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Transcriptional Regulation of *Drosophila* Tracheal and Neural Development

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Abstract

Drosophila melanogaster is an ideal system for investigating the functions of genes and is one of the predominant models for the study of branching morphogenesis in the tracheal system. Transcription factors are a unique class of proteins with the ability to bind DNA to recruit RNA polymerases to regulate gene expression directly. In *Drosophila,* a relatively small number of transcription factors are used during tracheogenesis and some of these are recycled and reused to direct regulation of different organ systems including the central nervous system (CNS). The purpose of investigating the re-usage of transcription factors is to reveal differential and similar mechanisms of gene regulation between organ systems. Some modes of regulation which could confer differences include chromatin remodelling and alternate splicing. In addition transcription factors often act in combination with other transcription factors at different time points for different functions. This study reveals the functions of sixteen transcription factors shared between the trachea and CNS through analyses of high-throughput experiments and current knowledge. The characteristics of these transcription factors were also investigated to determine whether differential or similar methods of regulating transcription factor activity are the driving force for organogenesis of the CNS and trachea.

Contents

Abbreviations

AEL	After egg laying
AJ	Adherens junction
AP	Anterior-posterior
BDGP	Berkley *Drosophila* Genome Project
bHLH	Basic helix loop helix
BMP	Bone morphogenic protein
BTB	Broad *tramtrack* bric-a-brac
bZIP	Basic leucine zipper
CME	CNS midline element
CNS	Central nervous system
DB	Dorsal branch
DTa	Dorsal trunk, anterior
DTp	Dorsal trunk, posterior
DV	Dorsal-ventral
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GB	Ganglionic branch
GMC	Ganglion mother cell
ISN	*in statu nascendi*
LTa	Lateral trunk, anterior
LTp	Lateral trunk, posterior
MAPK	Mitogen activated protein kinase
MGA	Midline glia, anterior
MGM	Midline glia, middle
MNB	Median neuroblast
MP1	Medial precursor 1
NB	Neuroblast
NMJ	Neuromuscular junction
PAS	Per ARNT Sim
pNR	Procephalic neurogenic region
POU	Pit Oct Unc
STAT	Signal transducer and activator of transcription
TIF	The Inter*active* Fly
UMI	Unpaired median interneuron
VB	Visceral branch
VML	Ventral midline
VNC	Ventral nerve cord
vNR	Ventral neurogenic region
ZF	Zinc finger

1. Introduction

Understanding the molecular mechanisms which drive organogenesis is one of the many goals of developmental biology. The simplicity of and the ability to manipulate *Drosophila melanogaster* makes it a useful organism for the study of the mechanisms that lead to organ formation. The tracheal system of *Drosophila* is composed of a branched network of approximately 10,000 epithelial tubes that allows oxygen to diffuse through exterior spiracles to reach terminal branches at tissues. Despite the relatively large number of tubes in the *Drosophila* tracheal system, the architecture of the network is relatively simplistic as development of each tracheal hemisegment follows a stereotyped pattern of cell migration, branch formation, branch elongation and finally branch fusion or branch termination. The major complexities are in the genetics, both embryo-wide and cellular-specific, which underlie tube formation controlling all aspects of tracheogenesis from the location of branching points to the length and size of the tubes. *Drosophila* is an ideal eukaryotic *in vivo* system for investigating the genetic and cellular basis of branching morphogenesis with studies extrapolated to the development of the more complex human branched structures of the vascular system, lungs and kidneys (Warburton et al., 2001).

The central nervous system (CNS) is the most complex organ in any organism. Loosely speaking the *Drosophila* CNS is composed of the brain proper, derived from the procephalic neurogenic region, and the ventral nerve cord (VNC), derived from the ventral neurogenic region (vNR), which under certain definitions includes the ventral midline (VML) derived from the mesectoderm (Hartenstein, 1993). The ventral neurogenic region is also known as the neuroectoderm later in development, from which cells are selected for delamination into neuroblasts. Neuroblasts are the precursors which further differentiate into glia and neurons for constructing the axonal tracts. Due to the complexity and wider prevalence of CNS related diseases, there has been a lot of progress made into discovering the mechanisms governing the development of the CNS.

While the *Drosophila* tracheal system and the CNS are both derived from the ectoderm, they are two very distinct and separate organs systems with different morphology, structure and function. Similarities may be found at the cellular or subcellular level especially in regards to gene functions. Approximately 1000 genes in the tracheal system have been identified of which approximately 150 of those genes have been characterised and approximately 60 of those are transcription factors. Only 1/3 of those transcription factors have been characterised in the tracheal system. Meanwhile studies on neural development have identified over 400 transcription factors.

2. Tracheogenesis overview

Tracheogenesis follows the general pattern of cell specification, sac invagination to form tracheal pits, primary branching, secondary branching and ends with branches undergoing either tube fusion or tube termination (Figure 1). Tracheal system organogenesis begins at approximately stage 10 of development (~5 h after egg laying (AEL)). At this time point, segments T2 to A8 of the dorsal ectoderm each form two placodes of tracheal precursors either side of the ventral midline forming a sagittal bilateral symmetry. Each of the 20 placodes will eventually consist of approximately 80 tracheal cells. The major genes involved in inducing a tracheal cell fate are *trachealess (trh), tango (tgo)* and *ventral veins lacking* (*vvl* also known as *drifter*) (Wilk et al., 1996; Zelzer and Shilo, 2000). *trachealess* and *tango* both encode basic helix loop helix (bHLH) proteins, which bind together to form the functional transcriptional activator and also interacts with *ventral veins lacking* for expressing the necessary branching genes later in tracheogenesis. In their absence, placodes fail to specify and do not invaginate properly nor do branches form. Prior to invagination, the population of placode tracheal cells further subdivide themselves into each of the specialised branches for the next step in tracheogenesis which will form the characteristic pattern of tracheal tubes. This occurs through segment polarity genes, of which *engrailed (en), hedgehog (hh), decapentaplegic (dpp), rhomboid (rho)* and *wingless (wg)* are among those implicated in this process. *hedgehog* is also required for allocating the correct number of placode cells (Glazer and Shilo, 2001). At stage 10, tracheal placodes invaginate from the ectoderm to form the tracheal pit, an epithelial sac still connected to the surface via a spiracle. It is at this point that

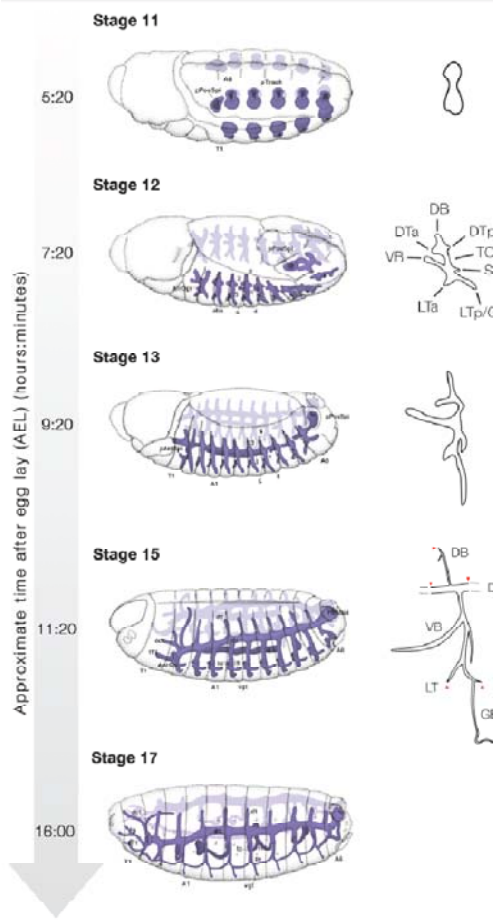


Figure 1 – Five discontinuous stages of tracheal system development during *Drosophila* embryogenesis. Up to stage 10, placodes of progenitors cell accumulate and divide before invaginating at stage 11. Six branches sprout in the process of primary branching at stage 12, the six branches are: the dorsal branch (DB), dorsal trunk anterior (DTa) and posterior (DTp), visceral branch (VB), lateral trunk anterior (LTa) and lateral trunk posterior (LTp). LTp is also called the ganglionic branch (GB). SB denotes the spiracular branch and TC is the transverse connective. The red arrows at stage 15 denote branches which undergo fusion with branches from adjacent hemisegments. Images courtesy of Richard Pollock, originally adapted from Hartenstein (1993) and Ghabriel, *et al.* (2003).

cells undergo the last round of division and no further cells are formed beyond this point (Ghabrial et al., 2003).

At approximately stage 11-12 (~7 h AEL) when tracheal pits have formed, primary branching occurs at six predefined bud sites of each sac. Two cells per bud site migrate which are followed by between four to twenty cells dependant on the branch in question (refer to Figure 1 for branch nomenclature). These cells undergo a process involving cell rearrangement, intercalation and intracellular tube formation where an epithelial monolayer is created with a lumen allowing air to pass. The major genes involved in primary branching are *branchless (bnl)* and *breathless* (*btl)* which are homologues of mammalian fibroblast growth factor (FGF) and fibroblast growth factor receptor (FGFR) respectively. *breathless* expression in the extending branch of tracheal cells is under control of *trachealess* and *ventral veins lacking,* and is responsive to Branchless signalling from nearby tissues in a chemotropic fashion. The stimulation of downstream genes through the FGF signalling induces cell changes for migration, intercalation and morphology changes necessary for primary branching (Ghabrial et al., 2003; Sutherland et al., 1996).

