normal development or physiology, its products must be present in cells that respond to Trh::Tgo signaling. Our complementary experiments demonstrated the presence of *jing* mRNA expression in the embryonic tracheal system, which expresses *trh*.

C. 1. Regulation of the jing expression in trachea

An important question is how *jing* expression in the trachea is regulated. The analysis of a 1.5 kb fragment of the *jing* promoter region revealed that it contained three central midline elements (CMEs: ACGTG), a SOX consensus site (TACAAT), binding sites for Drifter (Dfr), which is a POU domain transcription factor (CATAAT), and GGATCC binding sites for ETS proteins (Pnt and Aop) (Sedaghat et al., 2002; Sedaghat et al., 2005; Wharton et al., 1994; Ma et al., 2000; Anderson et al., 1996; Ohshiro et al., 2002). Trh::Tgo heterodimers regulate transcription of their target genes through binding to CMEs (Ohshiro and Saigo, 1997). The presence of three CMEs in the *jing* 5′ flanking region together with our *in vivo* finding suggest a possible regulatory effect of Trh::Tgo complexes on *jing* transcription (Sedaghat et al., 2002). Transient transfection of S2 cells

with the 1.5 kb *jing* promoter construct in the presence of Tgo and Trh resulted in the activation of the *jing* regulatory sequence by 17 fold. This activity was almost abolished by co-transfection with Jing, suggesting that Jing suppressed the Trh::Tgo-mediated transactivation of the *jing* responsive element.

To elucidate the role of Jing in regulating its own expression, further *in vivo* analysis of the *jing* promoter is required. The reporter gene expression of *1.5jing-lacZ* should be studied in the homozygous $jing^3$ mutant background. Mutants of the 1.5 kb *jing* promoter fragment that lack the putative Jing binding site with the core sequence of CAAT should also be tested in wild type background. Based on the transcription assay data, Jing should be able to repress its own transcription; therefore in $jing^3$ background *lacZ* expression should be increased compared with the expression of the reporter in wild type embryos. Deleting the putative Jing binding sites should result in an up-regulation of the reporter in wild type embryos.

To show that the transcriptional regulatory effect of Trh::Tgo on *jing* is through the CMEs, and that the self regulatory effect of Jing on itself might be through binding to the TACAAT sequence (Murphy et al., 1995; Liu and Montell, 2001), four different deletion constructs lacking each of the CMEs or the SOX sequence were made. The transcriptional activity of Trh::Tgo on *jing* was significantly reduced after removing the CMEs, demonstrating the requirement for these elements for Trh::Tgo transactivation of the *jing* promoter (Ohshiro and Siago, 1997; Sedaghat et al., 2005). The auto-inhibitory effect of Jing on the 1.5 kb *jing* promoter was significantly suppressed by removing the SOX consensus TACAAT site from the promoter, suggesting that TACAAT motif is a Jing putative binding site (Sedaghat et al., 2005; Liu and Montell, 2001). Deleting the

SOX sequence does not significantly effect the Trh::Tgo regulatory effect on the *jing* promoter. Electrophoretic mobility shift assay (EMSA) will confirm the importance of TACAAT sequence as Jing binding site.

In vivo experiments were performed to confirm the specific *jing* regulation by *trh*, and to show that the *jing* 5′ flanking region has tracheal-specific enhancer activity. *jing1.5-lacZ, Δjing-A1* and *Δjing-A3* (deletion constructs of the *jing* regulatory region lacking the CMEs) transgenic flies were generated and the *lacZ* reporter expression was investigated *in vivo*. The *jing1.5-LacZ* expression was observed in trh^{+} cells first at stage 12, with a strong appearance in the cells at the tip of the primary branches in the region where MAPK activity is present (Sedaghat et al., 2005; Gabay et al., 1997). The reporter expression was also observed in the secondary branch cells in the dorsal and ganglionic branches, lateral trunk anterior, and lateral trunk posterior. Since it seems that *jing* is regulated by Trh::Tgo heterodimers and its expression is strongly observed in the dorsal trunk, it confirms our previous data showing that *jing* expression is necessary for the proper formation of the trachea because the mutation in *jing* resulted in the breaks in the secondary branches (Sedaghat et al., 2002, Sonnenfeld et al., 2004; Sedaghat et al., 2005).

In wild-type embryos, *jing* reporter expression was not detected in the visceral branch at stage 12, but *jing1.5-lacZ* is activated at stage 12 in the same branch in the *aop* homozygous mutant suggesting that, *aop* is also required for the regulation of *jing* expression during the embryonic development. It has been shown that Aop and Pnt, two well-known ETS proteins, compete with each other for binding to the inverted repeat sequence GGATCC. Pnt binds to this site and activates the *btl* promoter, however, Aop

binding results in the repression of the *btl* transcription at later stages of tubule formation (Ohshiro et al., 2002). It is possible that Aop binds to the inverted repeat site on the 1.5 kb *jing* promoter fragment and represses *jing* expression in the visceral branch. To determine whether Aop represses *jing* transcription through its ETS binding sites in the 1.5 kb *jing* promoter fragment, this site should be deleted by site-directed mutagenesis. Transactivation of *jing* by Aop should be examined in S2 cells in the presence and absence of the ETS consensus binding site. Aop should be able to decrease reporter activity when it is fused to the entire 1.5 kb *jing* promoter fragment. Levels of the reporter activity should be increased after removing ETS binding sites from the *jing* promoter.

Interestingly, the reporter expression, which is representing the activation of 1.5 kb regulatory region of *jing*, is not observed at earlier stages of the tracheal development in the tracheal pits. However, our previous data had demonstrated the expression of *jing* in the tracheal pits and the necessity of the *jing* function in the formation of the pits at the beginning of the tracheal network formation (Sedaghat et al., 2002). It seems that Trh, Tgo, Aop and Pointed are not the only factors regulating *jing* transcription and the proper and complete expression of *jing* depends on other regulators as well, which was not possible to evaluate in our experiment since only 1.5 kb of the cis regulatory sequences upstream of the *jing* open reading frame was tested. In order to have the complete *jing* expression, a larger area should be considered as the *jing* promoter.

The 1.5 kb *jing* promoter fragment also contains a Dfr binding site (Wilk et al., 1996), another factor that was previously shown to be able to regulate downstream targets of Sim::Tgo and Trh::Tgo (Boube et al., 2000; Llimargas and Casanova, 1997). Therefore,

we monitored the effect of Dfr on *jing* transcription in cell culture and we found that Dfr is also able to cooperate with Jing to inhibit the Trh::Tgo-mediated transactivation of the *jing* responsive element (Sedaghat et al., 2005).

As mentioned earlier, our *in vivo* data showed the requirement for *jing* in the proper formation of the tracheal network. Trh::Tgo heterodimers apply their effect during tracheal development through *btl* transcriptional regulation, and our *in vivo* results have shown the possible function of Jing in the activation of *btl* expression (Ohshiro and Saigo, 1997; Sedaghat et al., 2002). To study the role of Jing in transactivation of the *btl* promoter, *btl* transcriptional regulation was examined in cell culture. Co-expression of Trh and Tgo in the presence of the *btl* promoter resulted in a 27-fold induction in luciferase reporter gene activity, which is consistent with previous results published by Jin et al. 2001. Co-transfection with Jing resulted in an increase in the expression of the reporter, suggesting that Jing acts cooperatively with Trh::Tgo in *btl* transcriptional regulation.

Based on these findings, we monitored the requirement for *jing* in *btl* expression *in vivo* by *in situ* hybridization. $jing^{3}$ homozygous embryos were hybridized with a digoxigenin-labeled *btl* RNA probe and *btl* mRNA expression was studied at different stages of the tracheal development. *btl* mRNA expression was significantly reduced at stages 11 and 13 compared to the strong expression in the tracheal pits and CNS midline in wild type embryos, suggesting that *jing* is required for *btl* expression *in vivo* (Sedaghat et al., 2005). We also monitored *btl* expression in embryos that over-expressed *jing* in the btl^{+} cells and observed that *btl* mRNA expression is spread out in the tracheal pits at

stage 11 and 12 compared with the controls (Sedaghat et al., 2005). Based on these findings, we propose that *jing* is required for *btl* expression during tracheal formation.

Our *in vivo* experiments showed *jing1.5-lacZ* expression in the cells at the tips of the lateral and dorsal branches, which express *btl* (Glazer and Shilo, 1991; Sedaghat et al., 2005). The *Drosophila* fibroblast growth factor (FGF)-like gene, *branchless (bnl)* (Sutherland et al., 1996), has a critical role in tracheal branch patterning. Prior to primary branching, *branchless (bnl)* is expressed in clusters of cells around the tracheal sacs where primary branches will grow and binds the FGF receptor, Btl, to establish the pattern of primary branching (Sutherland et al. 1996; Affolter et al., 2003). Therefore, we also analyzed *bnl* mRNA expression in $jing^3$ mutants *in vivo*. In $jing^3$ homozygous embryos, *bnl* mRNA expression was reduced compared to wild type at stage 11, which is the beginning of the tracheal branching. Reduction of just one copy of *jing* does not affect the tracheal formation to the same extent as removing one copy of *bnl*. However, in compound *jing/bnl* heterozygous embryos, visceral, dorsal, lateral anterior and lateral posterior branches were defective (Sedaghat et al., 2005). This is a strong indication that *jing* and *bnl* work in the same pathway.

FGF is one of the angiogenesis-inducing factors and angiogenesis plays an important role in early metastasis in cancer (Fontanini et al., 2002). The FGF family is involved in migration, invasion and endothelial tubulogenesis (Ingber et al., 1995). *jing* regulates *bnl* and *btl* mRNA expression as well as downstream target genes in Btl-Bnl pathway such as *crumbs* and *De-cad.* Several repressors, such as Sprouty, inhibits signaling mediated by the FGF receptor and the epidermal growth factor (EGF) receptor during tubule formation in *Drosophila* (Casci *et al.* 1999, Kramer *et al.* 1999, Reich *et al.* 1999). Four mammalian

genes (Sprouty1–4) have been identified with sequence similarity to *Drosophila* Sprouty (Tefft *et al.* 1999). *In vitro* studies have demonstrated that after tyrosine phosphorylation of Mammalian Sprouty, these proteins interact with components of the Ras/MAPK and Ras/Raf/ERK, which are involved in Cancer (Dikic & Giordano 2003). Jing gain-of-function increases the mRNA expression of the genes involved in FGF pathway. These changes could be partially controlled by the regulatory factors including Sprouty, however, complete recovery might not happen, resulting in the formation of extra tubule branching. Meanwhile, the precise function of AEBP2, the most similar mammalian protein to Jing, has been shown to stimulate the EED-EZH2 complex-mediated histone methyltransferase (HMTase) activity (Cao and Zhang, 2004). Histone methyltransferase (HMTase) activity of the EED-EZH2 complex has an important role in Hox gene silencing, X inactivation, and cancer metastasis. Therefore, identification of inhibitors for the EED-EZH2 HMTase activity may provide us a valuable tool for the development of therapeutic approaches for cancer (Simon and Tamkun, 2002; Francis and Kingston, 2001). Studying the FGF pathway in simple model system such as *Drosophila* can provide more information about tube formation, which is the key structure of angiogenesis.

C. 1. Primary and secondary branching

Immediately after the beginning of the branch migration, single cells at the tip of each branch express *Escargot (esg)* and will become fusion cells. *notch* limits the expression of *esg* to just one cell in each fusion branch. Notch signaling itself is affected by the Bnl-Btl pathway, which stimulates *delta* (*dl)* expression at the tip of the primary branches in the cells where *notch* expression appears to be suppressed (Tanaka-Matakatsu, 1996). In

homozygous and hemizygous *jing*^{3} embryos, a significant reduction in the number of *esg*$^{+}$ cells was observed in the dorsal branch (Sedaghat et al., 2005). *esg*$^{+}$ cells are normally observed at the dorsal midline at stage 16. At this stage tubules of the contralateral metameres make contact with each other at the dorsal midline in a process known as anastomosis (Tanaka-Matakatsu et al., 1996). One of the major regulators in this process is *DE-Cad* (Tepass et al., 1996; Tanaka-Matakatsu et al., 1996). Since *jing* is required for the normal expression of *esg* and differentiation of fusion cells in the dorsal trunk, we expected to find a reduction in *DE-cad* expression.

Our microarray analysis revealed that DE-cad mRNA levels were reduced in *jing*^{3} homozygous embryos and increased in *jing* gain-of-function in trachea. Therefore, we looked for the proper contact of the tubes from the contralateral metameres. Consistent with the phenotype associated with mutation in *DE-cad* (Tepass et al., 1996), *jing* loss-of-function resulted in the appearance of breaks in the dorsal trunk and absence of anastomosis, suggesting that the dorsal branches are not able to turn at the dorsal midline and contact with the contralateral branches. Removing one copy of *jing* and *DE-cad* resulted in the same phenotype, while a reduction of one copy of each alone did not effect the process (Sedaghat et al., 2005). Together, with our previous results, we suggest that *jing* function is required for the tracheal primary branching, lumen growth, and fusion cell fates, which result in the fusion of the tracheal tubules. Other genes functioning with *DE-cad* such as *arm* and *crb* were also affected by *jing* loss- and gain-of-function (Sedaghat et al., 2005). To validate the microarray results, expression of *DE-cad* should be studied by *in situ* hybridization in *jing* gain- and loss-of-function mutants *in vivo*.

jing is also required for initiation of border cell migration in the fly ovaries (Liu and Montell, 2001). In the ovaries of wild type embryos, when the border cell migration begins, expression of *DE-cad* increases within the border cells, and C/EBP is required for this elevation (Niewiadomska et al., 1999). *arm*, the *Drosophila* β-catenin, also co-localizes with *DE-cad* in both wild type and mutant egg chambers (Niewiadomska et al., 1999). *slbo* is an essential factor for the initiation of border cell migration and increases the expression of *DE-cad* and *arm* through activation of C/EBP (Murphy et al., 1995). Since *slbo* also regulates *jing* expression in the border cells, the possible regulation of *DE-cad* and *arm* by *jing* was investigated (Liu and Montell, 2001). However, in *jing* homozygous mutant embryos, border cells showed normal expression of both *DE-cad* and *arm*. Therefore, unlike in the tracheal system, expression of *DE-cad* or *arm* in the ovary is not *jing* dependent (Liu and Montell, 2001). It seems that *jing* can be deployed for different purposes in different tissues: for example, CNS midline cell survival and differentiation, cell adhesion and cell survival in the tracheal system and border cell migration in the ovaries.

Taken together, the original studies described in this thesis support the following conclusions:

i) Trh::Tgo heterodimers control the transcription of *jing*, a *Drosophila* gene that encodes a protein consisting of C2H2 zinc finger motifs.

ii) *jing* function is required for CNS midline and tracheal cell survival and differentiation.

iii) *jing* is functionally related to the *ras1* pathway.

iv) Jing acts cooperatively with Trh::Tgo in *btl* transcriptional regulation and plays an important role in the expression of genes involved in FGF-mediated tubule formation.

Chapter Six

References

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (2000). The genome sequence of *Drosophila melanogaster*. Science 287, 2185-2195.

Affolter, M., Bellusci, S., Itoh, N., Shilo, B., Thiery, J-P., Werb, Z. (2003). Tube or not tube: Remodeling epithelial tissues by branching morphogenesis. Dev. Cell 4: 11-18.

Affolter M., Nellen D., Nussbaumer U., Basler K. (1994). Multiple requirements for the receptor serine/threonine kinase thick veins reveal novel functions of TGF beta homologs during Drosophila embryogenesis, Development, Volume: 120, pp. 3105--3117

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

Austin R. J. and Biggin M. (1995). A domain of even-skipped protein represses transcription by preventing TFIID binding to the promoter: repression by cooperative blocking. Mol. Cell Biol. **15:** 4683–4693

Avery, L. and Wasserman, S. (1992). Ordering gene function: the interpretation of epistasis in regulatory hierarchies. Trends Genet. 8, 312-316.

Aza-Blanc P, Ramirez-Weber FA, Laget MP, Schwartz C, Kornberg TB. (1997) Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. Cell. Jun 27;89(7):1043-53

Baker N.E., Rubin G.M. (1989). Effect on eye development of dominant mutations in Drosophila homologue of the EGF receptor, Nature, Volume: 340, pp. 150—153

Bang A.G., Kintner C. (2000). Rhomboid and Star facilitate presentation and processing of the Drosophila TGF-α homolog Spitz, Genes Dev., Volume: 14, pp. 177—186

Baonza A., Casci T., Freeman M. (2001). "A primary role for the epidermal growth factor receptor in ommatidial spacing in the *Drosophila* eye", *Curr. Biol.*, Volume: 11, pp. 396-404

Baonza A., Murawsky C.M., Travers A.A., Freeman M. (2002). Pointed and Tramtrack69 establish an EGFR-dependent transcriptional switch to regulate mitosis, Nat. Cell Biol.; Volume: 4, pp. 976—980

Basler K, Hafen E. (1991). Specification of cell fate in the developing eye of *Drosophila*. Bioessays. Dec;13(12):621-31

Battye R, Stevens A, Jacobs JR. (1999). Axon repulsion from the midline of the *Drosophila* CNS requires *slit* function. Development 126:2475-2481

Bellen, H. J., O'Kane, C. J., Wilson, C., Grossniklaus, U., Pearson, R. K. and Gehring, W. J. (1989). P-element-mediated enhancer detection, a versatile method to study development in *Drosophila*. *Genes Dev.* 3, 1288-1300.

Bentley D. and Caudy M. (1983). Pioneer axons lose directed growth after selective killing of guidepost cells, *Nature* 304, pp. 62–65

Bergmann A., Agapite J., McCall K., Steller H. (1998). The Drosophila gene hid is a direct molecular target of ras-dependent survival signaling, Cell, Volume: 95, pp. 331—341

Bergmann A., Tugentman M., Shilo B.-Z., Steller H. (2002). Regulation of cell number by MAPK-dependent control of apoptosis: a mechanism for trophic survival signaling, Dev. Cell, Volume: 2, pp. 159—170

Bhat KM. (2005). SLIT-ROBO signaling neutralizes Netrin-Frazzled mediated attractant cue to specify the lateral positioning of longitudinal axon pathways. Genetics. Feb 16; [Epub ahead of print]

Bier E., Jan L.Y., Jan Y.N. (1990). rhomboid, a gene required for dorsoventral axis establishment and peripheral nervous system development in Drosophila melanogaster, Genes Dev., Volume: 4, pp. 190—203

Boerner Julie L., Andrew Danielsen, and Nita J. Maihleb. (2003). Ligand-independent oncogenic signaling by the epidermal growth factor receptor: v-ErbB as a paradigm. 2003. Experimental Cell Research 284, 111–121

Boncinelli E, Gulisano M, Broccoli V. (1993). Emx and Otx homeobox genes in the developing mouse brain. J Neurobiol. Oct;24(10):1356-66

Booth G.E., Kinrade F.V., Hidalgo A. (2000). Glia maintain follower neuron survival during Drosophila CNS development, Development, Volume: 127, pp. 237—244

Bossing, T. and Technau, G. M. (1994). The fate of the CNS midline precursors in *Drosophila* as revealed by a new method for single cell labeling. *Development* 120, 1895-1906.

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

Boyan G, Therianos S, Williams L, Reichert H. (1995). Axonogenesis in the embryonic brain of the grasshopper *Schistocerca gregaria*: An identified cell analysis of early brain development. Development 121:75-86

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

Bulow HE, Hobert O. (2004). Differential sulfations and epimerization define heparan sulfate specificity in nervous system development. Neuron. Mar 4;41(5):723-36

Butler H., Levine S., Wang X., Bonyadi S., Fu G., Lasko P. (2001). Map position and expression of the genes in the 38 region of Drosophila, Genetics, Volume: 158, pp. 1597--1614

Campbell, G., Göring, H., Lin, T., Spana, E., Andersson, S., Doe, C. Q. and Tomlinson, (1994). RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. Development 120, 2957-2966.

Campos-Ortega J.A., Hartenstein V. (1997). Stages of *Drosophila* embryogenesis, in the Embryonic Development of Drosophila melanogaster, Springer, Berlin. pp. 3–84

Campos-Ortega, A. J. and Hartestein, V. (1985). *The Embryonic Development of* Drosophila melanogaster. New York: Springer-Verlag.

Casci T., Freeman M. (1999). Control of EGF receptor signalling: lessons from fruitflies, Cancer Metastasis Rev., Volume: 18, pp. 181--201

Cau, E., Gradwohl, G., Casarosa, S., Kageyama, R. and Guillemot, F. (2000). Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium. *Development* 127, 2323-2332.

Charron F, Stein E, Jeong J, McMahon AP, Tessier-Lavigne M. (2003). The morphogen Sonic hedgehog is an axonal chemoattractant that collaborates with Netrin-1 in midline axon guidance, *Cell* 113, pp. 11–23

Chen L, Feany MB. (2005). alpha-Synuclein phosphorylation controls neurotoxicity and inclusion formation in a *Drosophila* model of Parkinson disease. Nat Neurosci. May;8(5):657-63

Chihara, T. and Hayashi, S. (2000). Control of tracheal tubulogenesis by Wingless signaling. Development.127, 4433-42.

Chitnis AG, Kuwada JY. (1990). Axonogenesis in the brain of zebrafish embryos. J Neurosci 10:1892-1905

Cress W. D. and Triezenberg S. J. (1990). Critical structural elements of the VP16 transcriptional activation domain. Science **251:** 87–90

Crews ST. (1998). Control of cell lineage-specific development and transcription by bHLH-PAS proteins. Genes Dev 12:607-620

Crews ST and Fan CM. (1999). Remembrance of things PAS: regulation of development by bHLH-PAS proteins. Curr Opin Genet Dev. Oct;9(5):580-7

Crews ST, Thomas JB, Goodman CS. (1988). The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the per gene product. Cell 52:143-151

Cui X, Doe CQ. (1992). Ming is expressed in neuroblast sublineages and regulates gene expression in the *Drosophila* central nervous system. Development 116:943-952

DeCelis, J. F., Llimargas, M. and Casanova, J. (1995). *ventral veinless*, the gene encoding the Cf1a transcription factor, links positional information and cell differentiation during embryonic and imaginal development in *Drosophila melanogaster*. Development 121, 3405-3416.

Dickson B.J., Van Der Straten A., Dominguez M., Hafen E. (1996). Mutations modulating Raf signaling in Drosophila eye development, Genetics, Volume: 142, pp. 163—171

Dong R., Jacobs J.R. (1997). Origin and differentiation of supernumerary midline glia in Drosophila embryos deficient for apoptosis, Dev. Biol., Volume: 190, pp. 165--177

Duchek P., Rorth P. (2001). Guidance of cell migration by EGF receptor signaling during Drosophila oogenesis, Science, Volume: 291, pp. 131--133

Easter SS, Ross LS, Frankfurter A. (1993). Initial tract formation in the mouse brain. J Neurosci 13:285-299

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

Estes, P., Mosher, J. and Crews, S. T. (2001). Drosophila single-minded represses gene transcription by activating the expression of repressive factors. *Dev. Biol.* 232, 157-175.

Evans R.M. , Hollenberg S.M. Zinc fingers: Gilt by association. (1988). Cell 52: 1- 3

Fields R.D. and Stevens-Graham B., New insights into neuron–glia communication, *Science* **298** (2002), pp. 556–562

Folkman, J. and Shing, Y. (1992). Angiogenesis. J. Biol. Chem. 267, 10931-10934.Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. Cell 87, 651-660.

Fontanini G, Faviana P, Lucchi M. (2002). A high vascular count and overexpression of vascular endothelial growth factor are associated with unfavourable prognosis in operated small cell lung carcinoma. Br J Cancer;86:558–63

Fortini M.E., Simon M.A., Rubin G.M. (1992). Signalling by the sevenless protein tyrosine kinase is mimicked by Ras1 activation, Nature, Volume: 355, pp. 559—561

Freeman M.R. , Delrow J, Kim J, Johnson E, Doe CQ. (2003). Unwrapping glial biology: Gcm target genes regulating glial development, diversification, and function, *Neuron* **38** pp. 567–580

Fristrom D, Gotwals P, Eaton S, Kornberg TB, Sturtevant M, Bier E, Fristrom JW. (1994). Blistered: a gene required for vein/intervein formation in wings of Drosophila. Development. Sep;120(9):2661-71

Fujita, S. C., Zipursky, S. L., Benzer, S., Ferrus, A. and Shotwell, S. L. (1982). Monoclonal antibodies against the *Drosophila* nervous system. *Proc. Natl. Acad. Sci. USA* 79, 7929-7933.

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

Futter M, Uematsu K, Bullock SA, Kim Y, Hemmings HC Jr, Nishi A, Greengard P, Nairn AC. (2005). Phosphorylation of spinophilin by ERK and cyclin-dependent PK 5 (Cdk5). Proc Natl Acad Sci U S A. Mar 1;102(9):3489-94.

Gabay, L., Seger, R. and Shilo, B. Z. (1997). MAP kinase in situ activation atlas during *Drosophila* embryogenesis. Development 1214, 3535-3541.

Gabay L., Seger R., Shilo B.-Z. (1997). In situ activation pattern of Drosophila EGF receptor pathway during development, Science, Volume: 277, pp. 1103—1106

Gavrieli Y, Sherman Y, Ben-Sasson SA. (1992). Identification of preprogrammed cell death in-situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493-501

Georgiou M. and Tear G. (2002). Commissureless is required both in commissural neurones and midline cells for axon guidance across the midline, *Development* 129 , pp. 2947–2956

Gerber H.-P., Seipel K., Georgiev O., Hofferer M., Hug M., Rusconi S. (1994). Transcriptional activation modulated by homopolymeric glutamine and proline stretches. Science **263:** 808–811

Gill G., Pascal E., Tseng Z. H. and Tjian R. (1994). A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the *Drosophila* TFIID complex and mediates transcriptional activation. Proc. Natl. Acad. Sci. USA **91:** 192–196

Glazer, L. and Shilo, B.-Z. (2001). Hedgehog signalling patterns the tracheal branches. Development 128, 1599-1606.

Glazer L., Shilo B.-Z. (1991). The Drosophila FGF receptor homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension, Genes Dev., Volume: 5, pp. 697—705

Golding J.P. and Cohen J. (1997). Border controls at the mammalian spinal cord: late-surviving neural crest boundary cap cells at dorsal root entry sites may regulate sensory afferent ingrowth and entry zone morphogenesis, *Mol. Cell. Neurosci.* 9 , pp. 381–396

Golembo M., Schweitzer R., Freeman M., Shilo B.-Z. (1996). Argos transcription is induced by the Drosophila EGF receptor pathway to form an inhibitory feedback loop, Development, Volume: 122, pp. 223—230

Golembo M., Raz E., Shilo B.-Z. (1996). The Drosophila embryonic midline is the site of Spitz processing and induces activation of the EGF receptor in the ventral ectoderm, Development, Volume: 122, pp. 3363—3370

Gonzalez-Reyes A., Elliott H., St Johnston D. (1995). "Polarization of both major body axes in *Drosophila* by gurken-torpedo signalling", Nature, Volume: 375, pp. 654-658

Goodman CS. and Doe CQ. (1993). Embryonic development of *Drosophila melanogaster*, Cold Spring Laboratory Press. pp. 1131-1244

Grigliatti, T. (1986). Mutagenesis. In Drosophila: A Practical Approach (ed. D.B. Roberts), pp. 39-58. Oxford: IRL Press Limited.

Guichard A., Biehs B., Sturtevant M., Wickline L., Chacko J., Howard K., Bier E. (1999). rhomboid and Star interact synergistically to promote EGFR/MAPK signalling during Drosophila wing vein development, Development, Volume: 126, pp. 2663—2676

Halfon M.S., Carmena A., Gisselbrecht S., Sackerson C.M., Jimenez F., Baylies M.K., Michelson A.M. (2000). Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors, Cell, Volume: 103, pp. 63--74

Hallam, S., Singer, E., Waring, D. and Jin, Y. (2000). The *C. elegans* NeuroD homolog *cnd-1* functions in multiple aspects of motor neuron fate specification. *Development* 127, 4239-4252.

Halter DA, Uban J, Rickert C, Ner SS, Ito K, Travers AA, Technau GM. (1995). The homeobox gene *repo* is required for the differentiation and maintenance of glia function in the embryonic nervous system of *Drosophila melanogaster*. Development 121:317-332

Harris, K., F., Christensen, J. B., Radany, E. H. and Imperiale, M., J. (1998). NovelMechanisms of E2F Induction by BK Virus Large-T Antigen: Requirement of both the pRb-Binding and the J Domains. Mol Cell Biol, 18, 1746-1756.

Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in *Drosophila*. Development 120, 2121-2129.

He GP, Muise A, Li AW, Ro HS. (1995), A eukaryotic transcriptional repressor with carboxypeptidase activity. Nature. Nov 2;378(6552):92-6

He, G. P., Kim, S. and Ro, H.-S. (1999). 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.

Hemphala J, Uv A, Cantera R, Bray S, Samakovlis C. (2003). Grainy head controls apical membrane growth and tube elongation in response to Branchless/FGF signalling. Development 130, 249-258.

Herrera R, Ro HS, Robinson GS, Xanthopoulos KG, Spiegelman BM. (1989), A direct role for C/EBP and the AP-I-binding site in gene expression linked to adipocyte differentiation. Mol Cell Biol. Dec;9(12):5331-9

Hidalgo, A. and Brand, A. H. (1997). Targeted neuronal ablation: the role of pioneer neurons in guidance and fasciculation in the CNS of *Drosophila*. *Development* 124, 3253-3262.

Hidalgo and Booth G.E. (2000). Glia dictate pioneer axon trajectories in the *Drosophila* embryonic CNS, Development **127** , pp. 393–402

Huang Z., Shilo B.Z., Kunes S. (1998). "A retinal axon fascicle uses spitz, an EGF receptor ligand, to construct a synaptic cartridge in the brain of *Drosophila*", *Cell*, Volume: 95, pp. 693-703

Hui CC, Slusarski D, Platt KA, Holmgren R, Joyner AL. (1994). Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. Dev Biol. Apr;162(2):402-13

Hummel T, Schimmelpfeng K, Klämbt C. (1999a). Commissure formation in the embryonic CNS of *Drosophila*. I. Identification of the required gene functions. Dev Biol 209:381-398

Hummel T, Schimmelpfeng K, Klambt C. (1999b). Commissure formation in the embryonic CNS of *Drosophila*. II. Function of the different midline cells. Development 126:771-779

Hummel T, Leifker K, Klambt C. (2000). The *Drosophila* HEM-2/NAP1 homolog KETTE controls axonal pathfinding and cytoskeletal organization, *Genes Dev.* **14**, pp. 863–873

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

Isaac, D. D. and Andrew, D. J. (1996). Tubulogenesis in *Drosophila*: a requirement of the *trachealess* gene product. Genes Dev. 10, 103-117.

Isshiki T, Pearson B, Holbrook S, Doe CQ. (2001). *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell 106:511-521

Ito K. *et al.*, (1995). Distribution, classification, and development of *Drosophila* glial cells in the late embryonic and early larval ventral nerve cord, *Roux's Arch. Develop. Biol.* **204** pp. 284–307

Jacobs JR. (1993). Perturbed glial template formation precedes axon tract malformation in *Drosophila* mutants. J Neurobiol 24:611-626

Jacobs JR, Goodman CS. (1989). Embryonic development of axon pathways in the *Drosophila* CNS. I. A glial scaffold appears before the first growth cones. J Neurosci 9:2402-2411

Jacobs J.R. (2000). The midline glia of *Drosophila*: a molecular genetic model for the developmental functions of glia, *Prog. Neurobiol.* **62**, pp. 475–508

Janody, F., Reischl, J., Dostatni, N. (2000). Persistence of hunchback in the terminal region of the *Drosophila* blastoderm embryo impairs anterior development. Development 127, 1573-1582.

Jarecki, J., Johnson, E. and Krasnow, M. A. (1999). Oxygen regulation of airway branching in *Drosophila* is mediated by branchless FGF. Cell 99, 211-220.

Joanna C. Jen,[1*] Wai-Man Chan,[7] Thomas M. Bosley,[10] Jijun Wan,[1] Janai R. Carr,[1] Udo Rüb,[11] David Shattuck,[1] Georges Salamon,[2] Lili C. Kudo,[3] Jing Ou,[1] Doris D. M. Lin,[12] Mustafa A. M. Salih,[13] Tülay Kansu,[14] Hesham al Dhalaan,[15] Zayed al Zayed,[16] David B. MacDonald,[15] Bent Stigsby,[15] Andreas Plaitakis,[17] Emmanuel K. Dretakis,[18] Irene Gottlob,[19] Christina Pieh,[20] Elias I. Traboulsi,[21] Qing Wang,[21] Lejin Wang,[21] Caroline Andrews,[7,9] Koki Yamada,[7,9] Joseph L. Demer,[1,4] Shaheen Karim,[4] Jeffry R. Alger,[2] Daniel H. Geschwind,[1] Thomas Deller,[11] Nancy L. Sicotte,[1] Stanley F. Nelson,[5] Robert W. Baloh,[1,6] Elizabeth C. Engle[7,8,9*] . (2004). Mutations in a Human *ROBO* Gene Disrupt Hindbrain Axon Pathway Crossing and Morphogenesis. Science, 4 June. Vol 304, Issue 5676, 1509-1513

Jiang L, Crews ST. (2003). The *Drosophila* dysfusion basic helix-loop-helix (bHLH)-PAS gene controls tracheal fusion and levels of the trachealess bHLH-PAS protein. Mol Cell Biol. Aug;23(16):5625-37.

Jin M.H., Sawamoto K., Ito M., Okano H. (2000). "The interaction between the *Drosophila* secreted protein argos and the epidermal growth factor receptor inhibits dimerization of the receptor and binding of secreted spitz to the receptor", *Mol. Cell. Biol.*, Volume: 20, pp. 2098-2107

Jin, J., Anthopoulos, N., Wetsch, B., Binari, R. C., Isaac, D., D., Andrew, D., Woodgett, J. R. and Manoukian, A. S. (2001). Regulation of *Drosophila* tracheal system development by protein kinase B. Dev.7 Cell 817-827.

Jurata, L. W., Thomas, J. B. and Pfaff, S. L. (2000). Transcriptional mechanisms in the development of motor control. *Curr. Opin. Neurobiol.* 10, 72-79.

Karpen, G. H. and Spradling, A. C. (1992). Analysis of subtelomeric heterochromatin in the *Drosophila* minichromosome *Dp1187* by single P element insertional mutagenesis. *Genetics* 132, 737-753.

Kidd T, Bland K, Goodman CS. (1999). Slit is the midline repellant for the Robo receptor in *Drosophila*. Cell 96:785-794

Kinrade EF, Brates T, Tear G, Hidalgo A. (2001). Roundabout signalling, cell contact and trophic support confine longitudinal glia and axons in the *Drosophila* CNS. Development 128 pp. 207–216

Klaes A., Menne T., Stollewerk A., Scholz H., Klämbt C. (1994). The Ets transcription factors encoded by the *Drosophila* gene pointed direct glial cell differentiation in the embryonic CNS, Cell, Volume: 78, pp. 149—160

Klämbt C. (1993). The *Drosophila* gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells, Development, Volume: 117, pp. 163—176

Klämbt C., Goodman C.S. (1991). Role of the midline glia and neurons in the formation of the axon commissures in the central nervous system of the *Drosophila* embyro, Ann. N.Y. Acad. Sci., Volume: 633, pp. 142—159

Klämbt C., Jacobs J.R., Goodman C.S. (1991). The midline of the *Drosophila* CNS. Model and genetic analysis of cell lineage, migration, development of commissural axon pathways, Cell, Volume: 64, pp. 801—815

Klämbt C., Glazer L., Shilo B.Z. (1992). breathless, a Drosophila FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells, Genes Dev., Volume: 6, pp. 1668—1678

Klug A. , Rhodes D. (1987). 'Zinc fingers': A novel protein motif for nucleic acid recognition. Trends Biochem. Sci. 12: 464- 469

Kolodkin A.L., Pickup A.T., Lin D.M., Goodman C.S., Banerjee U. (1994). Characterization of Star and its interactions with sevenless and EGF receptor during photoreceptor cell development in Drosophila, Development, Volume: 120, pp. 1731—1745

Kolodziej PA, Timpe LC, Mitchell KJ, Fried SR, Goodman CS, Jan LY, Jan YN. (1996). *frazzled* encodes a *Drosophila* member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance, *Cell* **87** pp. 197–204

Kotarski M.A., Leonard D.A., Bennett S.A., Bishop C.P., Wahn S.D., Sedore S.A., Shrader M. (1998). The Drosophila gene asteroid encodes a novel protein and displays dosage-sensitive interactions with Star and Egfr, Genome, Volume: 41, pp. 295—302

Kubota K., Goto S., Eto K., Hayashi S. (2000). "EGF receptor attenuates Dpp signaling and helps to distinguish the wing and leg cell fates in *Drosophila*", *Development*, Volume: 127, pp. 3769-3776

Kurada P., White K. (1998). Ras promotes cell survival in Drosophila by downregulating hid expression, Cell, Volume: 95, pp. 319--329

Lanoue B.R., Gordon M.D., Battye R., Jacobs J.R. (2000). Genetic analysis of vein function in the Drosophila embryonic nervous system, Genome, Volume: 43, pp. 564--573

Lavista-Llanos S, Centanin L, Irisarri M, Russo DM, Gleadle JM, Bocca SN, Muzzopappa M, Ratcliffe PJ, Wappner P. (2002). Control of the hypoxic response in

Drosophila melanogaster by the basic helix-loop-helix PAS protein similar. Mol Cell Biol. Oct;22(19):6842-53

Lee, S., and Kolodziej, P. A. (2002). The plakin Short Stop and the RhoA GTPase are required for E-cadherin-dependent apical surface remodeling during tracheal tube fusion. Development 129, 1509-1520.