Secondary branching begins concomitantly as primary branching but differences between primary and secondary branches are not apparent until stage 14 (~10 h AEL). Secondary branches arise at various points along predefined locations of the primary branches and usually consist of a single cell with an intercellular junction to seal the lumen. Approximately twenty secondary branches can emerge from a single primary branch either along the branch or at the tip resulting in forked patterns. Again *branchless* and *breathless* are involved in secondary branching, however under different molecular regulatory mechanisms. Most secondary branches can further elongate and terminate as ramified cells which penetrate and innervate target tissues. Termination is an unstereotyped and oxygen dependent process which occurs during the early larval stages, again utilising *branchless* and *breathless* but under the control of *similar (sima)*, the *Drosophila* homologue of the mammalian *hypoxia inducible factor α (HIF α)* (Ghabrial et al., 2003; Nagao et al., 1996).

Some branches undergo fusion instead of termination, such as the dorsal branch (DB), dorsal trunk (DT), lateral trunk (LT) and ganglionic branch (GB). Tracheal fusion cells are specialised cells at the anterior of one segment and the posterior of the next segment which meet and fuse together. They form an autocellular junction held together by a ring of E-cadherin adhesion proteins through which the lumen will develop via a series of actin tracks. The most notable tracheal anatomical part to undergo fusion is the dorsal trunk which will form the main continuous lumen spanning the length of the embryo. The major gene involved with this process is *dysfusion (dys),* a transcription factor whose inactivation will cause fusion failure (Jiang and Crews, 2003; Jiang and Crews, 2006).

3. Overview of the central nervous system

The *Drosophila* CNS is a highly complex organ composed of multiple parts including the brain proper, the VNC and the VML. As determined by the fate map generated by the global patterning genes, development of the brain follows from the procephalic neurogenic region, the VNC follows from the vNR, and the VML follows from the mesectoderm (Figure 2). These CNS structures can be further subdivided into more discrete parts however the focus here will be on the VNC and VML.

3.1. Development of the CNS ventral midline

Although one may consider the VML as part of the CNS, its unique development from the mesectoderm warrants its own section. The *Drosophila* VML is a specialised area of the CNS with a key role in axonal guidance. When fully developed the VML in conjunction with the VNC exhibits a distinct ladder pattern, consisting of longitudinal interconnective axons and transverse commissural axons interspaced between its cells. As well as being structurally separate from the other areas of the CNS, the embryonic VML is under the distinct control of the master regulatory gene, *single-minded* (*sim*). Classification of VML developmental stages according to BDGP terminology include the mesectoderm *in statu nascendi* (ISN; stage 5-6), mesectoderm anlage (stage 7-8), mesectoderm midline primordium (stage 9-12), and mature midline cells (stage 12+). During blastoderm and gastrula stages of

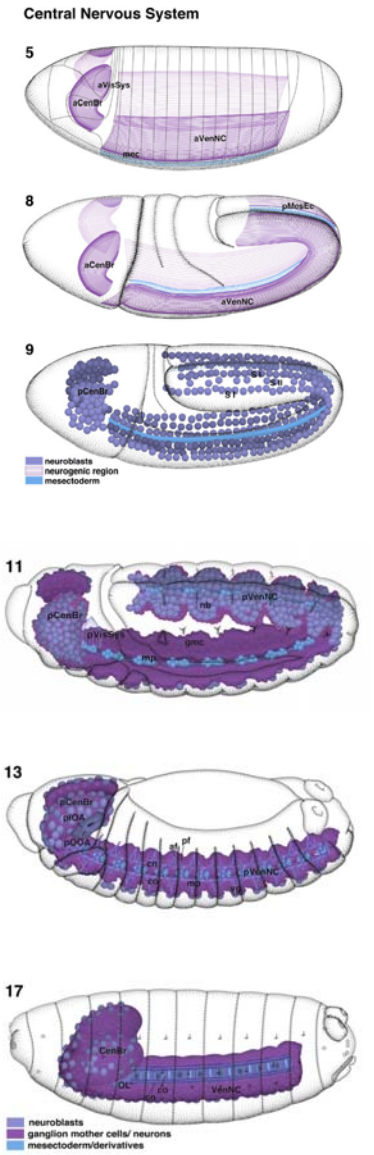


Figure 2 – Six discontinuous stages of CNS development in *Drosophila* embryogenesis. CNS neurogenic regions and the mesectoderm ISN are specified as early as stage 5. By stage 8, proneural clusters are formed in the neuroectoderm (labelled aVenNC) and the mesectoderm anlage is formed in the wake of gastrulation. Stage 9 sees the beginning of delamination of neuroblasts with possible asymmetric divisions in the neuroectoderm and the beginning of the midline primordium. At stage 11-12 both CNS and midline neuroblasts completely are almost fully delaminated and specification of cell fate occurs. Stage 13 heralds the end of midline divisions and the invasion of the midline by neuroectodermal cells. The final stages 14-17 show the condensation of the VNC and the distinct ladder pattern of axon tracts. Images from Hartenstein (1993).

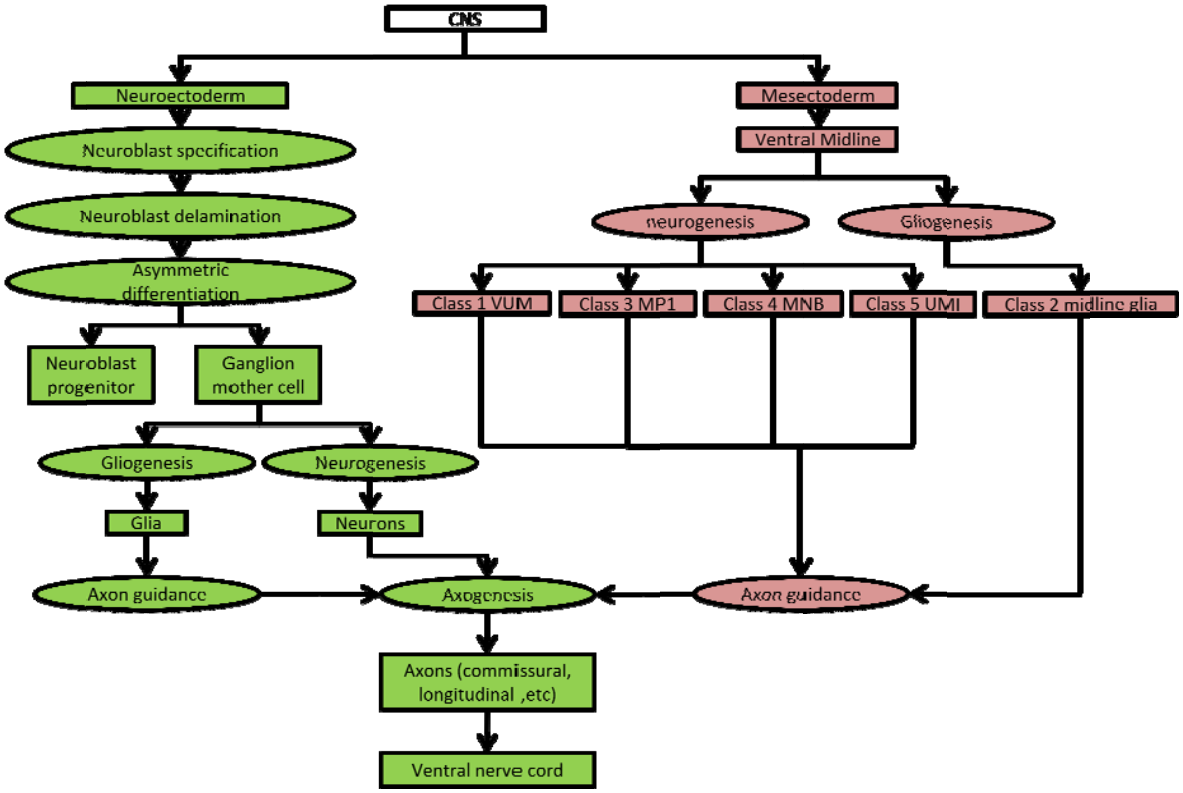


Figure 3 – Hierarchy of semantic terms for CNS developmental processes and anatomy that are frequently encountered in literature. Rectangular terms house anatomical parts whereas ellipses house processes. Those found in the neuroectoderm are coloured green and those found in the VML are coloured pink. Interactions between processes and anatomy are denoted by arrows with a general chronological order from top to bottom.

development, midline cells of the mesectoderm ISN can only be recognised by gene expression profile and not morphology (Brody, 1999).