Lee J.R., Urban S., Garvey C.F., Freeman M. (2001). Regulated intracellular ligand transport and proteolysis control EGF signal activation in *Drosophila*, Cell, Volume: 107, pp. 161--171

Lee, T., Hacohen, N., Krasnow, M. and Montell, D. J. (1996). Regulated Breathless receptor tyrosine kinase activity required to pattern cell migration and branching in the *Drosophila* tracheal system. Genes Dev. 10, 2912-2921.

LeHoux JG, Fleury A, Ducharme L, Hales DB. (2004). Phosphorylation of the hamster adrenal steroidogenic acute regulatory protein as analyzed by two-dimensional polyacrylamide gel electrophoreses. Mol Cell Endocrinol. Feb 27;215(1-2):127-34

Lin, D. M., Fetter, R. D., Kopczynski, C., Grenningloh, G. and Goodman, C. S. (1994). Genetic analysis of fasciclin II in *Drosophila*: defasciculation, refasciculation and altered fasciculation. Neuron 13, 1055-1069.

Lin, D. M., Auld, V. J. and Goodman, C. S. (1995). Targeted neuronal cell ablation in the *Drosophila* embryo: pathfinding by follower growth cones in the absence of pioneers. *Neuron* 14, 707-715.

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

Llimargas, M. (1999). The Notch pathway helps to pattern the tips of the *Drosophila* tracheal branches by selecting cell fates. Development 126, 2355-2364.

Llimargas, M. (2000). Wingless and its signalling pathway have common and separable functions during tracheal development. Development 127, 4407-4417.

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

Llimargas, M. and Casanova, J. (1999). EGF signalling regulates cell invagination as well as cell migration during formation of tracheal system in *Drosophila*. Dev. Genes Evol. 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. and 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. (1993). Development of the tracheal system. In: Bate, M., Martinez Arias, A. (Eds). The development of *Drosophila melanogaster*, v. 1. Cold Spring Harbor Laboratory Press, New York, NY 609-685.

Martin-Blanco E., Roch F., Noll E., Baonza A., Duffy J.B., Perrimon N. (1999). "A temporal switch in DER signaling controls the specification and differentiation of veins and interveins in the *Drosophila* wing". Development, Volume: 126, pp. 5739-5747

Matthews B.B., Crews S.T. (1999). Drosophila center divider gene is expressed in CNS midline cells and encodes a developmentally regulated protein kinase orthologous to human TESK1, DNA Cell Biol., Volume: 18, pp. 438—445

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

Mellerick DM, Kassis JA, Zhang S-D, Odenwald WF. (1992). Castor encodes a novel zinc finger protein required for the development of a subset of CNS neurons in *Drosophila*. Neuron 9:789-803

Michaud JL, Rosenquist T, May NR, Fan CM. (1998). Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1. Genes Dev. Oct 15;12(20):3264-75

Michaud JL, DeRossi C, May NR, Holdener BC, Fan CM. (2000). ARNT2 acts as the dimerization partner of SIM1 for the development of the hypothalamus. Mech Dev. Feb;90(2):253-61

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

Mitchell Kevin J, Jennifer L Doyle, Tito Serafini, Timothy E Kennedy, Marc Tessier-Lavigne, Corey S Goodman and Barry J Dickson. (1996). Genetic Analysis of *Netrin* Genes in Drosophila: Netrins Guide CNS Commissural Axons and Peripheral Motor Axons. Neuron 17, 203-215

Moran-Rivard, L., Kagawa, T., Saueressig, H., Gross, M. K., Burrill, J. and Goulding, M. (2001). *Evx1* is a postmitotic determinant of V0 interneuron identity in the spinal cord. *Neuron* 29, 385-399.

Morel, V. and Schweisguth, F. (2000). Repression by Suppressor of Hairless and activation by Notch are required to define a single row of *single-minded* expressing cells in the *Drosophila* embryo. Genes Dev. 14, 377-388.

Muqit MM, Feany MB. (2002). Modelling neurodegenerative diseases in Drosophila: a fruitful approach? Nat Rev Neurosci. Mar;3(3):237-43

Murphy, A., M., Lee, T., Andrews, C. M., Shilo, B.-Z. and Montell, D. J. (1995). The *breathless* FGF homolog, a downstream target of *Drosophila* C/EBP in the developmental control of cell migration. Development 121, 2255-2263.

Nambu JR, Franks RG, Hu S, Crews ST. (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.O., Wharton K.A., Crews S.T. (1991). The Drosophila single-minded gene encodes a helix–loop–helix protein that acts as a master regulator of CNS midline development, Cell, Volume: 67, pp. 1157-1167

Nelson W. J. Tube morphogenesis: closure, but many openings remain. (2003). TRENDS in Cell Biology, December, Vol.13 No.12, pp. 615-621

Nguyen H., Q., Hoffman-Liebermann, B., and Liebermann, D. A. (1993). The zinc finger transcription factor Egr-1 is essential for and restricts differentiation along the macrophage lineage. Cell 72, 197-209.

Noordermeer J.N., Kopczynski C.C., Fetter R.D., Bland K.S., Chen W.Y., Goodman C.S. (1998). Wrapper, a novel member of the Ig superfamily, is expressed by midline glia and is required for them to ensheath commissural axons in Drosophila, Neuron, Volume: 21, pp. 991—1001

Nussbaumer U, Halder G, Groppe J, Affolter M, Montagne J. (2000). Expression of the blistered/DSRF gene is controlled by different morphogens during Drosophila trachea and wing development. Mech Dev. Aug;96(1):27-36

Nüsslein-Volhard, C., Wieschaus, E. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. Wilhelm Roux's Arch. Dev. Biol. 193, 267-282.

Oda, H., Uemura, T., Harada, Y., Iwai, Y. and Takeichi, M. (1994). A *Drosophila* homolog of cadherin associated with Armadillo and essential for embryonic cell-cell adhesion. Dev. Biol. 165, 716-726.

Oellers N, Dehio M, Knust E. (1994). bHLH proteins encoded by the Enhancer of split complex of Drosophila negatively interfere with transcriptional activation mediated by proneural genes. Mol Gen Genet. Sep 1;244(5):465-73

Ohshiro, T., Emori, Y. and Saigo, K. (2002). Ligand-dependent activation of *breathless* FGF receptor gene in *Drosophila* developing trachea. Mech Dev. 114, 3-11.

Ohshiro T., Saigo K. (1997). Transcriptional regulation of *breathless* FGF receptor gene by binding of TRACHEALESS/dARNT heterodimers to three central midline elements in *Drosophila* developing trachea. Development 124:3975-3986

O'Neill E.M., Rebay I., Tjian R., Rubin G. (1994). The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway, Cell, Volume: 78, pp. 137-147

Orenic T, Chidsey J, Holmgren R. (1987). Cell and cubitus interruptus dominant: two segment polarity genes on the fourth chromosome in *Drosophila*. Dev Biol. Nov;124(1):50-6.

Oyhenart J, Le Goffic R, Samson M, Jegou B, Raich N. (2003). Phtf1 is an integral membrane protein localized in an endoplasmic reticulum domain in maturing male germ cells. Biol Reprod. Mar;68(3):1044-53

Patel N.H. (1994). Imaging neuronal subsets and other cell types in whole-mount Drosophila embryos and larvae using antibody probes, Fyrberg E., Goldstein L.S.G., (Ed.) Drosophila melanogaster : Practical Uses in Cell and Molecular Biology, Academic Press, San Diego, CA. pp. 446—488

Park JS, Wang M, Park SJ, Lee SH. (1999). Zinc finger of replication protein A, a non-DNA binding element, regulates its DNA binding activity through redox. J Biol Chem. Oct 8;274(41):29075-80

Pascual M, Pozas E, Barallobre MJ, Tessier-Lavigne M, Soriano E. (2004) .Coordinated functions of Netrin-1 and Class 3 secreted Semaphorins in the guidance of reciprocal septohippocampal connections. Mol Cell Neurosci. May;26(1):24-33

Patel, N. H., Schafer, B., Goodman, C. S. and Holmgren, R. (1989). The role of segment polarity genes during *Drosophila* neurogenesis. *Genes Dev.* 3, 890-904.

Pavletich and Pabo. (1991). Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. Science. May 10;252(5007):809-17.

Pavletich and Pabo. (1993). Crystal structure of a five-finger GLI-DNA complex: new perspectives on zinc fingers. Science. Sep 24;261(5129):1701-7.

Perrimon N., Perkins L.A. (1997). There must be 50 ways to rule the signal: the case of the *Drosophila* EGF receptor, Cell, Volume: 89, pp. 13—16

Peyrefitte S, Kahn D, Haenlin M. (2001). New members of the Drosophila Myc transcription factor subfamily revealed by a genome-wide examination for basic helix-loop-helix genes. Mech Dev. Jun;104(1-2):99-104

Price J.V., Clifford R.J., Schupbach T. (1989). The maternal ventralizing locus torpedo is allelic to faint little ball, an embryonic lethal, and encodes the Drosophila EGF receptor homolog, Cell, Volume: 56, pp. 1085—1092

Ptashne M. and Gann A. (1997). Transcriptional activation by recruitment. Nature 386: 569–577

Rajagopalan, S., Vivancos, V., Nicolas, E., and Dickson, B.J. (2000). Selecting a longitudinal pathway: Robo receptors specify the lateral position of axons in the Drosophila CNS. Cell 103: 1033-1045

Raz E., Shilo B.-Z. (1992). Dissection of the faint little ball (flb) phenotype: Determination of the development of the Drosophila central nervous system by early interactions in the ectoderm, Development, Volume: 114, pp. 113—123

Reich A., Shilo B.Z. (2002). "Keren, a new ligand of the *Drosophila* epidermal growth factor receptor, undergoes two modes of cleavage", *EMBO J.*, Volume: 21, pp. 4287-4296

Reichman-Fried, M. and Shilo, BZ. (1995). Breathless, a *Drosophila* FGF receptor homolog, is required for the onset of tracheal cell migration and tracheole formation. Mech Dev. 52, 265-73.

Ro, H-S. and Roncari, D. A. K. (1991). The C/EBP-binding region and adjacent sites regulate expression of the adipose P2 gene in human preadipocytes. Mol. Cell Biol. 11, 2303-2306.

Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D. M., Benz, W. K. and Engels, W. R. (1988). A stable source of P-element transposase in *Drosophila melanogaster*. *Genetics* 118, 461-470.

Roggel Ronald, Patricia J. Greenl, Jun Uranol, Shirley Horn-Saban2, Marek Mlodzik3, Ben-Zion Shilo2, Volker Hartenstein1 and Utpal Banerjeel, (1995). The role of yan in mediating the choice between cell division and differentiation. Development 121, 3947-3958

Rorth, P. and Montell, D. J. (1992). *Drosophila* C/EBP: a tissue-specific DNA-binding protein required for embryonic development. *Genes Dev.* 6, 2299-2311.

Rothberg J.M., Hartley D.A., Walther Z., Artavanis T.S. (1988). slit : an EGF-homologous locus of D. melanogaster involved in the development of the embryonic central nervous system, Cell, Volume: 55, pp. 1047—1059

Rubin GM, Spradling AC. (1982). Genetic transformation of Drosophila with transposable element vectors. Science. Oct 22;218(4570):348-53.

Ruiz i Altaba A. (1998). Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog. Development. Jun;125(12):2203-12

Rutledge B.J., Zhang K., Bier E., Jan Y.N., Perrimon N. (1992). The Drosophila spitz gene encodes a putative EGF-like growth factor involved in dorsal–ventral axis formation and neurogenesis, Genes Dev., Volume: 6, pp. 1503—1517

Sabatier C, Plump AS, Le Ma, Brose K, Tamada A, Murakami F, Lee EY, Tessier-Lavigne M. (2004). The divergent Robo family protein rig-1/Robo3 is a negative regulator of slit responsiveness required for midline crossing by commissural axons. Cell. Apr 16;117(2):157-69

Saha S., Brickman J. M., Lehming N. and Ptashne M. (1993). New eukaryotic transcriptional repressors. Nature **363:** 648–652

Salinas P. C. (2003). The morphogen sonic hedgehog collaborates with netrin-1 to guide axons in the spinal cord. Trends Neurosci. Dec;26(12):641-3

Samakovlis, C., Hacohen, N., Manning, G., Sutherland, D.X., Guillemin, K. and Krasnow, M. A. (1996). Development of *Drosophila* tracheal system occurs by a series

of morphologically distinct but genetically coupled branching events. Development 122, 1395-1407.

Samakovlis, C., Manning, G., Steneberg, P., Hacohen, N., Cantera, R. and Krasnow, M. (1996b). Genetic control of epithelial tube fusion during *Drosophila* tracheal development. Development 122, 3531-3536.

Schmid, A., Chiba, A. and Doe, C. Q. (1999). Clonal analysis of *Drosophila* embryonic neuroblasts: neural cell types, axon trajectories, and muscle targets. Development 126, 4653-4689.

Schnepp B., Grumbling G., Donaldson T., Simcox A. (1996). "Vein is a novel component in the *Drosophila* epidermal growth factor receptor pathway with similarity to the neuregulins", Genes Dev., Volume: 10, pp. 2302-2313

Scholz H., Sadlowski E., Klaes A., Klämbt C. (1997). Control of midline glia development in the embryonic Drosophila CNS, Mech. Dev., Volume: 64, pp. 137—151

Schulz C., Wood C.G., Jones D.L., Tazuke S.I., Fuller M.T. (2002). "Signaling from germ cells mediated by the rhomboid homolog stet organizes encapsulation by somatic support cells", Development, Volume: 129, pp. 4523-4534

Schweitzer R., Shilo B.-Z. (1997). A thousand and one roles for the Drosophila EGF receptor, Trends Genet., Volume: 13, pp. 191--196

Schweitzer R., Shaharabany M., Seger R., Shilo B.Z. (1995). Secreted spitz triggers the DER signaling pathway and is a limiting component in embryonic ventral ectodermal determination, Genes Dev., Volume: 9, pp. 1518—1529

Schweitzer R., Howes R., Smith R., Shilo B.Z., Freeman M. (1995). Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos, Nature, Volume: 376, pp. 699—702

Sedaghat Y., Sonnenfeld M.J. (2002). The jing gene is required for embryonic brain development in *Drosophila*, Dev. Genes Evol., Volume: 212, pp. 277—287

Sedaghat, Y., Miranda, W. and Sonnenfeld, M. J. (2002). The *jing* Zn-finger transcription factor is a mediator of cellular differentiation in the *Drosophila* CNS midline and trachea. Development 129, 2591-2606.

Seeger M, Tear G, Ferres-Marco D, Goodman CS. (1993). Mutations affecting growth cone guidance in *Drosophila*: genes necessary for guidance toward or away from the midline. Neuron 10:409-426

Sepp KJ, Schulte J, Auld VJ. (2000). Developmental dynamics of peripheral glia in *Drosophila melanogaster*, *Glia* 30, pp. 122–133

Shiga, Y., Tanada-Matakatsu, M. and Hayashi, S. A. (1996). Nuclear GFP/-galactosidase fusion protein as a marker of morphogenesis in living *Drosophila*. Dev. Growth Differ. 38, 99-106.

Silver J. (1993). Glia-neuron interactions at the midline of the developing mammalian brain and spinal cord. Perspect Dev Neurobiol 1:227-236

Simon M.A. (2000). Receptor tyrosine kinases: specific outcomes from generic signals, Cell, Volume: 87, pp. 13—15

Simon M.A., Bowtell D.D., Dodson G.S., Laverty T.R., Rubin G.M. (1991). Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase, Cell, Volume: 67, pp. 701—716

Skeath, J. B. and Doe, C. Q. (1998). Sanpodo and Notch act in opposition to Numb to distinguish sibling *Neuron* fates in the *Drosophila* CNS. Development 125, 1857-1865.

Skeath J. B. and Thor S. (2003). Genetic control of *Drosophila* nerve cord development. Current Opinion in Neurobiology. February 13(1); pp: 8-15

Skeath J.B. (1998). "The *Drosophila* EGF receptor controls the formation and specification of neuroblasts along the dorsal-ventral axis of the *Drosophila* embryo", Development, Volume: 125, pp. 3301-3312

Sonnenfeld M and Jacob JR. (1995). Apoptosis of the midline glia during Drosophila embryogenesis: a correlation with axon contact. Development. Feb;121(2):569-78

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

Sonnenfeld, M. J., Barazesh, N., Sedaghat, Y. and Fan, C. (2004). The *jing* and *ras1* pathways are functionally related during CNS midline and tracheal development. Mech. Dev. 121, 1531-1547.

Spana, E. P., Kopczynski, C., Goodman, C. S. and Doe, C. Q. (1995). Asymmetric localization of numb autonomously determines sibling neuron identity in the *Drosophila* CNS. Development 121, 3489-3494.

Spicer DB, Rhee J, Cheung WL, Lassar AB. (1996). Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. Science. Jun 7;272(5267):1476-80

Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. Science 218, 341-347.

Spradling, A. C. (1986). P element-mediated transformation. In *Drosophila*: *A Practical Approach* (ed. D. B. Roberts), pp. 175-197. Oxford-Washington DC: IRL Press.