Gastrulation is the event which formally begins VML formation and involves the invagination of the mesoderm to form the ventral furrow, thus bringing the lateral neuroectodermal tissues closer together. The mesectoderm is the border tissue which separates the invaginated mesoderm from the lateral neuroectoderm and is termed the mesectoderm anlage. In each segment of the anlage there is a single layer of four cells on either side of the ventral anterior-posterior (AP) axis, which become a set of progenitor cells fated to become the VML. These two rows of four cells form a single row of eight cells and subsequently each cell divides once. Some of these sixteen cells are terminally divided, however others will continue to divide with the progeny already fated to become a particular cell type.

The end of the anlage stage and beginning of the primordium is signalled by this last division. The mesectoderm midline primordium is characterised by various changes in the midline precursor cells which are also its defining characteristics. These characteristics include cell migration, differentiation, enlargement of cell diameters, shape changes, rotary movements, cell divisions and segregation. These cells remain integrated as epithelial cells of the surface ectoderm until around

stage 12 when the midline neuroblasts will have completely delaminated (Brody, 1999; Hartenstein, 1993).

Maturation of the midline cells is the final stage where neurogenesis and gliogenesis occurs to form cells classified into five groups: class 1 'ventral unpaired medial neurons' (VUM) which form interneurons and motoneurons; class 2 'midline glia, anterior' (MGA) and 'midline glia, middle' (MGM) which wrap commissures; class 3 'medial precursor 1 neurons' (MP1); class 4 'median neuroblasts' (MNB); and class 5 'unpaired median interneurons' (UMI). At this point accessory cells from the lateral neuroectoderm and mesoderm intermingle with the midline cells including neuroblast 6-4, channel glia and dorsal medial cells (Brody, 1999; Hartenstein, 1993).

As with all developmental processes, the VML is under control of a number of genes, over two hundred as described by various methodologies including microarrays (Kearney et al., 2004; Wheeler et al., 2006). During the ISN stage, thirty-seven genes are expressed, fourteen of which are transcription factors. Forty-one genes are expressed in the anlage stage, many of which are carried over from the ISN stage. Over a hundred genes are expressed in the midline primordium prior to maturation, forty of which are putative transcription factors (Brody, 1999; Kearney et al., 2004; Wheeler et al., 2006)

3.2. Development of the CNS ventral nerve cord

The VNC develops from the vNR of which the principle organ for development is the neuroectoderm, a two-dimensional organ structure which spans the floor of the embryo. Each segment of the neuroectoderm is termed a neuromere, and in one hemineuromere there are approximately 400 neuroectodermal cells which generate approximately 30 neuroblasts and the rest develop into the epidermis. The general order of embryonic events which govern neuroblast formation start with allocation of neuroectodermal clusters in each hemineuromere to a neural fate and selection of the neuroblast through lateral inhibition. This is followed by delamination of progenitor neuroblasts and asymmetric differentiation of the neuroblasts into progenitor cells and ganglion mother cells (GMC). While the progenitor cell will continue to divide iteratively, the GMC terminally differentiates with the final asymmetric division into either neurons and/or glia. The entire VNC structure undergoes condensation and construction of the axon tracts occur, the latter of which is a complex process involving the VML (Skeath and Thor, 2003). Note that these processes are only listed in a general chronological order and can occur concomitantly as with the branching processes during tracheogenesis.

3.2.1. Allocation, selection and specification of neuroblasts in the neuroectoderm

The initial selection and identity of a neuroblast amongst the neuroectodermal cells is mostly determined by the spatial location of the cell as determined by transplantation experiments (Brody, 1999). A temporal component to specification and selection also exists later during neurogenesis but the spatial coordinates are more important. Patterning allocates clusters of cells in each hemineuromere to express proneural genes: *achaete (ac), scute (sc), asense (ase)* or *lethal of scute (los).* These are necessary for a neuroblast fate and mutants of these genes do not develop as many neuroblasts as wildtype (Skeath and Thor, 2003).

What follows is a competition to be the sole neuroblast in each cluster of cells. It is believed that the cell which expresses the most proneural gene protein AC and SC upregulates and secretes the most *delta (Dl)*. Dl ligand is secreted which activates the Notch receptor in neighbouring neuroectodermal cells to downregulate proneural genes thereby preventing these cells from becoming neuroblasts. This is termed lateral inhibition and the result is to ensure only one neuroblast is selected from a particular cluster (Skeath and Thor, 2003).

As previously stated one of the determinants of the future fate of a particular neuroblast was pre-determined by its AP and DV location and later on, this is solidified by the timing of the asymmetric divisions. In the AP axis the pair-rule genes *wingless, hedgehog, gooseberry (gsb)* and *engrailed* subdivide the neuroectoderm along the axis into stripes termed rows (Brody and Odenwald, 2002). Meanwhile in the DV axis, expression compartments are formed by gradients of *dorsal (dl)*, *glass bottom boat* (*glass bottom boat*) and *spitz (spi)/rho* epidermal growth factor (EGF) signalling to determine the boundaries of the neuroectoderm. They further specify the three domains (termed columns) of the neuroectoderm. These columns are defined by the expression of a particular gene, *ventral nervous system defective* (*vnd*) in the ventral column, *intermediate neuroblasts defective* (*ind*) in the intermediate column and *muscle segment homeobox (msh)* in the dorsal column. Expression of these three columnar genes is compartmentalised and the resulting combination of AP segmentation and DV columnar genes cause upregulation of different target genes in each neuroblast to further specify neuronal and/or glial

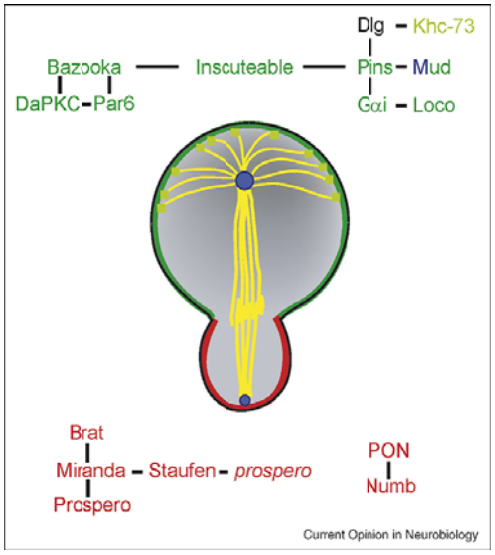


Figure 4 – Image of the asymmetrically dividing neuroblast. Proteins localised to the apical membrane include Inscuteable and accessory proteins and proteins localised to the basal side include Prospero and accessory proteins. Mitotic spindle orientation is maintained by *inscuteable*. Image from Zhong, et al. (2003).

identity (Skeath and Thor, 2003; Brody, 1999).

3.2.2. Delamination and asymmetric differentiation

The selected neuroblasts in each cluster bud-off and migrate dorsally towards the centre of the embryo in the process of delamination, which may simultaneously encompass the process of asymmetric differentiation. Neuroblasts selected from the neuroectoderm for delamination move towards the interior of the embryo into what is termed the subectodermal neuroblast layer. Delamination of neuroblasts occurs in five waves over three hours, starting at stage 9. As previously mentioned, the identity of neuroblasts is chiefly determined by their AP and DV position and, to some extent, their division time point but delamination time has little or no effect on neuroblast identity. Asymmetric differentiation occurs either shortly before or after delamination from the neuroectoderm. Early wave neuroblasts are not likely to divide before delamination whereas late wave neuroblasts always divide in the neuroectoderm (Skeath and Thor, 2003; Brody, 1999; Brody and Odenwald, 2002; Zhong and Chia, 2008).

At stage 9-13, each neuroblast undergoes up to eight asymmetric divisions to yield one progenitor neuroblast at the apical side and one GMC at the basal side. Although the generic term neuroblast can be used for all of the progenitor cells, two other names can be assigned to the progenitor at this time, glioblast and neuroglioblast depending upon the progeny they generate. The asymmetry of the mitotic cells arises from the localisation of the following proteins: Prospero, Numb, Partner of numb, and Miranda to the basal side for a GMC fate and Bazooka, Inscuteable, Partner of Inscuteable, G-protein subunit Gαi, *Drosophila* atypical protein kinase C, and Par-6 to the apical side. This occurs early in the cell cycle which causes the massive disparity in size between the progenitor and the GMC (Figure 4) (Zhong and Chia, 2008).

Figure 5 – The embryonic VNC at stage 15. Stained for monoclonal antibody BP102 for axon tracts depicting the segmented ladder pattern of commissures and longitudinal tracts. Image from (Hummel et al., 1999).

Although the GMC will continue to divide once more, it is this timing of the previous asymmetric division which consolidates cell fate. The progeny generated is temporally regulated by expressing five genes exclusively at different time windows during the divisions, these genes are in order: *hunchback (hb), kruppel (kr), POU domain protein (pdm), castor (castor)* and *grainyhead (grh)* (Brody, 1999; Brody and Odenwald, 2002). The GMC and its subsequent progeny continue to retain expression of the gene that was expressed at the point of division (Skeath and Thor, 2003). The GMC terminally divides once more to become two distinct neurons or glia or one of

each. The default differentiation programme is for the GMC to become neurons due to the expression of pro-neural genes, however, the key gene involved in gliogenesis is *glial cells missing (gcm)*. As its name implies and through mutant studies, the absence of *glial cells missing* causes defects in the development of CNS glia (Egger et al., 2002).

At stage 14-17 the previously segmented VNC structure condenses into one fluid organ system which is characterised by the deposition of extracellular matrix by migrating hemocytes and the commencement of neural activity (Figure 5)(Brody, 1999; Hummel et al., 1999).

3.2.3. Axogenesis

The main task of the VML is to provide guidance cues for CNS VNC neuronal axons. As with all arthropods the *Drosophila* VNC forms a distinctive segmented ladder pattern of axonal nerve tracts, which develop concomitantly during later stages of neurogenesis (Figure 5). Each segment consists of longitudinal axon tracts flanking each side of the midline, also called intersegmental tracts, and two commissural tracts, one located anterior and one posterior of each segment, which cross the midline laterally to connect each side of the neuromere. Tracts are formed initially by chemotropism, a complex interplay involving the chemoattractants, *Netrin (Netrin)* and *frazzled (fra)*, and chemorepellents, *roundabout (robo)* and *slit (sli)*. With specific reference to commissural growth cones, the posterior commissural tracts are guided across large distances and then across the midline to meet the VUM and MP1 neurons of the VML. The VUM neuron and the MGA glia have roles in attracting tracts that form the anterior commissure. Both the anterior and posterior commissural tracts form together as one tract and it is the role of the MGM glia to migrate over the MGA glia and VUM neuron to separate the commissures (Brody, 1999; Anderson et al., 1995).

4. Shared mechanisms and shared transcription factors of the CNS and tracheal system

The CNS and the tracheal system both have very different mechanisms of development. Branching morphogenesis of the tracheal system has a relatively simple process compared to the CNS, however, similarities can be observed at the cellular and subcellular level. From a tracheal point of view these include cell allocation and specification, cell migration, cell guidance, and cell morphology changes. Cell specification is the initial phase of organogenesis which assigns future identities to the stem cells of the ectoderm and is present in both CNS and tracheal system. Cell migration and cell morphology play a huge part in tracheal development because of the lack of cell divisions, which mean that cells must migrate, intercalate and elongate to form tubes. Meanwhile in

the CNS, cell migration is involved with axon tract formation. Cell guidance is shared between the two organ systems, with the chief chemotropic mechanism for tracheal cells being Bnl/Btl signalling and for axons, the main chemotropic mechanisms are through Netrin/Fra and Robo/Sli. There are also indications that Robo/Sli also has tissue interactions with branching morphogenesis of tracheal tubes (Englund et al., 2002; Lundstrom et al., 2004). Similarities may also exist at the molecular level such as the formation of actin cytoskeletal structures for cell morphology changes during lumen formation and axogenesis.

In addition, control of gene expression is another possibility for finding similarities, of which transcription factors constitute a class of genes, which have a vital role during organogenesis. Transcription factors are far too limited in number to each drive separate organ and suborgan development which leads to the notion of combinatorial usage of transcription factors to express differing combinations of genes. *tango* is an example of a transcription factor with aspecific expression in both the VML and the tracheal system. The ability of *tango* to form unique heterodimers with *single-minded* and *trachealess* in each respective organ gives *tango* its ability to drive differential gene expression. Analyses of transcriptional regulatory differences in the two organs are bound to reveal different modes of regulating transcription factor activity, binding efficacy, target genes, and so forth. However, similarities may also be present and may drive the same aspects of molecular development in the both the CNS and tracheal system.

It is this method of regulation by transcription factors which garners the interest of this review because transcription factors, with their ability to directly bind DNA and express genes directly, are among the most important genes in regulating development. Approximately sixty transcription factors have been identified in the *Drosophila* tracheal system (Cheng, 2009, unpublished observations) gleaned from a variety of sources including databases and review articles. Meanwhile the CNS, being a far more complex organ, expresses thousands of genes and a lot more transcription factors, some of which have been identified using high-throughput methods (Altenhein et al., 2006; Brody et al., 2002; Egger et al., 2002; Kearney et al., 2004; Wheeler et al., 2006). Analyses of transcription factors in databases such as Flymine, Flybase and the BDGP have found that sixteen of these transcription factors are expressed both in the embryonic trachea and CNS (Table 1).

Symbol	Name	Structural Motifs	CNS Microarray Detection[1]	Possible Tracheal Targets	Possible CNS Targets	Tracheal System Function	Neuroectoderm Function	VML Function	Notes
ci	*cubitus interruptus*	Zn Finger	Yes	*en*		Spiracle morphogenesis			Proteolytic processing
en	*engrailed*	homeodomain	Yes	*hh*			Row 6-7 specification	Specification of VUM and MNB	
esg	*escargot*	Zn Finger	Yes	*shg, hh*	*insc*	Fusion branching	Medial column specification, asymmetric division		
grh	*grainy head*	bHLH	Yes	*hh*	*Ubx, en*	Tube size regulation	Asymmetric division		Alternate splicing
jing	*jing*	Zn Finger	Yes	*rho, spi*		Placode specification	Glia migration, axogenesis	Specification, Apoptosis	
Mad	*Mothers against dpp*	MH1	No	*kni, knrl*		Dorsal branching	NMJ synaptogenesis		
peb	*pebbled*	ZF	No			Epithelium integrity, lumen integrity			
pnt	*pointed*	*ets*	Yes	*sty, kni, knrl, btl, bs, esg*		Secondary/terminal branching, DB migration	Asymmetric division, glia migration	Gliogenesis	
rib	*ribbon*	BTB	Yes	*crb, Moe, serp, verm*		Primary branching	N/A		
Stat92E	*Signal-transducer and activator of transcription protein at 92E*	SH2	Yes	*ken, vvl*	*vvl*	Placode specification, DT branching, spiracle morphogenesis	Neuron specification, axogenesis		
tdf	*tracheal defective*	bZIP	Yes			Primary branching, cell migration	NMJ		
tgo	*tango*	bHLH	Yes	---	---	---	---	---	
trh	*trachealess*	bHLH	No	*btl, sms, rho, tdf, peb*		Cell specification, branching			
ttk	*tramtrack*	BTB, Zn Finger	Yes	*pyd, bnl, mmy, esg*	*ase, dpn*	Cell intercalation, tube size regulation, lumen formation	Glia migration	Gliogenesis	Alternate splicing
unpg	*unplugged*	homeodomain	No			GB fate			
vvl	*ventral veins lacking*	POU, homeodomain	Yes	*btl, kni, knrl*		Branching	Cell specification, axogenesis	Glia migration	

Table 1 – List of shared CNS and tracheal system transcription factors.

[1] CNS detection in one of the following four papers: Brody, et al. (2002); Wheeler et al. (2004); Kearney et al. (2004); Altenhein et al. (2006).

4.1. Transcription factors of the tracheal placodes and pits also found in the CNS

The most important processes in this early stage of tracheogenesis are cell specification and cell migration during the invagination process. The shared CNS and tracheal system transcription factors found to be present here in the placodes and pits include *cubitus interruptus* (*ci*), *engrailed*, jing, *Mothers against dpp (Mad)*, *Signal-transducer and activator of transcription protein at 92E* (*Stat92E*), *unplugged (unpg*), *tramtrack (ttk*), *trachealess, tango* and *ventral veins lacking*.

4.1.1. *cubitus interruptus* specifies CNS and tracheal cells through segment polarity genes

cubitus interruptus is a zinc finger transcription factor classed as a segment polarity gene found mainly in anterior compartments of segments during early embryogenesis. It is involved somehow in tracheogenesis (Warburton et al., 2001) including spiracle morphogenesis through interactions with *hedgehog* (Merabet et al., 2005) and has been detected in the CNS only by high-throughput methods, specifically in the VML primordium (Kearney et al., 2004) and VNC (Brody et al., 2002).

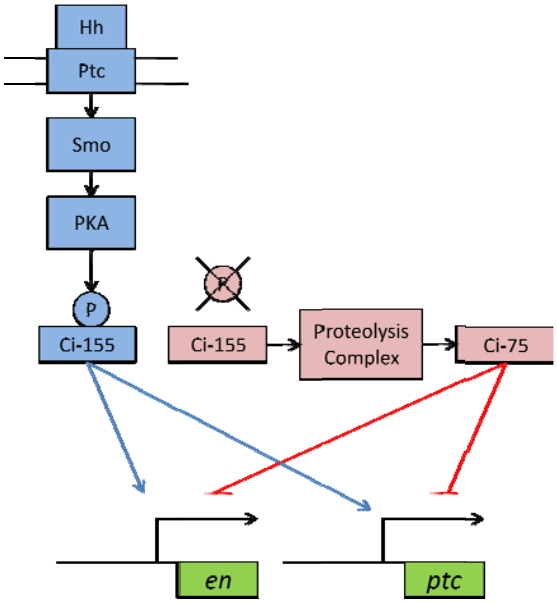


Figure 6 – Model of the interactions of the segment polarity gene *cubitus interruptus*. Ci-155 normally regulates *engrailed* and *ptc* in its phosphorylated state in response to Hh/Ptc/Smo/PKA signalling. However, in the absence of Hh, the desphosphorylated state of Ci-155 is prone to recruitment by the proteolysis complex, which cleaves Ci-155 to generate Ci-75. Ci-75 negatively regulates *engrailed* and *ptc*.