Spradling, A. C., Stern, D., Beaton, A., Rhem, E. J., Laverty, T., Mozden, N., Misra, S. and Rubin, G. M. (1999). The Berkeley *Drosophila* Genome Project gene disruption project: Single P-element insertions mutating 25% of vital *Drosophila* genes. Genetics 153, 135-177.

Steindler DA. (1993). Glial boundaries in the developing nervous system. Annu Rev Neurosci 16:445-470

Stemerdink C., Jacobs J.R. (1997). Argos and Spitz group genes function to regulate midline glial cell number in Drosophila embryos, Development, Volume: 124, pp. 3787—3796

Steneberg, P., Hemphala, J. and Samakovlis, C. (1999). Dpp and Notch specify the fusion cell fate in the dorsal branches of the *Drosophila* trachea. Mech. Dev. 87, 153-163.

Sturtevant, M. A., Roark, M., O'Neill, S. W., Biehs, B., Colley, N. and Bier, E. (1996). The Drosophila rhomboid protein is concentrated in patches at the apical cell surface. Dev. Biol. 174, 298-309.

Sugimoto Y, Taniguchi M, Yagi T, Akagi Y, Nojyo Y, Tamamaki N. (2001). Guidance of glial precursor cell migration by secreted cues in the developing optic nerve, Development 128, pp. 3321–3330

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

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

Tautz, D. and Pfeifle, C. (1989). A non radioactive in situ hybridization method for the localizaton of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. Chromosoma 98, 81-85.

Tayler TD, Robichaux MB, Garrity PA. (2004). Compartmentalization of visual centers in the Drosophila brain requires Slit and Robo proteins. Development. Dec;131(23):5935-45

Tepass, U., Theres, C. and Knust, E. (1990). Crumbs encodes an EGF-like proteinexpressed on apical membranes of *Drosophila* epithelial cells and required for organization of epithelia. Cell 61, 787-799.

Tepass, U. (1996). Crumbs, a component of the apical membrane, is required for zonula adherens formation in primary epithelia of *Drosophila*. Dev. Biol. 177, 217-225.

Therianos S, Leuzinger S, Hirth F, Goodman CS, Reichert H. (1995). Embryonic development of the *Drosophila* brain: formation of commissural and descending pathways. Development 121:3849-3860

Thomas JB, Crews ST, Goodman CS. (1988). Molecular genetics of the *single-minded* locus: a gene involved in the development of the *Drosophila* nervous system. Cell 52:133-141

Tsruya R., Schlesinger A., Reich A., Gabay L., Sapir A., Shilo B.Z. (2002). "Intracellular trafficking by Star regulates cleavage of the *Drosophila* EGF receptor ligand Spitz", Genes Dev., Volume: 16, pp. 222-234

Uemura, T., Oda, H., Kraut, R., Hayashi, S., Kotaoka, Y., Takeichi, M. (1996). Zygotic *Drosophila* E-cadherin expression is required for processes of dynamic epithelial cell rearrangement in the *Drosophila* embryo. Genes Dev. 10, 659-71.

Urban S., Lee J.R., Freeman M. (2001). Drosophila Rhomboid-1 defines a family of putative intramembrane serine proteases, Cell, Volume: 107, pp. 173—182

Uv, A., Cantera, R. and Samakovlis, C. (2003). *Drosophila* tracheal morphogenesis: intricate cellular solutions to basic plumbing problems. Trends in Cell Biol. 13, 301-309.

van der Geer P., Hunter T., Lindberg R.A. (1994). Receptor-protein tyrosine kinases and their signal transduction pathways, Ann. Rev. Cell Biol., Volume: 10, pp. 251—337

Van Vactor, D., Sink, H., Fambrough, D., Tsoo, R. and Goodman, C. S. (1993). Genes that control neuromuscular specificity in *Drosophila*. Cell 73, 1137-1153.

Vincent, S. Ruberte, E., Grieder, N. C., Chen, C. K., Haerry, T., Schuh, R. and Affolter, M. (1997). DPP controls tracheal cell migration along the dorsoventral body axis of the *Drosophila* embryo. Development 124, 2741-2750.

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

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

Wassarman J.D., Freeman M. (1997). Control of EGF receptor activation in *Drosophila*, Trends Cell Biol., Volume: 7, pp. 431—436

Wassarman J.D., Therrien M. (1997). RAS1-mediated photoreceptor development in *Drosophila*, Adv. Dev. Biol., Volume: 5, pp. 1-41

Wassarman J.D., Urban S., Freeman M. (2000). A family of rhomboid-like genes: Drosophila rhomboid-1 and roughoid/rhomboid-3 cooperate to activate EGF receptor signaling, Genes Dev., Volume: 14, pp. 1651-1663

Wharton, K. A. and Crews, S. T. (1993). CNS midline enhancers of the *Drosophila slit* and *Toll* genes. Mech. Dev. 40, 141-154.

Wharton, J., K. A., Franks, R. G., Kasai, Y. and Crews, S. T. (1994). Control of CNS midline transcription by asymmetric E-box elements: similarity to xenobiotic responsive regulation. Development 120, 3563-3569.

Whiteley, M., Noguchi, P. D., Sensabaugh, S. M., Odenwald, W. F. and Kassis, J. A. (1992). The *Drosophila* gene *escargot* encodes a zinc finger motif found in snail-related genes. Mech. Dev. 36, 117-127.

Wilk, R., Weizman, I. and Shilo, B.-Z. (1996). *trachealess* encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. Genes Dev. 10, 93-102.

Wittmann CW, Wszolek MF, Shulman JM, Salvaterra PM, Lewis J, Hutton M, Feany MB. (2001). Tauopathy in Drosophila: neurodegeneration without neurofibrillary tangles. Science. Jul 27;293(5530):711-4

Wodarz, A., Hinz, U., Engelbert, M. and Knust, E. (1995). Expression of *crumbs* confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. Cell 82, 67-76.

Wolff, T., Ready, D.F. (1991). The beginning of pattern formation in the Drosophila compound eye: the morphogenetic furrow and the second mitotic wave. Development 113(3):841-850

Wong K, Ren XR, Huang YZ, Xie Y, Liu G, Saito H, Tang H, Wen L, Brady-Kalnay SM, Mei L, Wu JY, Xiong WC, Rao Y. (2001). Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway. Cell. Oct 19;107(2):209-21

Xiao, H., Hrdlicka, L. A. and Nambu, J. R. (1996). Alternate functions of the *single-minded* and *rhomboid* genes in development of the *Drosophila* ventral neuroectoderm. Mech. Dev. 58, 65-74.

Xiong, W. C., Okano, H., Patel, N. H., Blendy, J. A. and Montell, C. (1994). *Repo*encodes a glial-specific homeo domain protein required in the *Drosophila* nervous system. Genes Dev. 8, 981-994.

Yang L., Baker N.E. (2003). Cell cycle withdrawal, progression and cell survival regulation by EGFR and its effectors in the differentiating Drosophila eye, Dev. Cell, Volume: 4, pp. 359—369

Zak N.B., Wides R.J., Scheijter E.D., Raz E., Shilo B.-Z. (1990). Localization of the DER/flb protein in embryos: implications on the faint little ball lethal phenotype, Development, Volume: 109, pp. 865—874

Zelzer, E. and Shilo, B.-Z. (2000). Interactions between the bHLH-PAS protein TRACHEALESS and the POU-domain protein DRIFTER, specifies tracheal cell fates. Mech. Dev. 91, 163-173.

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

Zhou, L., Xiao, H. and Nambu, J. R. (1997). CNS midline to mesoderm signaling in *Drosophila*. Mech. Dev. 67, 59-68.

Chapter Seven

Appendices

Sonnenfeld et al. (2004). The *jing* and *ras1* pathways are functionally related during CNS midline and tracheal development.

The following outlines the experimental contributions of the authors of this manuscript. RNA isolation for microarray analysis and new experimental procedures for in situ hybridization was established by Y. Sedaghat. Whole-mount in situ hybridization using *Egfr* and *jing* riboprobe (figure 2) was performed by Y. Sedaghat. Other parts of this paper were done by Dr. Sonnenfeld , N. Barazesh and C. Fan. Dr. M. Sonnenfeld wrote the manuscript.

The *jing* and *ras1* pathways are functionally related during CNS midline and tracheal development

Margaret J. Sonnenfeld , Nasrin Barazesh, Yalda Sedaghat, Cathy Fan

Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Ont., Canada K1H 8M5

Mechanism of Development, vol: 121 Issue: 12, December, 2004 pp:1531-1547

Abstract

The *Drosophila jing* gene encodes a zinc finger protein required for the differentiation and survival of embryonic CNS midline and tracheal cells. We show that there is a functional relationship between *jing* and the *Egfr* pathway in the developing CNS midline and trachea. *jing* function is required for *Egfr* pathway gene expression and MAPK activity in both the CNS midline and trachea. *jing* over-expression effects phenocopy those of the *Egfr* pathway and require *Egfr* pathway function. Activation of the *Egfr* pathway in loss-of-function *jing* mutants partially rescues midline cell loss. *Egfr* pathway genes and *jing* show dominant genetic interactions in the trachea and CNS midline. Together, these results show that *jing* regulates signal transduction in developing midline and tracheal cells.

Introduction

The *Drosophila jing* gene functions in multiple developmental processes and encodes a nuclear protein with zinc fingers and regions suggestive of transcriptional regulation (Sedaghat et al., 2002; Liu and Montell, 2001). In the ovary, *jing* is required for border cell migration downstream of the vertebrate homolog of the basic region/leucine zipper transcription factor CCAAT enhancer-binding protein (C/EBP) (Liu and Montell, 2001). During embryogenesis, *jing* function is required for the differentiation and survival of cells in the CNS midline, trachea and brain downstream of the master regulators *single-minded* and *trachealess* (Sedaghat et al., 2002; Sedaghat and Sonnenfeld, 2002). JING shows a nuclear localization in CNS midline glia and tracheal cells during early and late stages of differentiation suggesting that it may have a regulatory role in these cells. However, the mechanism of *jing* function and its downstream targets are unknown.

Previous studies show that tyrosine kinase signaling through the *Drosophila* epidermal growth factor receptor (EGFR) regulates midline glial differentiation and survival as well as tracheal patterning (Sonnenfeld and Jacobs, 1994; Scholz et al., 1997; Stemerdink and Jacobs, 1997; Wappner et al., 1997). Tracheal activation of the *Egfr* pathway controls the formation of the dorsal trunk and visceral branches (Wappner et al., 1997). The tracheal and CNS midline phenotypes of embryos homozygous mutant for *jing* and *Egfr* pathway genes are similar suggesting that these genes may function in the same pathway (Sonnenfeld and Jacobs, 1994; Sedaghat et al., 2002). To address this and in order to understand the regulatory mechanisms that control midline glial and tracheal cell

differentiation we have characterized the functional relationship between the *jing* and *Egfr* pathways during embryogenesis.

Egfr signaling is required in multiple developmental processes including *Drosophila* oogenesis, embryogenesis and adult eye development (Casci and Freeman, 1999; Wassarman and Freeman, 1997; Schweitzer and Shilo, 1997; Perrimon and Perkins, 1997). Receptor tyrosine kinases (RTKs) activate RAS1 which in turn activates protein kinases in the Ras1/mitogen activated protein kinase (MAPK) signaling cascade. MAPKs phosphorylate transcription factors and result in the regulation of gene expression and cellular development. Data indicate that RTKs all act through a common pathway involving MAPK and generate a generic signal that is interpreted in responding cells according to their developmental history (Halfon et al., 2000; Simon, 2000; van der Geer et al., 1994; Gabay et al., 1997a,b).

Tissue-specific activities of the EGFR in the CNS midline and trachea are regulated by the ligand SPI, which is similar to the mammalian TGF-α (Rutledge et al., 1992). Activation of SPI requires two proteins, Rhomboid-1 (RHO-1) (a seven transmembrane serine protease) and STAR (S) (a single-pass transmembrane protein) which regulate the intracellular trafficking and proteolysis of SPI (Lee et al., 2001; Urban et al., 2001; Bier et al., 1990; Kolodkin et al., 1994; Guichard et al., 1999; Schweitzer et al., 1995a; Bang and Kintner, 2000; Wassarman et al., 2000).

Differentiation of CNS midline glia during *Drosophila* embryogenesis requires activation of EGFR (Zak et al., 1990; Raz and Shilo, 1992). EGFR is widely expressed and is activated in the midline due to expression of *rho* (Raz and Shilo, 1992; Zak et al., 1990;

Gabay et al., 1997a,b). Regulation of EGFR activity in the CNS midline occurs in the presence of soluble Spi and by induction of the inhibitory protein Argos (Schweitzer et al., 1995a,b; Golembo et al., 1996a,b). Membrane-SPI is processed into secreted-SPI in CNS neurons and therefore axon contact is required for EGFR activation in midline glia (Bergmann et al., 2002). EGFR activates the conserved MAPK pathway which then activates the ETS domain transcription factor PNT and inactivates YAN (O'Neill et al., 1994; Scholz et al., 1997).

Loss or reduction of function in genes of the *Egfr* pathway leads to the premature death of the midline glia during stage 12 (Sonnenfeld and Jacobs, 1994; Scholz et al., 1997; Lanoue et al., 2000). In contrast, an increase in EGFR/ras1/raf signaling correlates with an increased survival of midline glia (Sonnenfeld and Jacobs, 1995; Scholz et al., 1997; Stermerdink and Jacobs, 1997; Lanoue and Jacobs, 1999; Dong and Jacobs, 1997). There is now strong evidence that this survival event is mediated through Ras1, which in turn inhibits apoptosis by phosphorylating head involution defective (Hid) and inactivating its proapoptotic effects (Bergmann et al., 1998, 2002; Kurada and White, 1998).

In this study, we show that the *jing* and *Egfr* pathways are functionally related in the embryonic CNS midline and trachea. The results demonstrate that *jing* is required for MAPK activity and *Egfr* pathway gene expression. *jing* over-expression can induce extra *Egfr* -expressing glia in the CNS midline and therefore resembles the over-expression phenotype of *Egfr* pathway genes (Klaes et al., 1994; Sonnenfeld and Jacobs, 1995; Stemerdink and Jacobs, 1997). Dominant genetic interactions reveal that *jing* and *Egfr*

function is critical for proper CNS midline and tracheal cell development. Together, these results show that *jing* is required for proper EGFR signaling.

Results

jing function is required for MAPK activity in the CNS midline and tracheal placodes

In homozygous *jing* mutant embryos, many cells in the CNS midline and trachea undergo apoptosis which is first detectable by TUNEL labeling during stage 12 and 10, respectively (Sedaghat et al., 2002). Since EGFR signaling is essential for the survival of midline glia we thought it may be possible that EGFR-mediated RAS1 signaling is down-regulated in *jing* mutant embryos (Scholz et al., 1997; Sonnenfeld and Jacobs, 1994; Lanoue et al., 2000). The activity of the Ras1 pathway was investigated in the absence of *jing* function by monitoring the presence of the activated, diphosphorylated form of MAPK (ERK) in CNS midline and tracheal cells in homozygous *jing* mutant embryos. These embryos were stained with a monoclonal antibody that recognizes diphospho-ERK representing the total activities of RTK signaling in *Drosophila* embryos (Gabay et al., 1997a,b).

Wild-type and homozygous *jing* mutant embryos were stained with anti-dp-ERK and anti-TRH to identify tracheal cells or anti-SIM to identify midline cells. At stage 10, MAPK was strongly activated in SIM-positive midline cells in wild-type embryos but not in homozygous *jing* mutant embryos (Fig. 1A–B′). It is possible that the MAPK reductions are not due to cell death since there were no defects in midline cell numbers

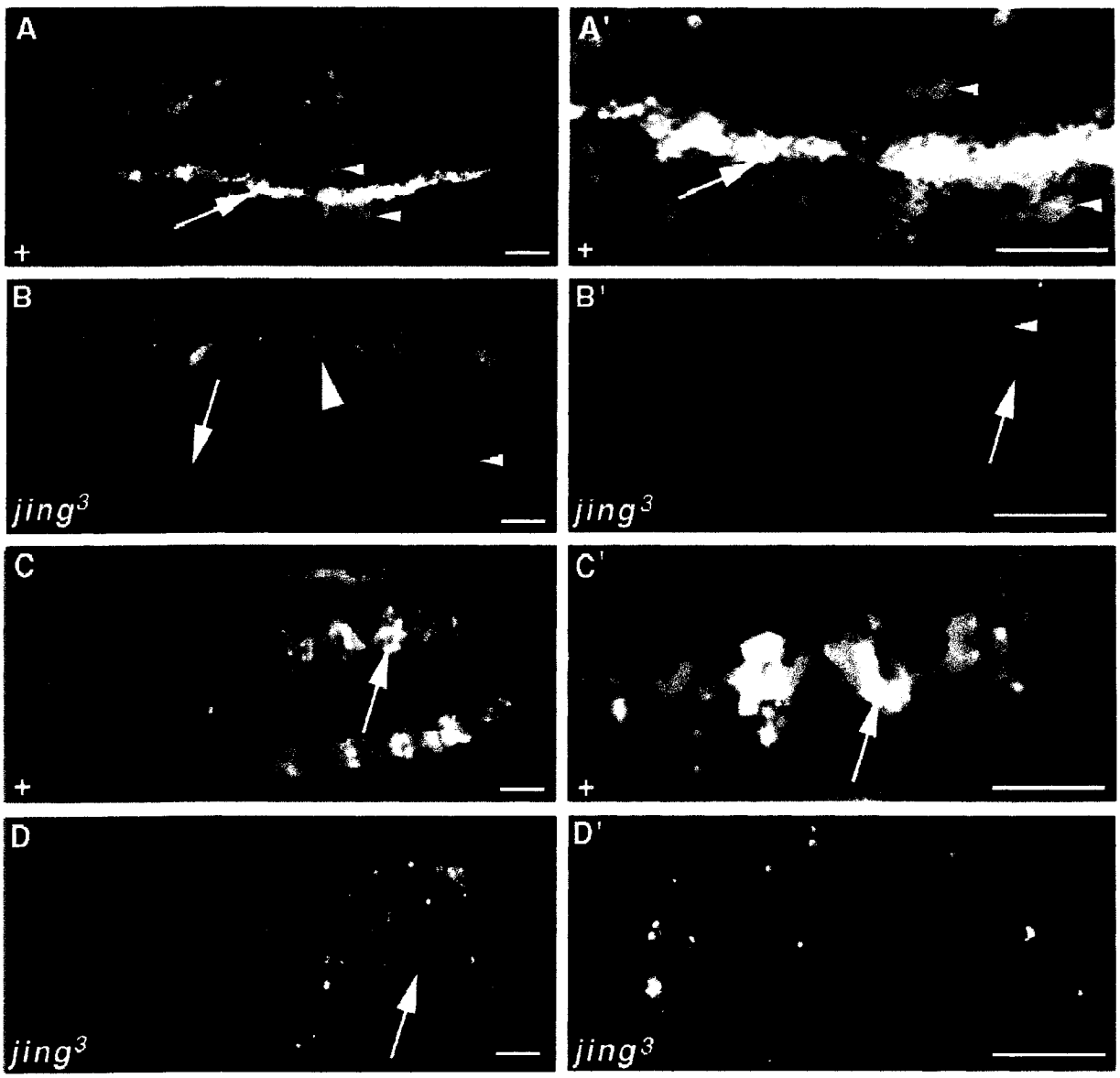

A
+
A'
+
B
jing3
B'
jing3
C
+
C'
+
D
jing3
D'
jing3

Fig. 1. *jing* is required to maintain MAPK activity in CNS midline and tracheal cells. Whole-mount embryos were double labeled with anti-dpErk and anti-SIM (A–B′) or anti-trachealess (TRH) (C–D′). (A) Midline cells show MAPK activity at stage 10 (arrow). The adjacent ventral ectoderm also shows MAPK activity (arrowheads). (A′) Close-up view of the midline cells in (A) showing that the majority of cells express MAPK (arrow). (B,B′) A stage 10 homozygous $jing^3$ mutant embryo with reduced MAPK activity in the midline (arrow) and ventral ectoderm (small arrowhead). In segments containing more MAPK in the midline (B′, arrow) there is more MAPK in the ventral ectoderm (arrowhead) consistent with the inductive capabilities of midline cells. The large arrowhead in (B) denotes reduced EGFR-dependent MAPK activity in the tracheal pits. (C) A wild-type stage 11 embryo showing MAPK activity in the tracheal pits (arrow). (C′) Close-up view of embryo in (C) showing that many TRH^+ cells have MAPK activity. (D) An homozygous stage 11 $jing^3$ mutant embryo showing a severe phenotype. Most TRH^+ cells do not have *btl* -dependent MAPK activity in the tracheal pits (arrow). (D′) Close-up view of the embryo in (D). All images are merged. Scale bars: 30 μm.

and nuclear size in *jing* mutants with reduced MAPK (Fig. 1 B,B′). MAPK activity was also reduced in the ventral ectoderm in regions where midline MAPK activity was weak, consistent with its dependence on active SPI from the midline (Golembo et al., 1996b; Schweitzer et al., 1995a; Gabay et al., 1997a).