Ci protein comes in two forms, a full length and truncated form, an activator Ci^{A} and repressor Ci^{R} respectively, also called Ci-155 and Ci-75. Both have the same binding affinity for the same sequence of DNA making them direct competitors of each other and the two forms come about through post-translation proteolysis of the C-terminus of Ci-155 where the cytoplasmic localisation signal is cleaved off (Figure 6) (Brody, 1999).

The specific gene targets of *cubitus interruptus* are not known for the CNS or tracheal system, although it is definitely regulated by *hedgehog* signalling through *patched* (*ptc*) and *smoothened (smo)*. Hh paracrine signalling originates in posterior compartment cells and diffuses into anterior cells, where the primary role of Hh is to repress proteolysis of Ci-155 to Ci-75. Roles for various adapter proteins and protein kinases in proteolysis are inferred to be involved through

analysis of development of other tissues (Akimaru et al., 1997; za-Blanc and Kornberg, 1999), which have been included in Figure 6. However, in a generic role *patched* and *engrailed* are implied as targets of *cubitus interruptus* (Hepker et al., 1997).

Because of *cubitus interruptus*'s general role as a segment polarity gene and also the technical difficulties faced by the lack of useful mutant alleles (Brody, 1999), only conjectures on its effects in the tracheal or CNS systems can be made. For the posterior spiracle, the effect of a *cubitus interruptus* mutant is likely to be similar but not as serious as a *hedgehog* mutant which causes deformations (Merabet et al., 2005). For the tracheal system and CNS as whole, cell specification and identity may be affected due to the requirements of compartmentalisation of specific gene combinations.

4.1.2. *engrailed* specifies CNS and tracheal cells through segment polarity genes

engrailed is a pair-rule gene active only in the posterior compartments of segments during early embryogenesis and is linked to *hedgehog* and *cubitus interruptus* signalling. There are fourteen stripes of *engrailed* posterior compartments on either side of each segment formed by the pair-rule gene expression of *even-skipped (eve), fushi tarazu* (*ftz,* an activator of *engrailed*), *sloppy paired (slp), runt (run), paired (prd) and odd-paired* (*opa,* a repressor of *engrailed*)(Brody, 1999). It appears to be very important in both CNS and tracheal system development, however, specific information on its mechanisms of action are lacking due to pleiotropic effects. *engrailed* acts on anterior compartments by upregulating *hedgehog* in posterior cells and the resulting Hh protein diffuses. This in turn regulates *cubitus interruptus* (shifting the Ci155:Ci75 ratios) and what it does in the trachea is inferred from studies linked to *hedgehog* and *cubitus interruptus* signalling (Figure 6). These indirect effects of *engrailed* in the tracheal system are postulated to be necessary for cell specification and establishing cell fates (Merabet et al., 2005).

Later in development within the VML, *engrailed* has an important role in specifying the fate of subsets of midline cells. There are five sets of midline cells in each segment: VUM, MNB, UMI, MP1 and glia. Two of these types of midline cells, the VUM and MNB neurons form the *engrailed*-positive population, whereas UM1, MP1 and glia form the *engrailed*-negative population. This subtle difference in specifying midline cells comes about from a temporally differential regulation by *wingless* and *hedgehog* signalling, which serve to cluster these two cell populations. The functional unit appears very complex where early *engrailed* (stage 9) is maintained by *wingless* and around stage 10, *wingless* also acts to repress *lethal of scute.* It appears *lethal of scute* provided a positive feedback mechanism for *engrailed* (Bossing and Brand, 2006; Glazer and Shilo, 2001).

In the neuroectoderm, *engrailed* in conjunction with its redundant partner *invected (inv)* has a function in specification of neural fates in the AP axis rows. *engrailed* and *invected* are expressed in rows 6-7 of the neuroectoderm and exerts its effects on row 5. Double mutants for *engrailed* and *invected* are required to induce phenotypes and such double mutants cause neuroblasts in row 5 identities to assume row 4 identities. The mutations in *engrailed* and *invected* disrupt *ptc* expression possibly via *hedgehog* signalling and this allows for *gooseberry* expression. *gooseberry* causes row 4 cells to be insensitive to *wingless* signalling which is a requirement for row 4 identities (Bhat and Schedl, 1997; Merabet et al., 2005).

In summary, *engrailed* is a pair-rule gene involved with early segmentation processes to determine the fates of cells. It appears that the fates of all CNS and tracheal cells utilise a similar mechanism involving a segmented gradient of paracrine Hh signalling to relay signals.

4.1.3. *jing* specifies cells in the CNS and tracheal system through regulation by *single-minded* and *trachealess*

jing is a transcription factor involved in the tracheal system where it specifies cell number in the placode and regulates apoptosis. In the VML it also regulates cell number through apoptosis and disruption to cell numbers here leads to inappropriate axonal tract formation in the VNC (Sedaghat et al., 2002).

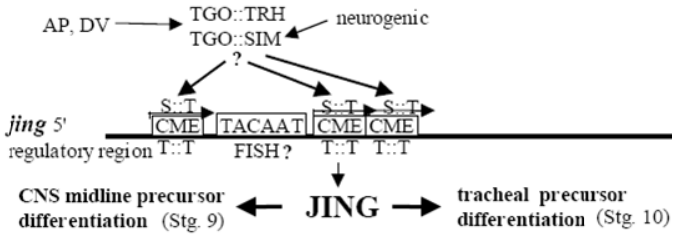


Figure 7 – Model of *jing* function in the VML and tracheal system. Trh/Tgo and Trh/Sim dimers, who both have similar binding specificities, regulate *jing* expression at the CMEs of the promoter region through an unknown mechanism denoted by the "?". The VML jing promoter may also include a *Dichaete* binding site (FISH on the diagram). Image from Sedaghat et al. (2002).

In the tracheal system, the earliest function of *jing* is the allocation of the correct number of cells to the placode starting around stage 10. *jing* mutations result in inappropriate apoptosis of placode cells which in turn results in missing parts of the DT, DB and transverse connective tubes. In addition the VB is completely missing. The DT and VB exhibit the most severe phenotypes in *jing* mutants and both branches are responsive to Spi/Rho EGF signalling in the central placode. This is contrasted to the DB which is dependent on *decapentaplegic* signalling. The EGF receptor is probably affected downstream of *jing* through an uncharacterised mechanism (Sonnenfeld et al., 2004).

The development of the lateral neuroectoderm neuroblasts is relatively unaffected with *jing* mutants however the VML is severely affected. *jing* expression normally begins from stage 9

onwards however inactivating mutants of *jing* result in inappropriate apoptosis of precursors of *single-minded*-positive midline cells and only 50% of neuroblasts differentiate into the proper glia and neurons. Again the role of EGF signalling is implicated in *jing* activity possibly through phosphorylation of *jing* by the EGF/Ras/MAPK pathway (Sedaghat et al., 2002; Sonnenfeld et al., 2004). The role of the VML as a guidance organ for axon scaffold formation is severely compromised due to the lack of cells and thus chemotropic molecules for growth cones. Commissures are extremely disrupted with *jing* loss-of-function mutants. On the other hand a *jing* gain-of-function mutant also disrupted the longitudinal axon tracts in addition to the commissures (Sedaghat and Sonnenfeld, 2002; Sedaghat et al., 2002).

jing is regulated by *single-minded* and *trachealess* in the VML and tracheal system respectively through binding of E-boxes and regulatory elements termed CNS midline elements (CME) in the 5' regulatory region. The transcription and/or maintenance of *jing* may also require the actions of *ventral veins lacking* and/or *dichaete* (*D*, also known as *fish-hook;* Figure 7). The latter of which can bind to the TACAAT sequence located in the *jing* 5' regulatory region. The gene targets of *jing* are not clearly evident but as previously implied the suspects are components of the EGF cascade and cell survival factors possibly by exerting its effects through modifications to chromatin accessibility (Sun et al., 2006).

It would appear that *jing* may have almost identical subcellular roles in the VML and trachea. Namely that EGF signalling is both implied as necessary for modifying *jing* function to act on genes. Targets of *jing* may still be different however. In addition the establishment of cell identity is a common function of *jing* in the VML and trachea although at different time points.

4.1.4. *Signal-transducer and activator of transcription protein at 92E* is a generic signal transduction pathway involved in specification of tracheal cells by upregulating *trachealess*

Signal-transducer and activator of transcription protein at 92E (S*tat92E*) also known as *Marelle* is part of a Janus kinase / signal-transducer and activator of transcription (JAK/STAT) pathway and is found in the tracheal system and in the CNS.