Activation of EGFR by SPI in the tracheal placodes is required for formation of the dorsal trunk and visceral branch of the tracheal network (Wappner et al., 1997; Gabay et al., 1997a). During stage 10, MAPK activity in the tracheal placodes is dependent upon proper *Egfr* pathway function (Gabay et al., 1997b). EGFR-dependent MAPK activity in the tracheal placodes was reduced in homozygous stage 10 *jing* mutant embryos (Fig. 1 B, arrowhead). During stage 11, MAPK activity is dependent on the FGF receptor, *breathless* (*btl*) in wild-type embryos (Gabay et al., 1997b; Glazer and Shilo, 1991; Klämbt et al., 1992; Reichman-Fried and Shilo, 1995) but MAPK was not activated in homozygous *jing* mutant embryos (Fig. 1 C–D′). Therefore, *jing* function is required during both *Egfr* - and *btl* -dependent stages of MAPK activity in the developing trachea.

jing is required for Egfr pathway gene expression

To determine whether *jing* is required for *Egfr* pathway transcription, we analyzed the mRNA levels of *Egfr* pathway genes in *jing* homozygous mutant embryos and after over-expression in the CNS midline and trachea. As shown by microarray analysis, the levels of *ras1* mRNA were reduced by 50% in *jing* homozygous mutant embryos compared to the levels of control w^{118} and midline glial-expressed genes *wrapper* and *asteroid* (*ast*) ($P < 0.05$) (Fig. 2A) (Kotarski et al., 1998; Noordermeer et al., 1998).

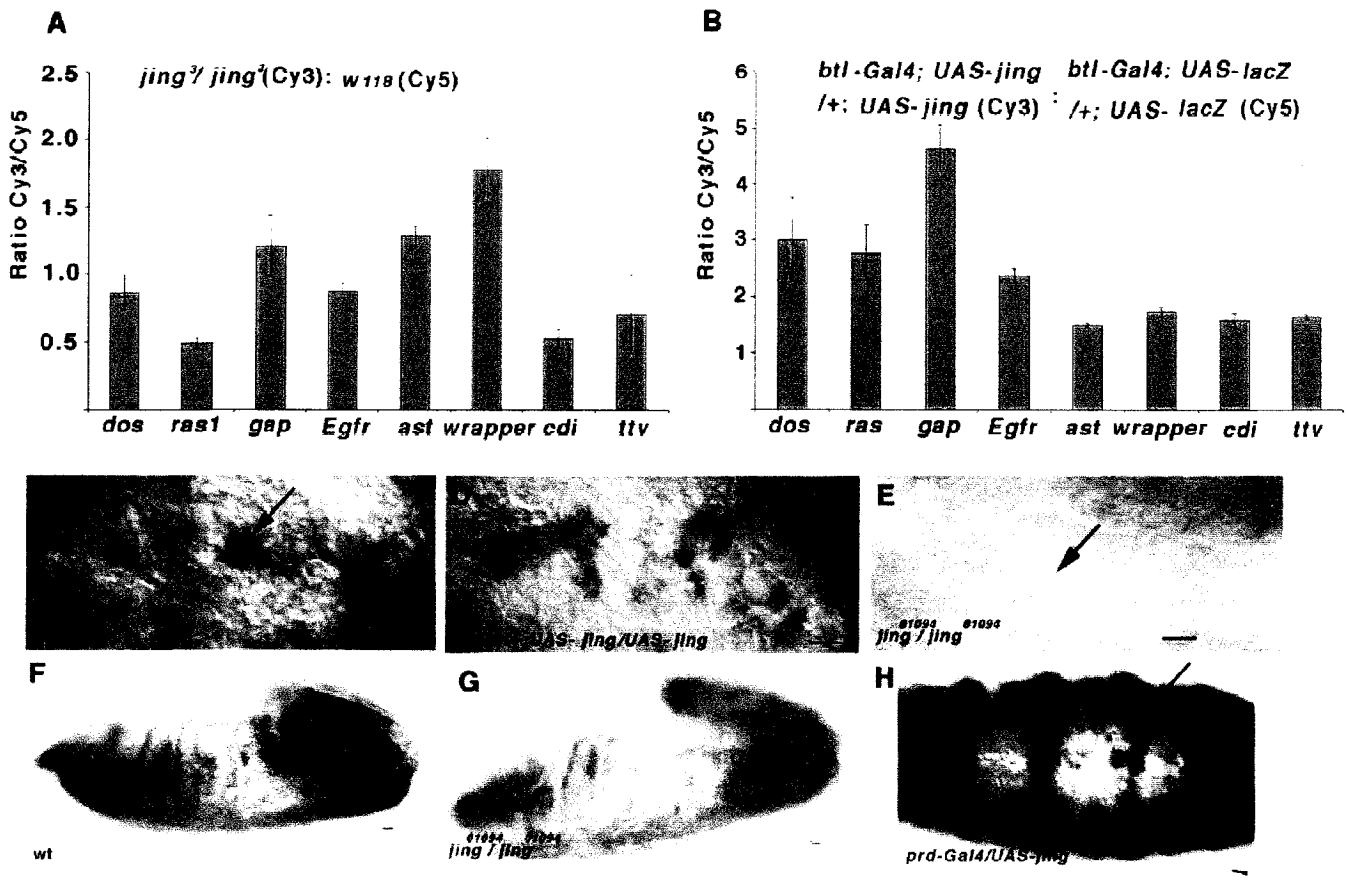

A
jing3/ jing3(Cy3): w118(Cy5)
Ratio Cy3/Cy5
2.5
2.0
1.5
1.0
0.5
dos
ras1
gap
Egfr
ast
wrapper
cdi
ttv
B
btl-Gal4; UAS-jing /+; UAS-jing (Cy3)
btl-Gal4; UAS-lacZ /+; UAS-lacZ (Cy5)
Ratio Cy3/Cy5
6
5
4
3
2
1
dos
ras
gap
Egfr
ast
wrapper
cdi
ttv
E
jing e1094 / jing e1094
F
wt
G
jing e1094 / jing e1094
H
prd-Gal4/UAS-jing

Fig. 2. Effects of *jing* on *Egfr* pathway gene expression. (A,B) Microarray analysis of total RNA extracted from embryos homozygous mutant for $jing^{3}$ (A) and over-expressing *jing* in CNS midline glia and trachea (B). Ratios of experimental (Cy3) to control (Cy5) mRNA fluorescent intensities are shown. (A) In homozygous *jing* mutants, the levels of *ras* and *cdi* mRNA are most significantly reduced (50% reduction, $P < 0.05$) from control (w^{118}) levels. (B) *jing* was over-expressed in CNS midline glia and in the trachea using the *btl -Gal4* driver. mRNA levels of all indicated *Egfr* pathway genes increased by at least two-fold over control levels and over those of other midline or tracheal-expressed genes ($P < 0.05$). (C–G) Whole-mount in situ hybridization using an *Egfr* riboprobe. Anterior is to the left with ventral views (C–E,H) and sagittal views of the trachea (F,G). (C) In wild-type stage 12/3 embryos, *Egfr* is expressed in 1–2 midline glia (arrow). (D) There is an increase in the number of *Egfr* -expressing glia (arrow) after *jing* over-expression in midline glia, as driven by *sli -Gal4*. (E) There is an absence of CNS midline *Egfr* expression (arrow) in stage 12/3 homozygous $jing^{01094}$ mutant embryos. (F) *Egfr* expression in the trachea (arrow) of control stage 12 embryos. (G) *Egfr* expression is reduced in the trachea of $jing^{01094}$ homozygous mutant stage 12 embryos. (H) Strong expression of *jing* mRNA in *prd-expressing* stripes in a stage 13 embryo carrying P[*prd -Gal4*] and P[*UAS -jing*]. Scale bars: 10 μm.

Other genes expressed in the CNS midline (such as *center divider*, *cdi*) or in the trachea (such as *thick veins*, *ttv*), also showed reduced mRNA levels in comparison to control levels (Affolter et al., 1994; Matthews and Crews, 1999).

When *jing* was over-expressed in the CNS midline and trachea, using the *Gal4 /UAS* targeted gene expression system and the *btl -Gal4* driver (Brand and Perrimon, 1993; Shiga et al., 1996), small but statistically significant two-fold increases in *Egfr* pathway mRNA levels were observed except for *gap* which increased almost four-fold ($P < 0.05$) (Fig. 2 B). Other CNS midline- or tracheal-expressed genes, such as *ttv*, *ast*, *wrapper* and *cdi*, did not show significant changes in mRNA levels in comparison to controls after *jing* over-expression (Fig. 2 B).

To further address whether *jing* activates *Egfr* expression, embryos homozygous mutant for *jing* and those over-expressing *jing* were subjected to in situ hybridization using an *Egfr* riboprobe. As shown in Fig. 2 D, extra *Egfr* -expressing glia were observed when *jing* was over-expressed in the CNS midline using a *Gal4* fusion to the promoter of the *slit* (*sli*) gene (*sli -Gal4*). *sli -Gal4* drives expression specifically in midline glia from stage 11 until the end of embryogenesis (Scholz et al., 1997; Wharton et al., 1994). Similar results were obtained after driving *jing* expression in all CNS midline cells using a *sim -Gal4* driver (data not shown). Conversely, there were reductions in midline *Egfr* expression in homozygous $jing^{01094}$ and $jing^{3}$ (data not shown) embryos (Fig. 2 E). Tracheal *Egfr* expression was also reduced in *jing* homozygous mutant embryos (Fig. 2 G) compared to controls (Fig. 2 F). *jing* function may thus be considered essential for *Egfr* pathway gene expression in both the CNS midline and trachea.

Ectopic expression studies were used to determine whether *jing* can ectopically activate the *Egfr*. *jing* was expressed in the ectodermal stripes of the *paired* (*prd*) gene using a *prd -Gal4* driver and one copy of *UAS -jing*, as this was sufficient to obtain strong *jing* activation (Fig. 2 H). in situ hybridization using an *Egfr* riboprobe failed to detect any mRNA expression in *prd* -expressing cells suggesting that *jing* cannot ectopically activate *Egfr* transcription (data not shown).

Over-expression of jing in the CNS midline phenocopies gain-of-function phenotypes in EGFR signaling

Over-expression of *Egfr* pathway genes in the CNS midline leads to supernumerary glia and in some cases, to lack of commissural connections (Klaes et al., 1994; Sonnenfeld and Jacobs, 1995; Stemerdink and Jacobs, 1997; Scholz et al., 1997). We, therefore, reasoned that if *jing* regulates the *Egfr* pathway, then over-expression of *jing* should result in a similar phenotype. To address this, *jing* was over-expressed in the midline glia by crossing flies carrying two copies of a *UAS -jing* transgene to flies carrying the *sli -Gal4* driver. The progeny from this cross were stained with anti-Sim to determine the effect of *jing* over-expression on both midline neurons and glia. In addition, a reporter for *sli* expression (*sli -lacZ*) was used to determine the number of glia per VNC segment after *sli -Gal4* -induced *jing* over-expression. *sli-lacZ* is expressed specifically in midline glia from stage 11 until the end of embryogenesis (Ma et al., 2000).

Increases in midline cell numbers were observed after *jing* over-expression (Fig. 3B,E,H). Quantitative analysis revealed an average of 16 (±1) SIM-positive cells after *jing* over-expression (n =30 segments) compared to 11 (±2) in each VNC segment of

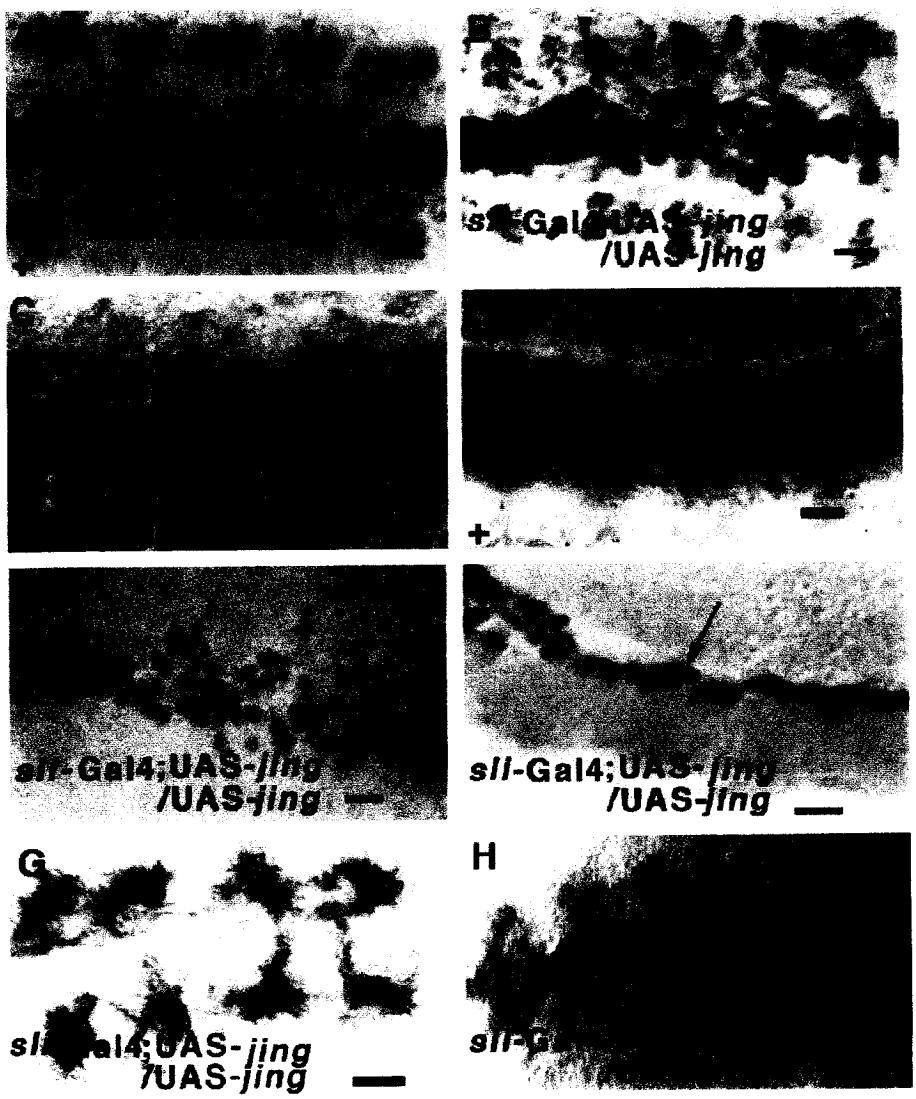

/UAS-jing
+
sll-Gal4;UAS-jing
/UAS-jing
G
H

Fig. 3. *jing* over-expression in midline glia alters cell numbers and commissure formation. Two copies of *UAS -jing* were placed under control of *sli -Gal4* and embryos were stained with anti-SIM to identify midline neurons and glia (A,B), with anti-β-Galactosidase to assess *sli -lacZ* expression (C–F,H) or with BP102 to visualize CNS axons (G,H). Ventral views (A–C,E,G,H) and sagittal views (D,F) of stage 13 embryos are shown with anterior to the left. (A) In wild-type embryos, SIM localizes to glia and neurons along the midline (arrow) and laterally to muscle precursors (arrowhead). (B) In an embryo over-expressing *jing* in midline glia, SIM localizes to additional ectopically distributed midline cells (arrow). The pattern of SIM staining in the muscle precursors is unaltered from wild-type (arrowhead). (C,D) In wild-type embryos, *sli -lacZ* expression is detected in approximately 6–7 glia along the midline in each segment (C, arrow) and in dorsal positions in the nerve cord (D, arrow). Additional small *sli-lacZ* -expressing cells outside the ventral and dorsal regions of the nerve cord are apoptotic glia (arrowheads). (E,F) After *sli -Gal4* -directed expression of *jing*, *sli -lacZ* is expressed in an average of twelve glia (arrow) that are dispersed laterally from the midline. The extra glia maintain a dorsal position in the nerve cord (F, arrow). Note the absence of *sli-lacZ* -expressing apoptotic glia surrounding the nerve cord. (G) Commissural axons do not properly approach the midline after *sli -Gal4* -directed expression of *jing*. The axons instead appear stalled along the longitudinal tracts. (H) A stage 12/5 embryo over-expressing *jing* in the CNS midline glia and carrying *sli-lacZ*. Arrowhead denotes CNS axons. The supernumerary glia migrate toward longitudinal axons (arrow). *n* is greater than 70 segments. Scale bars: 10 μm.

wild-type stage 13 embryos (n =45 segments) representing a statistically significant difference (P <0.05) (Fig. 3 B). Similar results were found using an independent *UAS -jing* strain (data not shown). The extra cells expressed *sli -lacZ* confirming their glial identities while *sli -Gal4* heterozygotes did not induce extra *sli -lacZ* expression (Fig. 3E). There was an average of 12 glia that were scattered laterally from the midline compared to eight in wild-type embryos (P <0.05) (Figs. 3C–E,4). Interestingly, the supernumerary glia were still located dorsally in the VNC (Fig. 3 F), similar to those found after ectopic expression of *pointed*^{P1} (*pnt*^{P1}) in the midline (Klaes et al., 1994). There was no cell death present in the glial population (Fig. 3 F) as compared to the small apoptotic glia found surrounding the nerve cord in wild-type embryos (Fig. 3 D, arrowheads) (Sonnenfeld and Jacobs, 1995). These results suggest that *jing* over-expression suppresses normal midline glial cell death and correlates with the increase in *Egfr*- expressing glia (see Fig. 2 D).

CNS axon formation is a sensitive indicator of midline cell function (Klämbt et al., 1991; Klämbt and Goodman, 1991). To determine whether the extra glia affected CNS axon formation, embryos over-expressing *jing* were stained with the CNS axon specific monoclonal antibody BP102. *jing* over-expression in midline glia was associated with a lack of commissural fascicle formation similar to its over-expression in all CNS midline cells (Fig. 3 G,H) (Sedaghat et al., 2002). There was an association between the laterally dispersed supernumerary glia and the lack of commissure formation as shown in Fig. 3 H (arrow). The supernumerary glia appeared to migrate towards the longitudinal axons as they have previously been observed to do in *commissureless* mutants and after ectopic expression of *pnt*^{P1} in the midline (Klaes et al., 1994; Sonnenfeld and Jacobs, 1995). In

summary, *jing* is able to induce supernumerary glia in a similar fashion as the pnt^{P1} transcription factor.

jing gain-of-function is suppressed by E*gfr* loss-of-function

The genetic data indicates that *jing* may have an upstream function in the *Egfr /ras1* pathway. To address this issue, the effect of a reduction in EGFR signaling on the *jing* gain-of-function phenotype was examined in the midline glia. The *sim -Gal4* and *sli -Gal4* drivers were used to over-express *jing* specifically in the CNS midline in heterozygous and homozygous *spi* and *S* mutant backgrounds. The number of *sli-lacZ* -expressing midline glia in each nerve cord segment was quantified during stage 13 and compared to that in wild-type embryos over-expressing *jing* (Fig. 4). Expression of two copies of the *UAS -jing* transgene in the midline glia of wild-type or heterozygous *spi* and *S* embryos resulted in an average of 12 midline glia instead of the normal 8 during stage 13 (Fig. 4; n >50 segments, P <0.05). In contrast, *UAS -jing* transgene expression was unable to induce 12 midline glia in homozygous *spi* and *S* mutant backgrounds (Fig. 4; n >52 segments, P <0.05). In these embryos, there was an average of 1.5 midline glia in each nerve cord segment after *jing* over-expression which is similar to the number of midline glia present in homozygous *spi* and *S* mutant embryos during stage 13 (Fig. 4) (Sonnenfeld and Jacobs, 1994).

To test the independent activity of jing, we chose to examine the effects of ectopic *jing* expression in the *Drosophila* eye which is a system that is functional for the *Egfr* pathway but not for *jing* or upstream regulators including *single-minded* (*sim*) or *trachealess* (*trh*) (Simon et al., 1991; Dickson et al., 1996).

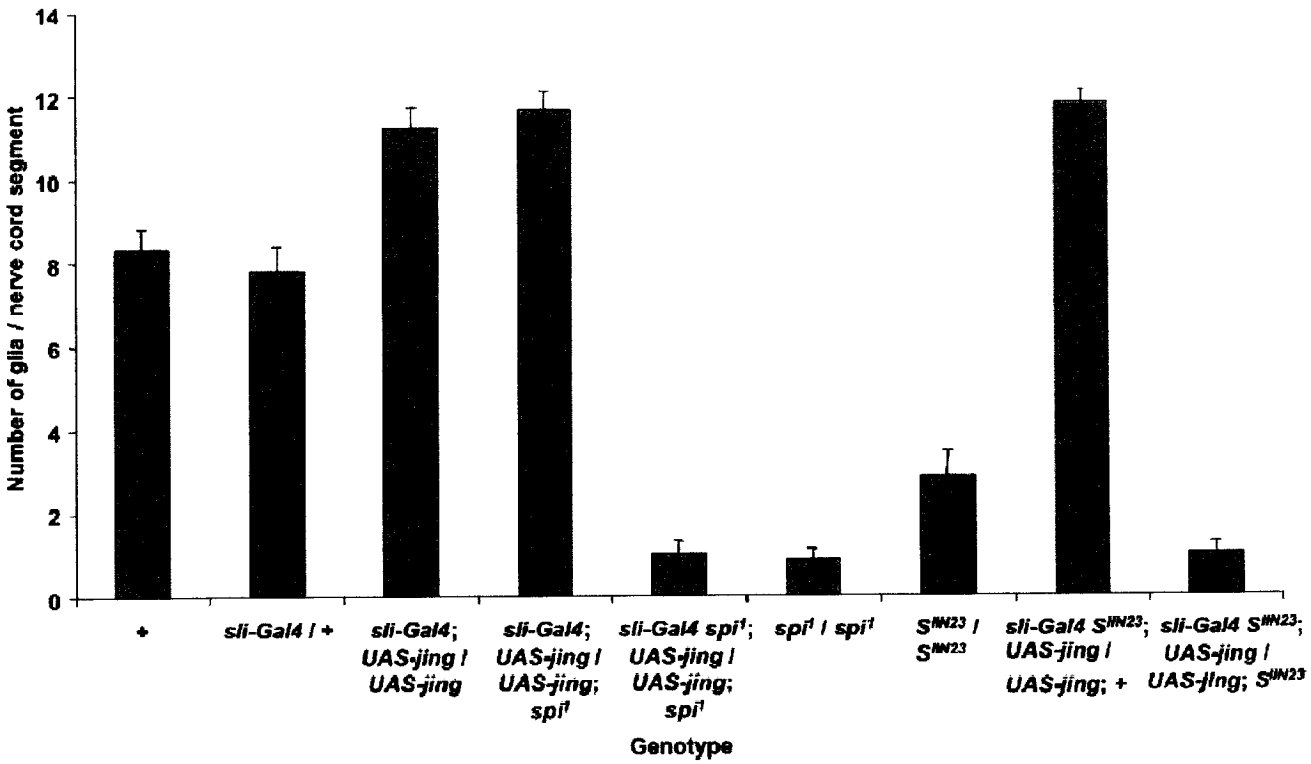


Figure 4. Induction of extra midline glia by *jing* over-expression is suppressed by reducing EGFR signaling. The *UAS -jing* transgene was expressed specifically in midline glia using the *slit* (*sli*) promoter (*sli -Gal4*). Midline glia were detected by *sli-lacZ* expression. Expression of two copies of *UAS -jing* induces 12 midline glia compared to the normal 8 glia present in wild-type stage 13 embryos. Induction of the extra glia is inhibited by the presence of homozygous but not heterozygous *spi* and *S* mutations. The induction of extra glia by *jing* over-expression is therefore dependent on *Egfr* pathway function. In all cases, $P < 0.05$ by unpaired *t* tests in comparison to w^{118} and *sli -Gal4* /+where *n* is greater than 100 segments.

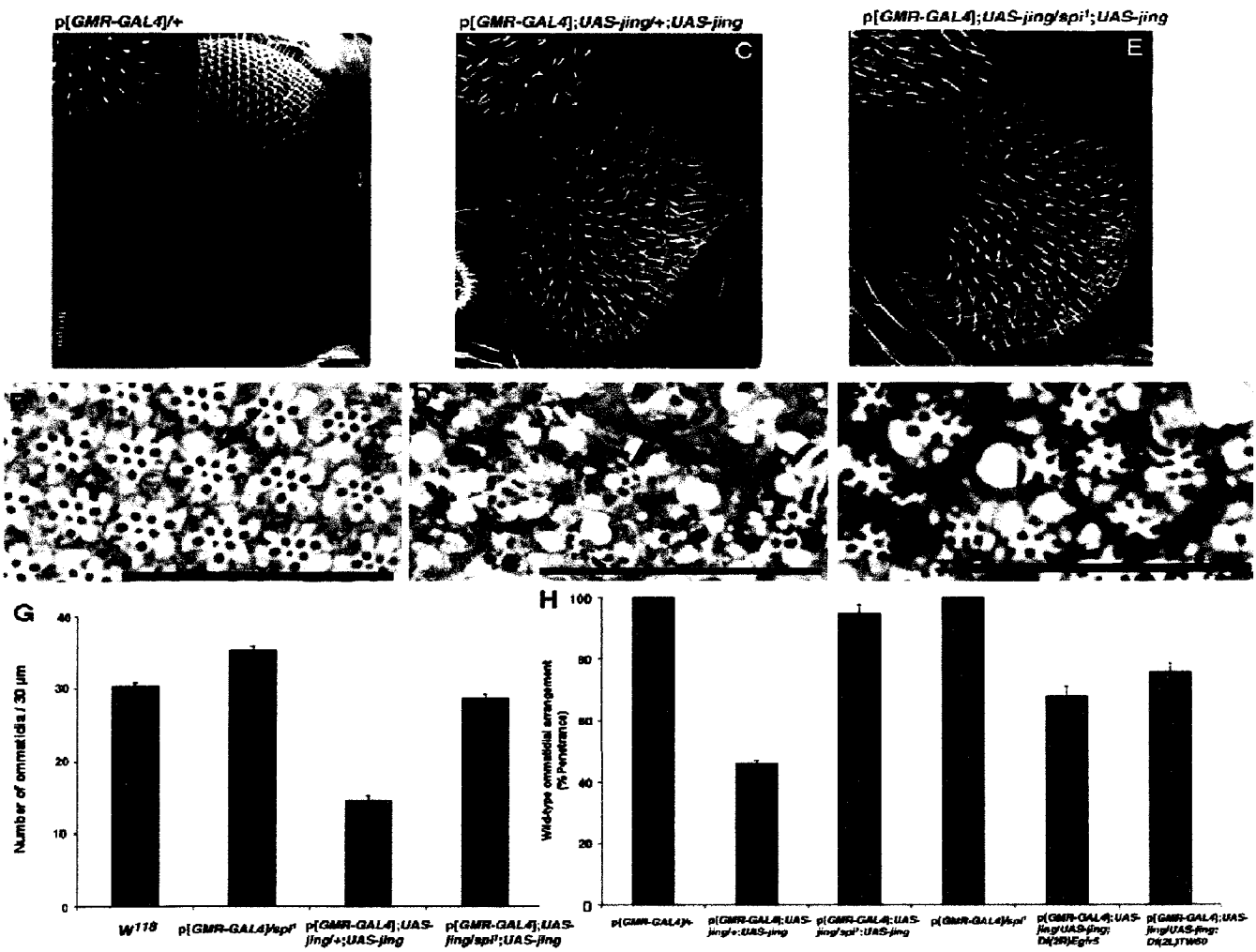

p[GMR-GAL4]/+
p[GMR-GAL4];UAS-jing/+;UAS-jing
p[GMR-GAL4];UAS-jing/spi¹;UAS-jing
C
E
G
Number of ommatidia / 30 µm
40
30
20
10
0
W¹¹⁸
p[GMR-GAL4]/spi¹
p[GMR-GAL4];UAS-jing/+;UAS-jing
p[GMR-GAL4];UAS-jing/spi¹;UAS-jing
H
Wild-type ommatidial arrangement (% Penetrance)
100
80
60
40
20
0
p(GMR-GAL4)/+
p(GMR-GAL4);UAS-jing/+:UAS-jing
p(GMR-GAL4);UAS-jing/spi¹:UAS-jing
p(GMR-GAL4)/spi¹
p(GMR-GAL4);UAS-jing/UAS-jing; Df(2R)Egf-5
p(GMR-GAL4);UAS-jing/UAS-jing; Df(2L)TW50

Fig. 5. EGFR pathway components lie genetically downstream of *jing* in the adult eye. (A–F) Scanning and light microscopic analysis of eyes from adults expressing the *UAS -jing* transgene under control of the *glass* promoter (P[GMR-*Gal4*]). (A,B) Flies heterozygous for P[GMR-*Gal4*] have a wild-type arrangement of ommatidia (B, arrow) and mechanosensory bristles (A, inset). (C, D) Expression of wild-type *jing* under control of the *glass* promoter causes a rough appearance. There is a disruption in the structure and arrangement of the ommatidia (D, arrow) and mechanosensory bristles (C, inset). (E,F) The *jing* over-expression phenotype is dominantly suppressed by heterozygosity for the *spi* allele. Note the less severe disruption in ommatidial structure and arrangement (F). (G) Quantification of ommatidia. (H) Quantification of *spi*, *Egfr* and *jing* interactions. Forty-five percent of flies over-expressing *jing* have a rough eye phenotype. Heterozygosity for *spi* (*spi*^{1}) or *spi* deficiency (Df(2L)TW50) and *Egfr* (Df(2R)*Egfr5*) deficiency suppresses the *UAS -jing* -mediated rough eye phenotype. Scale bars in (B), (D) and (F): 0.05 mm.

Analysis of *jing*^{01094} enhancer trap *lacZ* expression and of endogenous mRNA expression by in situ hybridization showed that *jing* is not expressed in third instar larval eye imaginal discs (data not shown). Expression of wild-type *jing* in the eye, under regulation of the *glass* promoter (P[GMR-*Gal4*]), was associated with a rough appearance (Fig. 5C) compared to P[GMR-*Gal4*] heterozygotes or wild-type (Fig. 5 A; data not shown). The rough eye consisted of highly disorganized ommatidia and mechanosensory bristles in 45% of flies (Fig. 5 C,D,H) and the number of ommatidia was reduced by 50% from that in wild-type and P[GMR-*Gal4*] heterozygous eyes (Fig. 5 G). Therefore, the gain-of-function phenotypes of *jing* and *Egfr* both result in a significant reduction in ommatidia (Fortini et al., 1992; Baker and Rubin, 1989). Consistent with similar pathways, the rough eye phenotype of *Egfr* gain-of-function was not enhanced by that of *jing*. Out of 1000 flies scored, carrying P[GMR-*Gal4*] and both UAS-*jing* and UAS-*ellipse*, 100% showed the same eye phenotype as flies carrying only P[GMR-*Gal4*] and UAS-*ellipse*.

The *jing* ectopic expression phenotype was dominantly suppressed by a 50% reduction in the levels of *spi* (*spi*^{1}) and Df(2L)TW50 or *Egfr* deficiency (Df(2R)*Egfr5*) (Fig. 5 E–H). After *spi* reduction, ommatidia were more organized and more abundant, although the position of the photoreceptors was not like that in controls (Fig. 5 F,G). This interaction was not influenced by activation of the *glass* promoter in the heterozygous *spi* background (P[GMR-*Gal4*]/*spi*^{1}) (Fig. 5 G). These results suggest that there is a dosage-sensitive interaction between the *Egfr* pathway and *jing* function in the eye, where increased *jing* activity can be suppressed by a reduction in downstream components such as *spi* and *Egfr*. Given that *sim* and *trh* are not expressed in third instar larval eye discs

(data not shown), these experiments suggest that *jing* can have an effect on the *Egfr* pathway in the absence of *sim* or *trh* and supports our model that *jing* works as an independent regulator in bHLH-PAS pathways (Sedaghat et al., 2002).

Modulating the Egfr pathway has strong effects on *jing*-induced midline cell death

If the Ras1/MAPK pathway is involved in midline cell survival downstream of *jing* then it may be possible to rescue midline cell reductions in *jing* mutants by providing a supply of activated RAS1 to the CNS midline. To address this, the number of embryos with reductions in midline cells was determined in control and homozygous or transheterozygous *jing* mutant embryos after expression of the *UAS -ras*^{V12} and *UAS -ellipse* transgenes. The *Egfr* pathway was activated specifically in the CNS midline using *sim -GAL4* to drive expression and *UAS -lacZ* to visualize the midline cells. Loss-of-function alleles of *jing* are associated with a reduced number of midline cells by stage 9 compared to control embryos (Sedaghat et al., 2002). By stage 14, an absence of *sim -GAL4* -expressing midline glia was apparent in homozygous *jing* mutants compared to control embryos (Fig. 6B–E). 37.8% of homozygous *jing* mutant embryos displayed the midline cell-loss phenotype (Fig. 6 A).

Expression of one copy of the *UAS -ras*^{V12} and *UAS -ellipse* transgenes suppressed the *jing* glial-loss phenotype so that only 11.3 and 20% of mutant embryos displayed reductions in midline glia, respectively (Fig. 6A). Midline-targeted expression of the *UAS -ras*^{V12} and *UAS -ellipse* transgenes was not associated with glial reductions in the absence of the *jing* mutation (Fig. 6A). Therefore, these results support a role for *jing* upstream of EGFR signaling in CNS midline glia.