The stereotypical model of JAK/STAT (Hopscotch/Stat92E in *Drosophila*) is the activation and dimerisation of cell surface receptors by the ligand (*unpaired; unp* in *Drosophila*), which in turn phosphorylates and activates the receptor tyrosine kinase. This phosphorylates tyrosine residues on the receptors which recruit STAT. STAT is subsequently phosphorylated by JAK and activated by dimerisation with another STAT molecule. Translocation of STAT and binding to TTCNNNGAA DNA sequences occurs next (Figure 8)(Li et al., 2003; Luo and Dearolf, 2001). *Stat92E* is a segmentation gene whose targets include *eve* and *run* but its detection in the tracheal system and CNS goes beyond that.

Through mutations studies of zygotic *Stat92E*, it was found to act upstream of *trachealess* expression, which is required for cell specification and cell numbers in the tracheal placode. Later on Stat92E is involved with invagination of tracheal pits at the correct time and also tracheal cell migration. Removal of maternal *Stat92E* in addition to zygotic *Stat92E* abolished tracheal pits completely (Li et al., 2003)(Li et al., 2003). *Stat92E* targets include the transcription factor *ken and barbie* (*ken)* in the DT and spiracles (Arbouzova et al., 2006).

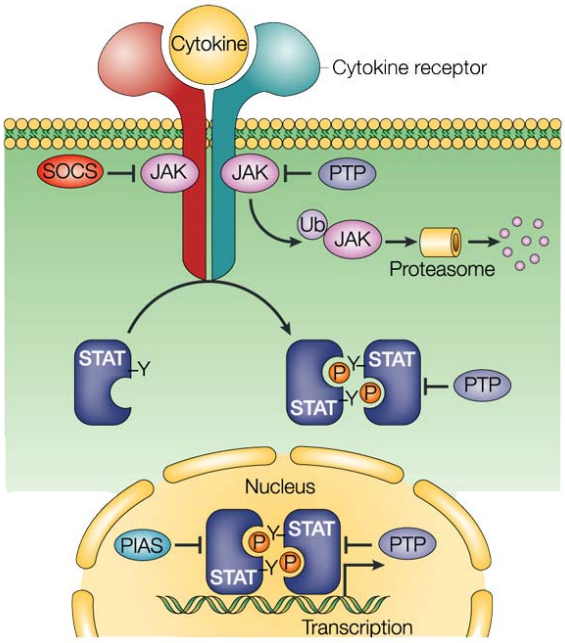


Figure 8 – General model of JAK/STAT signalling taken from the mammalian immune system. Image from (Shuai and Liu, 2003).

In the CNS, mutations of *Stat92E* cause deformations in the longitudinal tracts and missing commissures in images of stage 15 embryos and this has been attributed to defects in axogenesis, however, data is currently lacking in this area (Li et al., 2003). It is possible that the lack of CNS tracts has its roots in inappropriate cell specification, which would put the role of *Stat92E* in accordance with its role in the tracheal system. The activation of *ken and barbie* in the CNS by *State92E* is not determined.

4.1.5. *trachealess* is the master tracheal specification and branching gene with detection in the CNS by *in situ hybridisation*

The expression of *trachealess* begins at around stage 10 and subsists for the entire branching process of tracheogenesis. *trachealess* expression is one of the defining characteristic of a cell fated to become the trachea. Trh is a bHLH PAS protein which heterodimerises with Tgo to form the functional transcription factor, which is phosphorylated by PKB to promote translocation to the nucleus. *trachealess* expression is localised to the tracheal precursors by its AP and DV positioning and acts downstream of *Stat92E*. Targets of *trachealess* include *breathless, stumps (sms), rhomboid, tracheal defective (tdf), pebbled (peb)*, which are necessary for tracheal fate consolidation, invagination and branching. In conjunction with *ventral veins lacking, trachealess* also activates *knirps (kni),knirps-related (knrl)* and *thickveins (tkv)* (Boube et al., 2000; Ghabrial et al., 2003; Isaac and Andrew, 1996).

trachealess has also been detected in a subset of cells in the CNS by the BDGP starting at stage 13 using *in situ hybridisation*, and also in the supraesophageal ganglion of the CNS (Isaac and Andrew, 1996). However, due to the lack of information, there can only be speculation as to what *trachealess* does in the CNS and whether the same mechanisms are utilised.

4.1.6. *tango* is an aspecific bHLH transcription factor with specific effects through binding with *trachealess* and *single-minded*

tango is a bHLH transcription factor which is expressed throughout the embryo. It can form dimers with *single-minded* in the VML, *trachealess* and *dysfusion* in the tracheal system, and *similar* in oxygen-deprived tissues of the larva. These transcription factors all require dimerisation with *tango* for nuclear translocation and binding to DNA. However, *dysfusion* and *similar* are not found in the CNS. Of particular note the binding specificities of these *tango* heterodimers are extremely similar, for example, *trachealess* and *single-minded* both prefer ACGTG sequences and the preferred binding sequence of *dysfusion* is TCGTG (Brody, 1999; Jiang and Crews, 2007). *tango* is an example of a transcription factor which is aspecifically expressed but acts in concert with other transcription factors to drive specific functions.

4.1.7. *ventral veins lacking* is necessary for tracheal and CNS fates by interactions with *trachealess* and *single-minded*

ventral veins lacking, also known as *drifter,* is one of the most important transcription factors in the tracheal system and is also extensively studied in the CNS. It is involved with tracheal placode specification in conjunction with *trachealess,* and expression of *ventral veins lacking* shortly begins after *trachealess.* The POU domain of Vvl interacts directly with the PAS domain of Trh (Brody, 1999; Zelzer and Shilo, 2000) and the combination of *ventral veins lacking*, *tango* and *trachealess* induce expression of *breathless*. In *trachealess* mutants, some tracheal specific genes are still expressed and *ventral veins lacking* is responsible for driving the remnants of tracheal specification in the absence of *trachealess*. Redundant functions of *ventral veins lacking* and *trachealess* probably exist in their induction of gene expression of tracheal system cells but both are required for full tracheal specification and subsequent branching functions. Maintenance of *trachealess* and *ventral veins lacking* expression is probably via auto- or cross-regulation mechanisms (Boube et al., 2000; Ghabrial et al., 2003).

This gene is extensively studied in the CNS including cell specification (Certel and Johnson, 1996), identity and axon guidance of motoneurons (Certel and Thor, 2004), axon guidance in the VML by regulation of the *slit* gene (Ma et al., 2000), and midline glia migration (Anderson et al.,

1995). With specific reference to the latter process of migration, it was found that the MGM was unable to migrate posteriorly which is a necessary action for separating the commissures after they form as one single tract. The same defect in migration was found with tracheal cells attempting to migrate in preparation for branching. The presence of *breathless* in both midline and trachea suggests that it is very likely the molecular mechanism is conserved and that *ventral veins lacking* regulates *breathless* expression (Anderson et al., 1995).

4.1.8. *unplugged* begins with early GB fate specification and ends with termination of the GB into CNS tissues

unplugged is found in the tracheal system during branching, specifically promoting primary GB fate in each segment. It is not found in the CNS itself, however, the GB is directly involved in VNC development, since it is the primary branch which terminates and innervates CNS tissue through a migration process analogous to axonal pathfinding (Englund et al., 2002). Although *unplugged* was found to be expressed in early placode cells, it is not thought to play a major role in specification (Chiang et al., 1995).

It is at stage 12 where *unplugged* has major roles in branching morphogenesis, specifically cell migration and extension of the GB. However, its expression location is limited by homeotic genes with specific reference to the cerebral branch, technically a brain-innervating tubule and hence will not be discussed in this review. The regulatory mechanisms of and by *unplugged* are not conclusive with the only other gene postulated to be involved being *pointed* which acts before *unplugged* expression (Chiang et al., 1995).

While the GB follows the typical Bnl/Btl FGF signalling for initial branching, termination involves switching navigation substrates to one expressed by the VNC glial cells. *unplugged* in conjunction with *adrift* (*aft*) is responsible for this penetration into the VNC by switching the GB response from Bnl/Btl to CNS derived substrates. There may be as many as five different signalling molecules for penetration into the CNS including the Sli/Robo pathway , (Englund et al., 1999; Uv et al., 2003; Chiang et al., 1995; Ghabrial et al., 2003). *slit* is the chief chemorepellent of axogenesis produced by midline cells and it appears this role is reused by the GB to prevent crossing the midline (Englund et al., 2002).

Although *unplugged* is not a VNC or VML transcription factor, it is still important for the GB to penetrate the CNS during development. Its usage of the canonical axogenesis chemorepellent system of Sli/Robo is an excellent example of an almost identical function between two different organ systems.