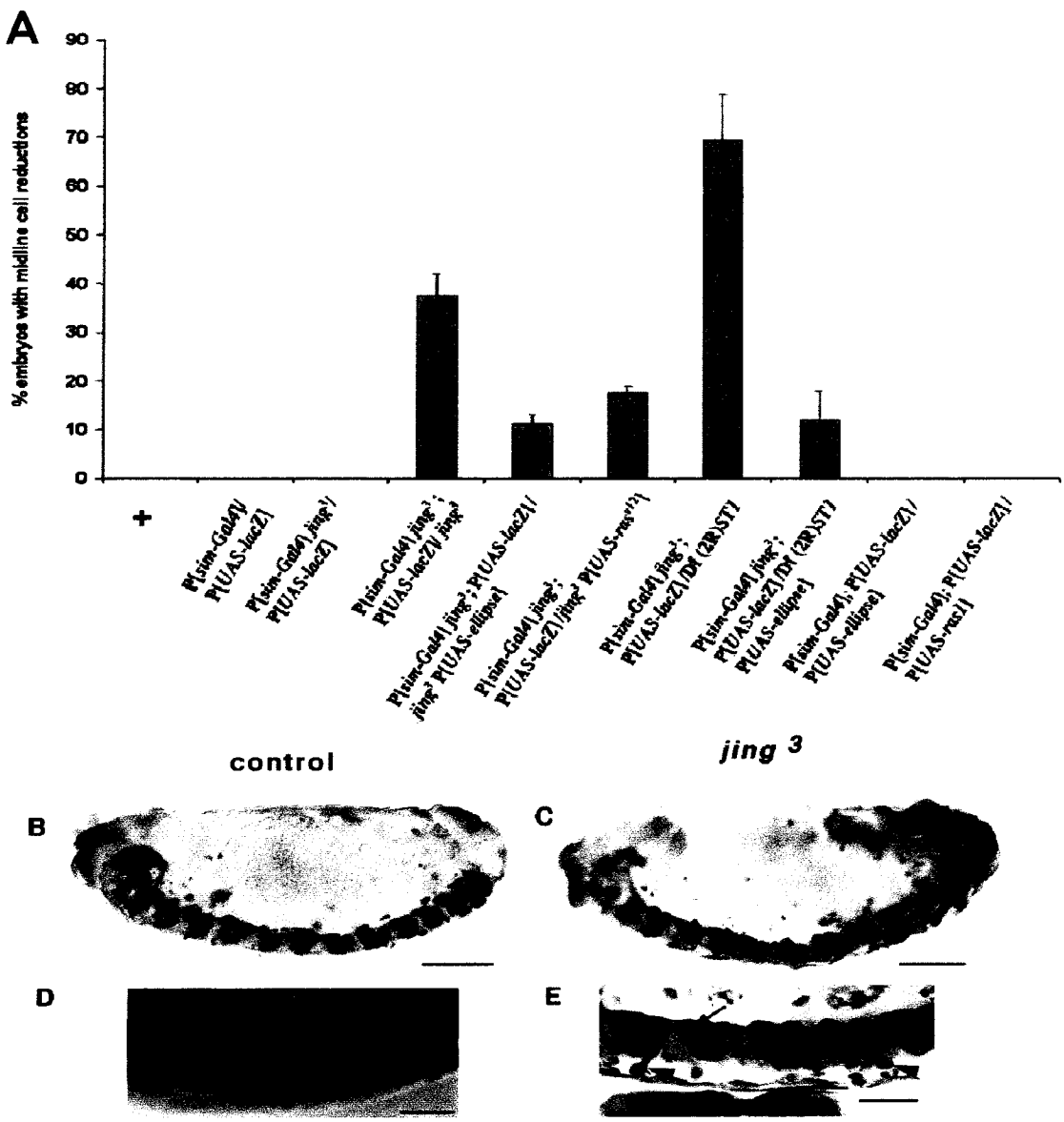


Fig. 6. (B–E) Wild-type (B,D) and *jing*^{3} homozygous (C,E) embryos carrying *sim* -Gal4/UAS-lacZ. Activation of the *Egfr* pathway allows midline cell survival in homozygous *jing* mutant embryos. Activated *ras* (*UAS -ras*^{V12}) and dominant *Egfr* (*UAS -ellipse*) were expressed specifically in the midline cells of homozygous *jing* mutant embryos using the *sim -Gal4* driver. Approximately 37.8% of *jing* mutant embryos show reductions in the number of midline glia. However, after expression of activated *ras* and *Egfr* (ellipse), only 11.3 and 20% of homozygous *jing* mutant embryos display midline glial loss. Expression of *UAS -ras*^{V12} or *UAS -ellipse* in a wild-type background is not associated with midline cell reductions.

The jing and Egfr pathways display dominant genetic interactions in the CNS midline and trachea

Gene dosage experiments were used to determine the effects of simultaneously altering the levels of *jing* and genes of the *Egfr* pathway. Mutations in *spi* and its regulator *Star*, have been previously characterized for their midline and tracheal phenotypes (Sonnenfeld and Jacobs, 1994; Wappner et al., 1997). To determine whether *jing* and *Egfr* function is inter-dependent, the development of the CNS midline and trachea was analyzed in double heterozygotes of *jing* and *S* or *spi*. The basis for this experiment is that if the *Egfr* and *jing* pathways are inter-dependent then simultaneous reduction of only one copy of each gene should alter CNS midline and tracheal function. Multiple *jing* alleles balanced with *wg-lacZ Cyo* were crossed to S^{IIN23} */wg-lacZ Cyo* or spi^{1} */wg-lacZ Cyo* flies and their progeny were double stained with anti-SIM or anti-TRH and anti-β-Gal.

The number of CNS midline cells was reduced from wild-type in embryos homozygous and double heterozygous for *jing*, *spi* or *S* and stained with anti-SIM (Fig. 7B–D,F). Since some of the SIM-positive nuclei appeared to be fragmenting (Fig. 7 D) their fate was determined by TUNEL labeling to identify apoptotic cells. In wild-type embryos, cell death is uncommon in the CNS midline during stage 12 with an average of 6(±2) SIM-positive apoptotic nuclei per embryo (n =30). In contrast, in homozygous *jing* stage 12 mutant embryos, there was an average of 35(±3) SIM-positive apoptotic nuclei per embryo, therefore, displaying a significant increase over that in wild-type embryos (n =35; P <0.05) (Sedaghat et al., 2002). In embryos double heterozygous for mutations in

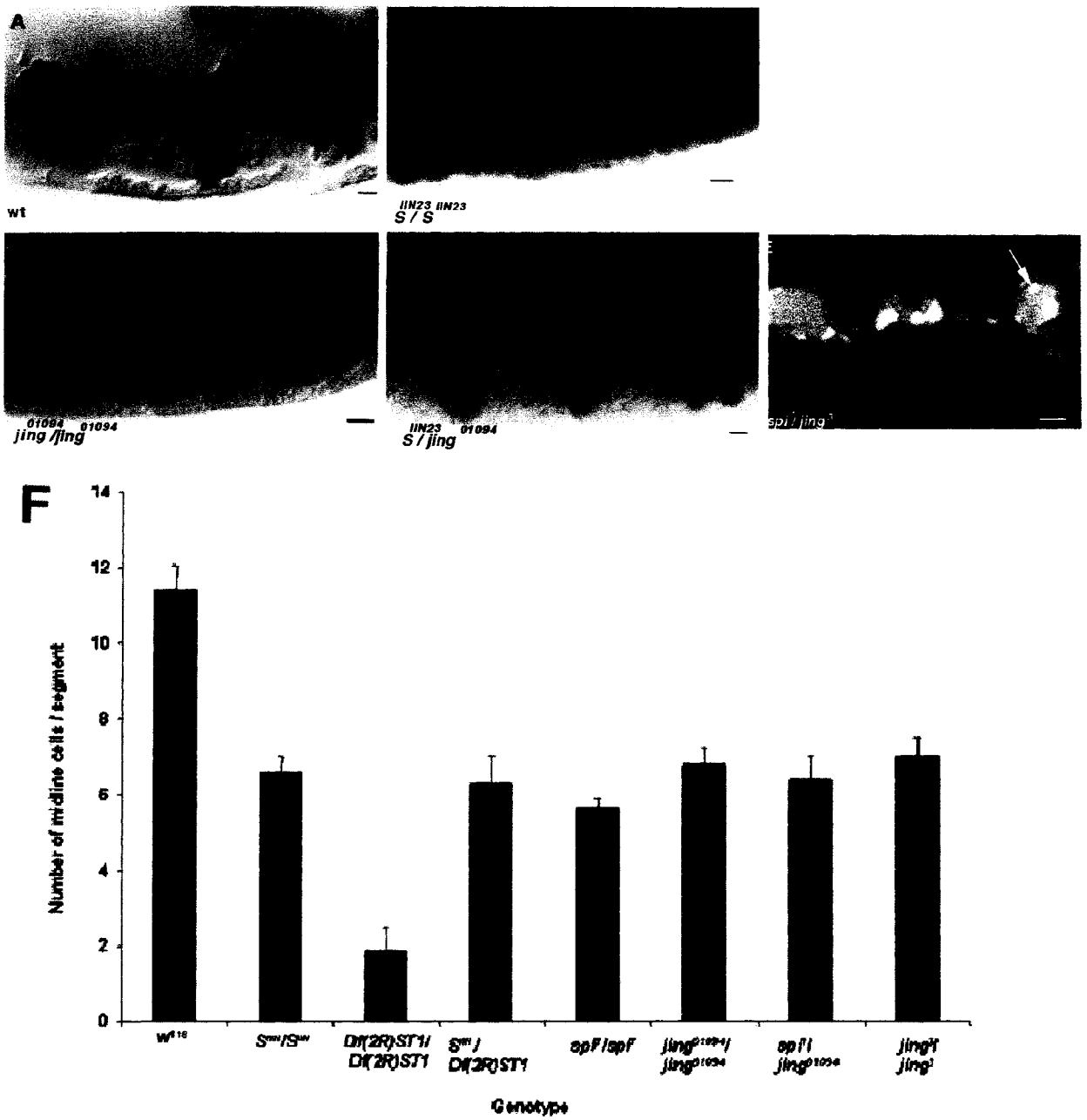


Fig. 7. The *jing* and *Egfr* pathways display dominant genetic interactions in the CNS midline. Midline cells were labeled with anti-SIM. Sagittal views of stage 14 VNCs are shown. (A) A wild-type embryo. (B) An embryo homozygous for the S^{IIN23} mutation showing an absence of glia. (C) An embryo homozygous for $jing^{01094}$ showing reduced SIM+cells. (D) An embryo double heterozygous for S^{IIN23} and $jing^{01094}$ with SIM+cell reductions. Fragmentation of nuclei is apparent (arrow). (E) Embryos double heterozygous for *spi* and *jing* have TUNEL+SIM+cells (arrow). (F) Quantification of midline cells. Scale bars: 10 μm.

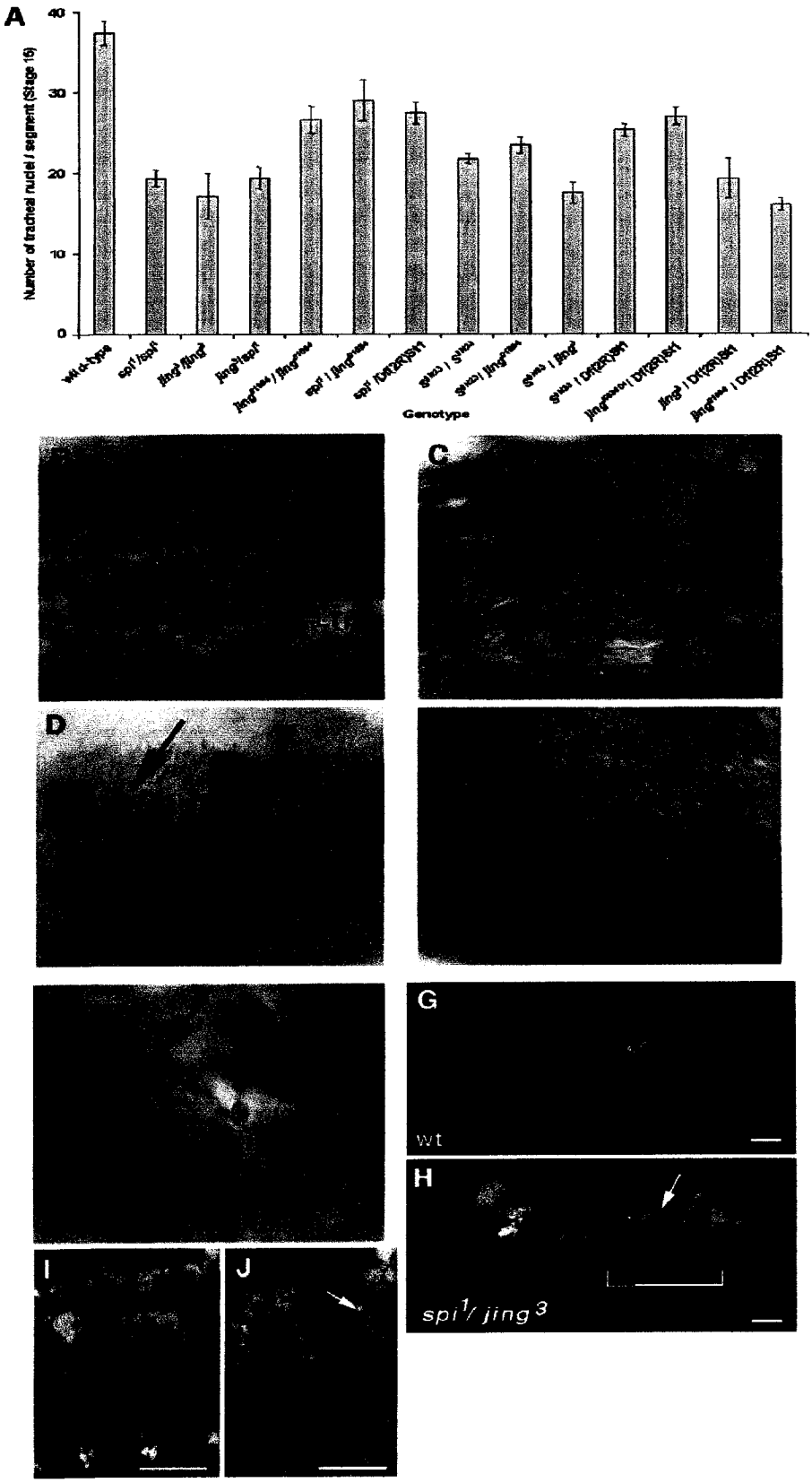

A
Number of tracheal nuclei / segment (Stage 16)
40
30
20
10
0
wt ct-type
spi1/spi1
jing3/jing3
jing3/spi1
jingnew/jingnew
spi1/jingnew
spi1/Df(2R)Sch1
gnu1/gnu1
gnu1/jingnew
gnu1/jing3
gnu1/Df(2R)Sch1
jingnew/Df(2R)Sch1
jing3/Df(2R)Sch1
jingnew/Df(2R)Sch1
Genotype
C
D
G
wt
H
spi1/jing3
I
J

Fig. 8. The *jing* and *Egfr* pathways display dominant genetic interactions in the trachea. Sagittal views of stage 15 embryos stained with anti-TRH are shown. (A) Quantitative analysis of tracheal nuclei in *jing* hemizygotes and *jing* and *Egfr* pathway double heterozygotes. (B) The wild-type trachea. (C–F) There are breaks in the dorsal trunk (arrows) in embryos hemizygous for *jing* (C), homozygous for S^{IIN23} (D) and double heterozygous for S^{IIN23} and $jing^{3}$ (E) or $jing^{01094}$ (F). Arrowheads denote unfused dorsal trunk and truncated transverse connectives. (G–J) Stage 15 embryos were stained with anti-TRH and TUNEL and viewed by confocal microscopy. (G) Wild-type whole-mount embryo. (H) A *spi* and *jing* double heterozygote showing breaks in the dorsal trunk (arrow). (I) Close-up view of wild-type trachea. (J) Close-up view of area denoted in (H). Note presence of TRH+TUNEL+cells (arrow). Scale bars in (B)–(F) are 10 μm and in (G)–(I) are 30 μm. DT, dorsal trunk; TC, transverse connective; VB, visceral branch; LTa, lateral trunk anterior; LTp, lateral trunk posterior. Fluorescent images are merged.

jing and *S* or *spi* there was an average of 25(±2) and 30(±3) SIM-positive apoptotic nuclei per embryo during stage 12, respectively (Fig. 7 E) (n =28, n =32; P <0.05). This is consistent with the time period for the requirement of *Egfr* function in CNS midline glia (Sonnenfeld and Jacobs, 1994; Scholz et al., 1997). Embryos heterozygous for either *jing*, *spi* or *S* mutations did not alter the normal events of midline cell apoptosis (data not shown). In summary, these results suggest that proper dosage of both *jing* and *spi* group gene function is required for midline cell survival.

In the stage 15 trachea, the number of TRH+ nuclei was reduced within each segment of embryos homozygous and double heterozygous for *jing* and *spi* or *S*, (Fig. 8A). In addition, breaks were observed in the dorsal trunk of double heterozygotes (Fig. 8 E,F,H), consistent with the homozygous phenotypes of *jing* and *Egfr* pathway mutants (Fig. 8 C,D) (Sedaghat et al., 2002). In *jing* homozygous and transheterozygous mutants, there also were truncated transverse connectives suggesting that *jing* is not only involved in Egfr signaling in the trachea (Fig. 8 C, arrowheads; data not shown) (Wappner et al., 1997).

In embryos double heterozygous for *jing* and *S* or *spi* mutations, apoptotic tracheal nuclei were detected in stage 15 embryos (Fig. 8 H,J; data not shown). The overall embryonic pattern of apoptosis was not significantly altered from wild-type suggesting that the tracheal defects did not result from gross perturbations to development (compare Fig. 8 G,H). Embryos heterozygous for *jing*, *spi* or *S* did not show any alterations from wild-type in tracheal cell numbers (data not shown).

Discussion

jing and *Egfr/Ras1* function is essential for midline cell differentiation and survival

The *jing* gene was identified in two independent genetic screens for regulators of CNS midline development and border cell migration, two processes which are regulated by the EGFR (Scholz et al., 1997; Sedaghat et al., 2002; Liu and Montell, 2001; Duchek and Rorth, 2001). RTK signaling pathways have been implicated in multiple cell biological processes including proliferation, migration, differentiation and survival (Baonza et al., 2002; Duchek and Rorth, 2001; Wassarman and Therrien, 1997; Casci and Freeman, 1999; Kurada and White, 1998). How one MAPK pathway controls such different outcomes is a major area of research. Studies of *Egfr* function in the *Drosophila* adult eye suggest that signaling levels dictate the multiple cellular responses to the EGFR, such that differentiation requires the highest levels of signaling while mitosis and cell survival require less (Yang and Baker, 2003). Therefore, it is important to understand the mechanisms that control the expression of positive and negative regulators of this family of signaling molecules.

Prior work has established the important role that the *Egfr* plays during the differentiation of midline glia (MG) and tracheal cells (Klämbt, 1993; Scholz et al., 1997; Sonnenfeld and Jacobs, 1994; Wappner et al., 1997; Stemerdink and Jacobs, 1997). The results in this paper indicate that the *jing* gene regulates Egfr signaling in the MG and trachea and there are several lines of supportive evidence. First, *jing* mutant embryos fail to maintain MAPK activity and *Egfr* expression in cells that clearly have midline and tracheal identities. Second, *jing* is required for and can induce *Egfr* pathway transcription in the

CNS midline and trachea. Third, *jing* over-expression promotes midline glial survival in a similar fashion as over-expression of *Egfr* pathway genes. Fourth, *jing* -mediated over-expression phenotypes require *Egfr* pathway function in CNS midline glia and the adult eye. Fifth, a transgenic copy of either activated *ras1*, secreted *spi* or gain-of-function *Egfr* can partially rescue midline cell death in homozygous *jing* mutants. Lastly, proper dosage of both pathways is essential for survival of midline glia and for proper tracheal morphogenesis. Together, these findings suggest that *jing* functions upstream in the *Egfr /ras1* pathway. Future studies will be aimed at elucidating the nature of the relationship between *jing* and *Egfr* pathway genes and may help in the design of therapeutics to regulate over-active RTK pathways in oncogenic cells.

To our knowledge *jing* is the only gene, other than those already characterized in the *Egfr* pathway, that can promote midline glial survival. *jing* over-expression, as driven by the *sim* and *sli* promoters, induces extra midline glia that express *Egfr*, *slit* and *sim* and appear to be rescued from apoptotic fates. The extra glia are observed during stage 13 which is consistent with the timing of *Egfr* pathway-induced extra glia (Sonnenfeld and Jacobs, 1995). The absence of apoptotic glia and the wild-type midline neuronal numbers after *jing* over-expression suggests that the supernumerary glia are not likely recruited from neuronal populations and may represent glia rescued from death due to inappropriate *Egfr* expression. This effect phenocopies gain-of-function phenotypes in EGFR signaling in the CNS midline and suggests that *jing* -mediated cell survival may be carried out by the EGFR/RAS1 signaling pathway (Klaes et al., 1994; Scholz et al., 1997; Stemerdink and Jacobs, 1997). In support, *jing* over-expression phenotypes in the CNS midline and eye are suppressed by reductions in *Egfr* function.

Our results suggest that *jing* is involved in both the differentiation and survival of cells in the embryonic CNS midline and trachea. In wild-type embryos, early MAPK activity controls midline glial (MG) differentiation through activation of the downstream Ets-type transcription factor *pointed* (*pnt*) (Klämbt, 1993; Scholz et al., 1997). The reductions in early MAPK activity and *Egfr* expression in the midline of *jing* mutants, therefore, reveals the requirement for *jing* function in MG differentiation. The similarities in gain- and loss-of-function midline glial phenotypes between *pnt* and *jing* are consistent with this model. In *jing* mutants, reduced MAPK activity occurs in midline and tracheal cells that express the *sim* and *trh* genes, respectively, indicating that the reductions in MAPK activity are not due to a general failure in cellular differentiation (Isaac and Andrew, 1996; Wilk et al., 1996; Nambu et al., 1991).

It is possible that improper MG differentiation in *jing* mutants could be due to cells being committed to death. However, we have detected a loss of MAPK activity prior to apoptosis in the CNS midline of homozygous *jing* mutants suggesting that early MAPK inactivity in the CNS midline is independent of the apoptotic machinery. In support, the MG initially form in MAPK mutants and it is not until later stages, which are dependent on repression of *hid*, that the MG die (Bergmann et al., 2002). MG death in *jing* mutants may be due to a combined lack of the axon-glial contacts that are necessary for MAPK-mediated inactivation of *hid* as well as from reduced MAPK activity within the MG (Bergmann et al., 2002).

Role of jing in the trachea

During stage 10, EGFR signaling is activated in the central region of the tracheal placode by transcription of *rhomboid* resulting in the formation of anteroposterior branches including the dorsal trunk and visceral branch (Raz and Shilo, 1992; Zak et al., 1990; Gabay et al., 1997a,b; Wappner et al., 1997; Zelzer and Shilo, 2000). Wingless (WG) signaling originates in ectodermal cells adjacent to the tracheal placodes and causes Egfr-induced cells to form the dorsal trunk (Chihara and Hayashi, 2000). *jing* is expressed in most tracheal cells and its protein product is localized to their nuclei suggesting that this $C_2 H_2$ -type zinc finger may have a regulatory role directly within these cells (Sedaghat et al., 2002). Additional evidence that *jing* may have a role directly in tracheal cells comes from its perturbation of tracheal morphogenesis and alteration of *Egfr /ras* pathway gene expression profiles when over-expressed specifically in the trachea (Sedaghat et al., 2002; this work).

jing affects branching morphogenesis and cellular survival in the tracheal system and its expression in the tracheal placodes coincides with that of *Egfr* pathway genes (Sedaghat et al., 2002; Bier et al., 1990; Gabay et al., 1997a,b). *jing* and *Egfr* pathway mutants have similar tracheal phenotypes which include breaks in the dorsal trunk and reduced visceral branch formation. The reductions in Egfr-induced cells may explain the defects in dorsal trunk formation in *jing* homozygous mutant embryos and possibly in *jing* and *Egfr* pathway double heterozygotes. Alternatively, the dorsal trunk defects may arise from perturbations in WG signaling in the ectoderm of *jing* mutants. *spitz* group tracheal mutant phenotypes do not reflect ectodermal patterning defects but this remains to be analyzed in more detail in *jing* mutants (Wappner et al., 1997).

Our results indicate that proper *Egfr* pathway and *jing* function is required for midline and tracheal cell survival. This is the first evidence, to our knowledge, of such a survival role in the trachea and requires further investigation. However, this does not rule out the possibility that other processes involved in tracheal morphogenesis are not affected in double heterozygotes and *jing* homozygotes. Furthermore, in *jing* homozygotes and hemizygotes, truncated tubules are present in the transverse connectives suggesting that the requirement for *jing* function is more global than that of *Egfr /ras1* (Wappner et al., 1997). In support, *jing* is expressed in embryonic tissues that are not active in MAPK suggesting that *jing* has additional functions (Sedaghat et al., 2002).

Compared to other midline and tracheal-expressed genes, those of the *Egfr* pathway are more highly expressed after *jing* over-expression but not more than three-fold. Nevertheless, the effects of *jing* over-expression in the CNS midline can be seen by extra glia and *Egfr* expression establishing the importance of regulating *jing* expression during embryogenesis. Ectopic expression analyses suggest that *jing* is not sufficient to activate *Egfr* pathway gene expression. Therefore, these results suggest that in order to induce gene expression *jing* may require another protein, such as a cell-specific chromatin remodeling protein, that is not present in *prd* stripes but is present in the CNS midline, trachea and eye. The exact relationship between *jing* and *Egfr* pathway genes requires further analysis.

Experimental procedures

Drosophila stocks

spitz (spi^{1}) (Nüsslein-Volhard et al., 1984), *Star* (*S^{IIN23}*) and the *jing* deficiency, *Df(2R)ST1* (Liu and Montell, 2001; Sedaghat et al., 2002), were obtained from the Bloomington *Drosophila* stock center. Df(2R)*Egfr5* (Price et al., 1989) and Df(2R)TW50 (Butler et al., 2001) are deficient in *Egfr* and *spi*, respectively, and were obtained from the DK2 kit (Bloomington *Drosophila* stock center). *jing^{3}*, *jing^{01094}* and *jing^{K03404}* were previously described (Sedaghat et al., 2002). *w^{118}* was used as a control strain. The *slit* (*sli*) reporter P[*sli* 1.0 HV-*lacZ*] was used to assess transcription of the *sli* gene (Rothberg et al., 1988; Wharton et al., 1994; Ma et al., 2000).

The following *Gal4* driver lines were used: P[*sim -Gal4*] (second chromosome; provided by S. Crews); P[*sli -Gal4*] (second chromosome; provided by R. Jacobs), P[*prd -Gal4*] and P[GMR-*Gal4*] (provided by J. Nambu; Ma et al., 2000); P[*btl -Gal4*] (Shiga et al., 1996). The following *UAS* lines were used: P[*UAS -lacZ*]; P[*UAS* -s*spi4a*] (Golembo et al., 1996b); P[*UAS -jing*] (Sedaghat et al., 2002); P[*UAS -ras^{Val12}*] (Fortini et al., 1992); and P[*UAS -ellipse*] (provided by J.R. Jacobs).

Genetics and generation of rescue lines

All crosses were performed at 25 °C. In rescue experiments, *lacZ* marked balancer chromosomes were used to distinguish transgenic embryos lacking endogenous *jing* from those that carried a functional copy of the *jing* gene (supplied by the balancer chromosome). For rescue in homozygous *jing* mutant backgrounds two stable lines were

produced. In the first line, P[*sim -Gal4*] was recombined onto *jing* mutant chromosomes by meiotic recombination and balanced with *CyO wg-lacZ*. To generate the second stock, P[*UAS -jing*] was incorporated into *jing* mutant backgrounds by standard genetic techniques. Single-copy rescue was assessed by collecting embryos from a cross of P[*UAS -jing*]; *jing /CyO wg-lacZ* females to P[*sim -Gal4*] *jing /CyO wg-lacZ* males.

jing was over-expressed in the CNS midline using the *sim -Gal4* and *sli -Gal4* drivers and two copies of P[*UAS -jingE*] or P[*UAS -jingU*]. For targeted expression of *Egfr* pathway genes in homozygous *jing* mutant embryos, P[*sim -Gal4*] *jing /CyO wg-lacZ* females were crossed to *UAS -ras*^{V12}*jing /CyO wg-lacZ*, P[*UAS -ellipse*] *jing /CyO wg-lacZ* or P[*UAS -sspi4a*] *jing/CyO wg-lacZ* males. For over-expression of *jing* in *S* and *spi* homozygous mutant backgrounds, the P[*sli -Gal4*] driver was recombined onto the *S* and *spi* mutant chromosomes and P[*UAS -jing*] was incorporated into these fly strains by standard genetic techniques. In addition, embryos of the genotype P[*UAS -jing*]; *spi /wg -lacZ* Cyo were generated. Embryos containing P[*sli -Gal4*], two copies of P[*UAS -jing*] and that were homozygous for *S* or *spi* were identified by their lack of expression of the *CyO wg-lacZ* balancer.

RNA isolation, microarray analysis and in situ hybridization

A microarray screen was performed to identify *jing* transcriptional targets (in preparation). Total RNA was prepared from control and experimental embryonic samples using an RNeasy kit (Qiagen). All embryos were collected at 25 °C. The RNA samples were labeled independently with Cy3 and Cy5 and then hybridized to an array by the Canadian *Drosophila* Microarray Centre (CDMC). The array includes the Berkeley DGC

EST set (approximately 5900 clones), B. Oliver's and J. Andrews non-DGC clones (approximately 750 clones from a testis library), genes from submitted lists (approximately 572 genes).

For control over-expression experiments, flies carrying *btl -Gal4* and one copy of *UAS -lacZ* were crossed to *UAS -lacZ* and RNA was extracted from their embryonic progeny. To analyze *jing* over-expression, RNA was extracted from embryos of a cross between flies carrying *btl -Gal4* and one copy of *UAS -jing* and flies carrying *UAS -jing*. One-half of all progeny over-expressed *jing* in the CNS midline and trachea whereas 1/4th expressed two copies. Four independent samples from each group were analyzed. In all samples, embryos were aged to 7–9 h after egg laying at 25 °C.

For loss-of-function experiments, the control samples included RNA extracted from w^{118} embryos to ensure a similar genetic background as the *jing* mutants (Sedaghat et al., 2002). Experimental samples included RNA extracted from embryos homozygous mutant for $jing^{3}$. Values represent averages of four independent samples. Embryos were aged as described above and un-fixed homozygous embryos were selected after staining with X-Galactosidase. Control w^{118} embryos were treated in a similar fashion. Microsoft excel was used to calculate the mean, standard error and statistical significance for the Cy3/Cy5 ratios of *Egfr* pathway genes.

In situ hybridization was carried out using digoxigenin-labeled riboprobes prepared from *Egfr* (Stemerdink and Jacobs, 1997) or *jing* cDNA (Sedaghat et al., 2002) according to Janody et al. (2000). Whole-mount embryos were collected from control w^{118}, $jing^{3}$, $jing^{01094}$, P[*prd -Gal4*]/P[*UAS -jing* E] and P[*sli -Gal4*]; P[*UAS -jing* E]/P[*UAS -jing* E].

The number of embryos with loss of *Egfr* expression was counted in experimental and control populations.

Antibodies

Monoclonal antibody BP102 was obtained from the Developmental Studies Hybridoma Bank. The following antibodies were used; mAb anti-β-galactosidase (anti-β-gal) (Promega); rabbit polyclonal anti-β-gaL (Promega); rat polyclonals anti-Single-minded and anti-Trachealess (obtained from S. Crews) (Sonnenfeld et al., 1997; Ward et al., 1998). Anti-diphospho-MAPK was obtained from Sigma (Gabay et al., 1997a,b).

Immunohistochemistry, confocal microscopy and TUNEL labeling

Embryo staging was according to Campos-Ortega and Hartenstein (1997) as modified by Klämbt and Goodman (1991). Processing for light microscopy was according to standard protocols (Patel, 1994). Balancer chromosomes carrying *lacZ* for the second (*CyO* P[*wg -lacZ*]) and third chromosomes (*TM3* P[*ubx -lacZ*]) were used to identify homozygous mutant embryos after anti-β-Gal staining. Antibody staining was visualized using HRP-, FITC- or rhodamine-conjugated secondary antibodies (Jackson). HRP-labeled embryos were analyzed by light microscopy using a Zeiss Axioskop. In the case of anti-diphospho-MAPK (Gabay et al., 1997a,b), peroxidase staining was enhanced using Tyramide Signal Amplification reagents (TSA, New England Nuclear). Fluorescent labeling was visualized on a Zeiss Axiovert 100 TV confocal microscope.

TUNEL (TMR red; Roche) staining was carried out according to previous procedures and was double stained with anti-Sim, anti-TRH or anti-β-GAL (Booth et al., 2000).

Fluorescently labeled embryos were mounted in 4% *n* -propyl gallate to inhibit photobleaching and analyzed on a Zeiss Axiovert 100 TV confocal microscope. Optical sections of 1 μm were recorded in line average mode and parameters were set by the TRH and SIM fluorescence. All figures were processed with Adobe Photoshop software.

Genetic interactions in the eye

To analyze genetic interactions in the eye, females carrying P[GMR-*Gal4*] and P[*UAS -jing*] were crossed to males carrying; (1) spi^{1}; P[*UAS -jing*], (2) Df(2R)*Egfr5*; P[*UAS -jing*] or (3) Df(2L)TW50; P[*UAS -jing*]. In addition, females carrying P[GMR-*Gal4*] and P[*UAS -jing*] were crossed to males carrying P[*UAS -ellipse*] and compared to progeny from crosses of P[GMR-*Gal4*] and P[*UAS-ellipse*] or P[*UAS -jing*]. Rough eye phenotypes were identified under a dissecting microscope at 25 °C where 1/16th of progeny carried two copies of P[*UAS -jing*] and one copy of P[GMR-*Gal4*] and *spi* or Df(2R)*Egfr5* or Df(2L)TW50. Crosses were repeated in triplicate.

The ommatidial arrangement was analyzed in images generated by scanning electron microscopy and light microscopy by the bioimaging centre at Mount Sinai Hospital (Toronto, Canada). For light and scanning electron microscopy, eye specimens were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, washed in buffer, then transferred into liquid 4% agarose. After the agarose cooled and solidified the eyes were cut out in cubes and dehydrated in a graded ethanol series followed by propylene dioxide and embedded in Spurr epoxy resin in flat molds. One micrometer serial sections were cut with glass knives on a RMC MT6000 ultramicrotome and were stained with Toluidine

Blue. The number of ommatidia in light-level sections was determined in two 30 μm areas in each section. Three sections from independent eyes were scored.

Quantification of CNS midline and tracheal cells

To quantify midline cells, the MG were identified using the P[*sli* 1.0 HV-*lacZ*] reporter whereas both MG and neurons were identified using anti-Sim antibody. For over-expression and rescue experiments, the number of MG per nerve cord segment was determined by light microscopy of HRP-stained stage 13 embryos. Midline cells labeled with MAPK and anti-SIM were counted on confocal microscopic images. To determine the effects of *ras1* and *Egfr* rescue on midline cell survival, the number of midline cells in homozygous and transheterozygous *jing* mutants was determined using *sim -GAL4 /UAS -lacZ* as a marker of cell identity. Microsoft Excel and unpaired *t* tests were used to determine the statistical significance compared with w^{118} values.

To determine the effects of *jing* and the *Egfr* pathway on cell survival, the number of midline cells in homozygous and double heterozygous embryos was counted in stage 15 embryos stained with anti-SIM. At least 30 segments were scored in each case. The number of TRH+cells was determined in each tracheal segment in stage 14/15 *jing* homozygotes, transheterozygotes and *jing* and *spi* or *S* double heterozygotes. At least 40 segments were scored in each case. Microsoft Excel and unpaired *t* tests were used to determine the statistical significance compared with w^{118} values.

To determine the extent of apoptosis in the CNS midline, the number of SIM^{+} and $TUNEL^{+}$ cells (yellow cells) was counted per embryo for a particular genotype. To

analyze apoptosis in the trachea, the number of TRH^+ and $TUNEL^+$ cells was counted per tracheal segment in stage 14/15 embryos. In each case, the average, standard error and statistical significance were calculated using Microsoft Excel for at least 50 tracheal segments and VNC segments. Scanning parameters during confocal microscopy were set by the SIM and TRH fluorescence.

Acknowledgments

We are grateful to Roger Jacobs for *Egfr* cDNA, *sli* 1.0HV-*lacZ*, *sli -Gal4*, *UAS -ras*^{V12}, *UAS -ellipse* and *UAS -sspi4a* and the Bloomington stock center for sending fly strains. We thank G. Boulianne, D. van de Hoef and D. Holmyard for eye sectioning and scanning electron microscopy. Thanks to S. Crews for anti-TRH and anti-SIM and S. Hayashi for *btl -Gal4*. This work was supported through a grant from the Canadian Institutes of Health Research to M.J. S and an Ontario Graduate Scholarship to Y.S.