4.1.9. *tramtrack* is a long-lasting transcription factor beginning with tracheal cell specification and important for gliogenesis

tramtrack is a BTB-domain zinc finger transcription factor which starts expression in the placodes for cell specification but subsists later for cell intercalation and lumen formation (Araujo et al., 2007). It is also present in the CNS with studies on VML glia and lateral glia (Badenhorst, 2001) and its detection by high-throughput methods (Brody et al., 2002). *tramtrack* is most known for its ability to bind to the promoter sequence of the pair-rule gene *ftz*, and exists as two isoforms. The two isoforms are Ttk88 (mostly activating) and Ttk69 (mostly repressing), who both share the same N-terminal BTB domain with a difference in C-terminal zinc finger identity (Brody, 1999)

Intercalation of tracheal cells involves the migration and rearrangement of tracheal cells as they bud and move towards the site of *branchless* signalling. *tramtrack* mediates this action most probably via adherens junctions. Genes which are regulated by *tramtrack* include *polychaetoid (pyd)* and possibly *breathless*. The anti-intercalation transcription factors *spalt* and *knirps* are not regulated by *tramtrack*. Regulating tracheal tube size is concomitant with lumen formation and central to the formation of any branch including fusion and termination. It requires the assembly of a luminal chitin filament which requires several enzymes for synthesis including *mummy (mmy)*, a target of *tramtrack*. However *tramtrack* is thought to be a repressor of chitin synthesis given that mutants of *tramtrack* result in abnormally high deposits of chitin and it is suggested that the vesicular delivery of luminal materials is affected. *tramtrack* is also thought to be a regulator of the septate junction's functions, however, there is only speculation as to what those functions are. Of further note *tramtrack* can also be targeted by Notch signalling in the tracheal system for controlling fusion (Figure 9A) (Araujo et al., 2007).

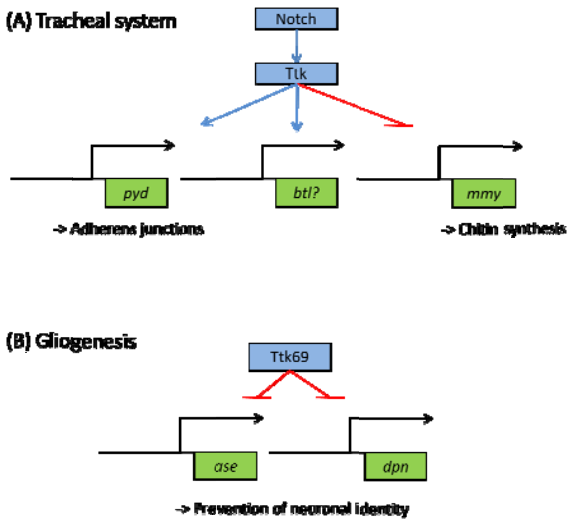


Figure 9 – Model of *tramtrack* interactions. (A) In the tracheal system, under the influence of Notch, Ttk may upregulate *pyd* and *breathless* for lumen formation, while limiting *mmy* synthesis to ensure the correct levels of protein are maintained. (B) During gliogenesis in the CNS, Ttk69 represses pro-neural genes *asense* and *dpn* thereby allowing pro-glial genes to predominate.

Glial cells are affected by *tramtrack* and mutants for *tramtrack* will cause the overdevelopment of neurons. The default programme for neuroblasts is to create neurons unless specified otherwise. The expression of *glial cells missing* is the key regulator for switching from a neural to glial fate in lateral neuroectodermal glia however pro-neural genes still need to be repressed, in

which Ttk69 is implicated as the instigator for repressing the pro-neuron genes *asense* and *deadpan (dpn)* (Figure 9B). In a similar role to Prospero, Ttk69 also regulates cell proliferation to ensure the terminal asymmetric division of a GMC by targeting Cyclin-E for degradation (Badenhorst, 2001).

With the inclusion of Notch in the *tramtrack* functional module, there could be some similar mechanisms in controlling CNS and tracheal development because Notch is also the main receptor used during neuroectodermal lateral inhibition.

4.1.10. *Mothers against dpp* specifies dorsal branches and regulates NMJ synaptogenesis

Mothers against dpp is a transcription factor which is activated during placode specification of the cells destined to become the dorsal branch. It has been detected in the process of synaptogenesis at the neuromuscular junction (NMJ)(McCabe et al., 2004) .

In the tracheal system *Mothers against dpp* is activated in response to *decapentaplegic* signalling and it is possible that *Mothers against dpp* directly activates *knirps* for upregulation during DB specification. *knirps* in turn represses *spalt*, which is for dorsal trunk specification thus ensuring that only the DB is specified (Chen et al., 1998; Myat et al., 2005).

At the NMJ, *Mothers against dpp* is part of a retrograde signalling cascade where the NMJ signals the cell nucleus to stimulate production of synapses. The order of events are *glass bottom boat*, a bone morphogenic protein (BMP) ligand, from the muscle cells activates three cell surface proteins receptors encoded by *wishful thinking (wit), saxophone* (*sax*) and *thickveins* which in turn indirectly activates two transcription factors, *medea (med)* and *Mothers against dpp ,* to signal transcription of new genes for synaptogenesis. The activation of the transcription factors is presumably via phosphorylation by one of the downstream targets of the cell surface receptors.

The activation of *Mothers against dpp* is perhaps the only common element in the CNS NMJ and trachea. In the NMJ it is definitely phosphorylated by Tkv for activation and in the tracheal system *thickveins* is also present in dorsal branches. Unless *knirps* is proven to fulfil a role in synaptogenesis it is not likely to be the target of *Mothers against dpp* in the NMJ unlike the tracheal system where it is used for cell specification.

4.2. Transcription factors active for primary and secondary branching also involved in the CNS

During the early primary branching phase with specific reference to the branch tree consisting of the DB, dorsal trunk, anterior (DTa) and dorsal trunk, posterior (DTp), visceral branch (VB), lateral trunk anterior (LTa) and GB, the following transcription factors are believed to be involved: *tracheal defective*, *pebbled*, *grainyhead* and *ribbon* (*rib)*. These may also be active during secondary branching, however, this part of branching morphogenesis has gathered little interest and

no specific secondary transcription factors were found that were not already present during primary branching. These transcription factors were also found in the CNS in one form or another. Again, the process of primary branching morphogenesis utilises cell migration, cell intercalation and cell morphology changes. It should be noted that expression of the following transcription factors, *Mothers against dpp, tramtrack, trachealess, tango, and ventral veins lacking*, were also carried over from early specification functions.

4.2.1. *grainyhead* undergoes alternate splicing to generate CNS and tracheal specific effects

grainyhead is a transcription factor which undergoes alternate splicing to form several isoforms, of which two will be presented - the N-form, which targets the palindromic be1 sequence, and the O-form, which targets the non-palindromic be2 sequence (Uv et al., 1997). The N-form is found in the tracheal system while the O-form is found in neuroblasts of the CNS. Each isoform targets different genes and exerts different effects, possibly by their dimerisation partner. The N-form is likely to form homodimers whereas, the O-form forms heterodimers with an unknown partner (Brody, 1999; Uv et al., 1997). Within the tracheal system *grainyhead* controls tube size and length. *grainyhead* loss-of-function mutants cause elongated and convoluted tubes whereas overexpression of *grainyhead* causes disruption to lumen formation despite cytoplasmic basal extensions still occurring. Grh is a target of the Bnl/Btl signalling cascade for increased activity and is believed to be effected through a post-translational mechanism since *grainyhead* is uniformly expressed in the tracheal system. Phosphorylation of Grh by mitogen activated protein kinases (MAPK) is a likely candidate. Gene targets of Grh in the tracheal system are unknown and genes which enhance termination or repress pro-apical membrane extension are suspected (Ghabrial et al., 2003; Hemphala et al., 2003).

During neuroblast asymmetric division *grainyhead* is switched on as part of the temporal network of five genes, *hunchback, kruppel, POU domain protein*, *castor* and *grainyhead*, which act to specify a different fate for each GMC during the divisions. The five genes act sequentially by activating the next gene and repressing the one after the next and the currently active gene is passed on to the progeny to continue cell fate determination, in this instance *grainyhead* will specify some motoneurons (Brody and Odenwald, 2000; Cenci and Gould, 2005; Skeath and Thor, 2003).

grainyhead is under control by different regulatory genes in each organ system for possibly different functions. Tracheal tube morphology is determined by phosphorylation state of *grainyhead* depending upon FGF signalling however in the CNS the presence of *grainyhead*, under the combinatorial control of the repressor *POU domain protein* and activator *castor*, could also be used later to regulate cell morphology in preparation for initiating axogenesis.

4.2.2. *ribbon* regulates branch elongation in the tracheal system

ribbon (also known as *pipsqueak)* and involved in branch elongation during primary branching of tracheogenesis. It has also been detected in neuroblasts of the CNS by one high-throughput analysis (Brody et al., 2002) however information about its involvement is sparse other than mutations prevent dorsal closure (Jack and Myette, 1997). *ribbon* encodes a protein with a broad *tramtrack* bric-a-brac (BTB) protein-binding domain and the fifty residue DNA binding pipsqueak domain (Siegmund and Lehmann, 2002).

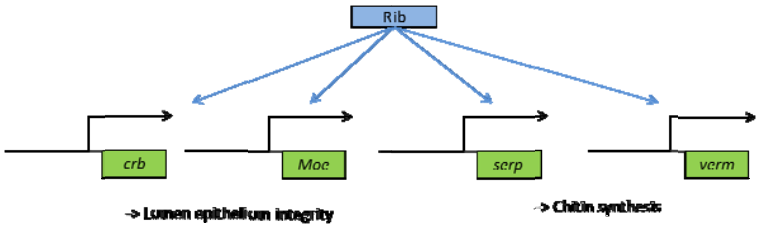


Figure 10 – Model of possible Rib transcriptional targets. Rib targets *crumbs* and *Moesin* for lumen epithelium integrity, in addition to *serpentine* and *vermiform* for chitin synthesis. Both are hallmarks of branching morphogenesis.

Primary branching impairment of *ribbon* mutants is similar to *branchless, breathless* or *stumps* mutants. Cell movement is impaired as are apical cytoplasmic processes (Kerman et al., 2008). Basal cytoplasmic processes however are not impaired and continue membrane extension towards *branchless* signalling (Shim et al., 2001). The targets of *ribbon* are not fully known but include *crumbs* (*crb*) and *Moesin* (*Moe*) which have roles in epithelium integrity (Kerman et al., 2008), *serpentine* (*serp*) and *vermiform* (*verm*)(Luschnig et al., 2006), genes with GAGAG sequences (Lehmann et al., 1998), genes mediating transmission of *breathless* signalling or cytoskeletal components (Blake et al., 1999).

The targets of rib in the CNS are unknown, however, conjectures may assume a role for *crumbs* and *Moesin* in maintaining apical-basal polarity as it does during branching because this is one of the important hallmarks of asymmetric divisions in the CNS.

4.2.3. *tracheal defective* has identical molecular mechanisms in tracheal and CNS cells

tracheal defective (also known as *apontic)* is important in primary branching during tracheogenesis. It has also been detected in the CNS by various methods including microarray (Brody et al., 2002; Egger et al., 2002; Kearney et al., 2004). Maternal *tracheal defective* is detected in a large number of tissues and is relatively unstudied compared to zygotic *tracheal defective* (Brody, 1999). Tdf is a basic leucine zipper domain (bZIP) transcription factor with established DNA-binding specificity and has a role in transcriptional regulation as determined by experiments with its co-

activator multiprotein bridging factor 1 (Mbf1). The preferred binding sequence is palindromic (A/G) TTC (C/T) (A/T) AT (T/A) (G/A) GA (A/T) (T/C) (Liu et al., 2003).

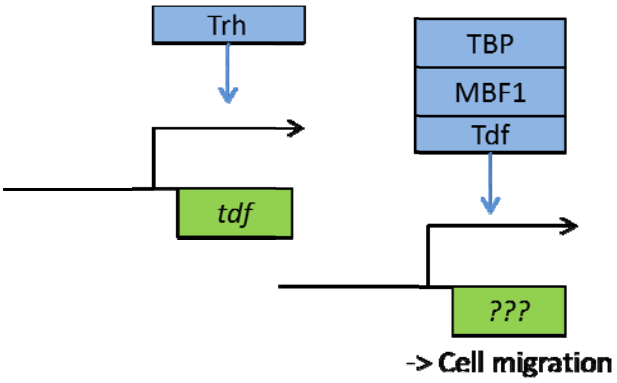


Figure 11 – Model of *tracheal defective* interactions. *tracheal defective* is regulated by Trh which causes its upregulation. Tdf has the ability to bind DNA and drive transcription, however, its efficacy at transcriptional activation is increased when coupled with the adapter proteins TBP and MBF1.

In the tracheal system, *tracheal defective* expression begins in the placodes before invagination and subsists throughout organogenesis of the tracheal system. It is primarily regulated by *trachealess* as inferred from analysis of the downstream targets of *trachealess* and *breathless* FGF signalling (Figure 11)(Boube et al., 2000; Ghabrial et al., 2003). Mutations of *tracheal defective* show that it is not important for tracheal cell identity but for cell migration especially during the primary branching phase. *tracheal defective* is a postulated regulator of a subset of genes necessary for cell migration (Eulenberg and Schuh, 1997).

In the CNS, *tracheal defective* expression can be detected in the following structures: embryonic NMJ (Takasu-Ishikawa et al., 2001) and VNC (Liu et al., 2003). Analysis of a mutant of *tracheal defective* in the NMJ show most impairment in the process of synaptic transmission specifically at neurotransmitter release although synapse formation was thought not to be affected (Takasu-Ishikawa et al., 2001), whereas in the VNC, Tdf is regulated by its Mbf1 co-activator which can increase Tdf activity by 500% and mutants of Mbf1 show conclusive deformations in the formation of the VNC tracts (Liu et al., 2003).

Images from CNS studies only show the overall deformations of tracts with disruptions to *tracheal defective* activity and/or expression. It is possible that these deformations are also a result of migration problems, which are evident in the tracheal system, bearing in mind the necessary role of midline cells in axogenesis by migrating and attracting axon tracts in their wake.

4.2.4. *pebbled* is involved with tracheal epithelium integrity and gliogenesis

pebbled (also known as *hindsight*) is a zinc finger transcription factor present in all tracheal cells and is involved during branching phases for epithelial integrity (Wilk et al., 2000). It has been detected in the CNS only by *in situ hybridisation* during germ band retraction (Yip et al., 1997). During branch elongation, the concomitant construction of the lumen for air passage requires an

epithelium with taenidia facing the interior. While mutants of *pebbled* will cause some cells to undergo apoptosis and also prevents secondary branching, the surviving cells form unusual air sacs caused by collapse or expansion of the tracheal tubes. Apoptosis is thought not to be responsible for the defects in tube architecture, and although mutants of the epithelial genes *crumbs, shotgun* and *stardust (sdt)* yields a similar phenotype to *pebbled* mutants, these are not the targets of *pebbled* (Wilk et al., 2000). Locating a common or differential process for *pebbled* in the CNS and trachea is not possible at this time due to a lack of information for both organs. The prevention of apoptosis is a remote possibility, however, the role of *pebbled* in epithelial integrity is not likely to be involved in the CNS.

4.3. Transcription factors active for fusion and termination also involved in the CNS

The end of branching morphogenesis is signalled by either fusion or termination. The following tracheal transcription factors *escargot (esg)* and *pointed (pnt),* definitely involved in fusion and termination, were also present in the CNS. It should be noted that expression of the following transcription factors: *unplugged*, *pebbled* and *trachealess*, were also carried over from primary and/or secondary branching into these final phases of branching morphogenesis.

4.3.1. *escargot* regulates a subset of *dysfusion* genes and has vital roles in CNS cell specification and asymmetric division

escargot is a zinc finger transcription factor operating in the tracheal system and regulates a subset of genes downstream of its parent transcription factor *dysfusion*. Although *dysfusion* is a transcription factor, it is thought that *dysfusion* regulates *escargot* indirectly to cause fusion (Jiang and Crews, 2006). In the CNS, *escargot* is also involved in asymmetric division of the neuroblasts into progenitors and GMCs (Cai et al., 2003) and has also been detected by high-throughput methods (Brody et al., 2002; Kearney et al., 2004). Both of these processes involve major remodelling of the cell structure.

dysfusion is a tracheal fusion cell specific transcription factor not found in the CNS. Mutants of *dysfusion* abolish fusion of the DB, LT, and GB with many genes targeted for downregulation including *trachealess* and many other transcription factors (Jiang and Crews, 2006), however, not all genes examined were directly regulated. *dysfusion* targets *escargot* indirectly and both transcription factors have been experimentally demonstrated as necessary for fusion events. Due to the number of genes regulated by *dysfusion* there could be a purely speculative role for *escargot* in regulating a subset of *dysfusion* genes (Figure 12B). In *escargot* mutants fusion cells generally undergo apoptosis with only the DT fusion branch remaining intact. *escargot* possesses the capability to repress genes

in other tissues (Brody, 1999) and is postulated to repress branch growth genes and therefore promote branch fusion (Jiang and Crews, 2006; Samakovlis et al., 1996), although *shotgun* (*shotgun)* is postulated to be upregulated by *escargot* (Tanaka-Matakatsu et al., 1996).

escargot has a role in specifying the neuroectodermal columns. In the neuroectodermal columns, Spi/Rho EGF signalling is active in the columns to suppress *escargot* and *scute* expression. However, in the medial column of the neuroectoderm, the expression of *ventral nervous system* is necessary for *escargot* expression by counteracting EGF mediated repression of *escargot*. It is thought that *ventral nervous system* acts to prevent activation of a negative regulatory region of the *escargot* gene (Brody, 1999), which is a direct interaction as opposed to the tracheal system's indirect action of *dysfusion* on *escargot* (Figure 12A). Due to the compartmentalisation of gene expression in the columns for cell specification, cell identity is most likely to be affected by disruptions to this mechanism.

During asymmetric division, the three snail genes *escargot, worniu (wor)* and *snail (sna)* have redundant functions in alignment of the mitotic spindle. The apical complex consists of various proteins previously outlined in Figure 3 which are necessary for basal localisation of the basal protein complex, and subsequently, the formation of the mitotic spindle alignment. Triple mutations

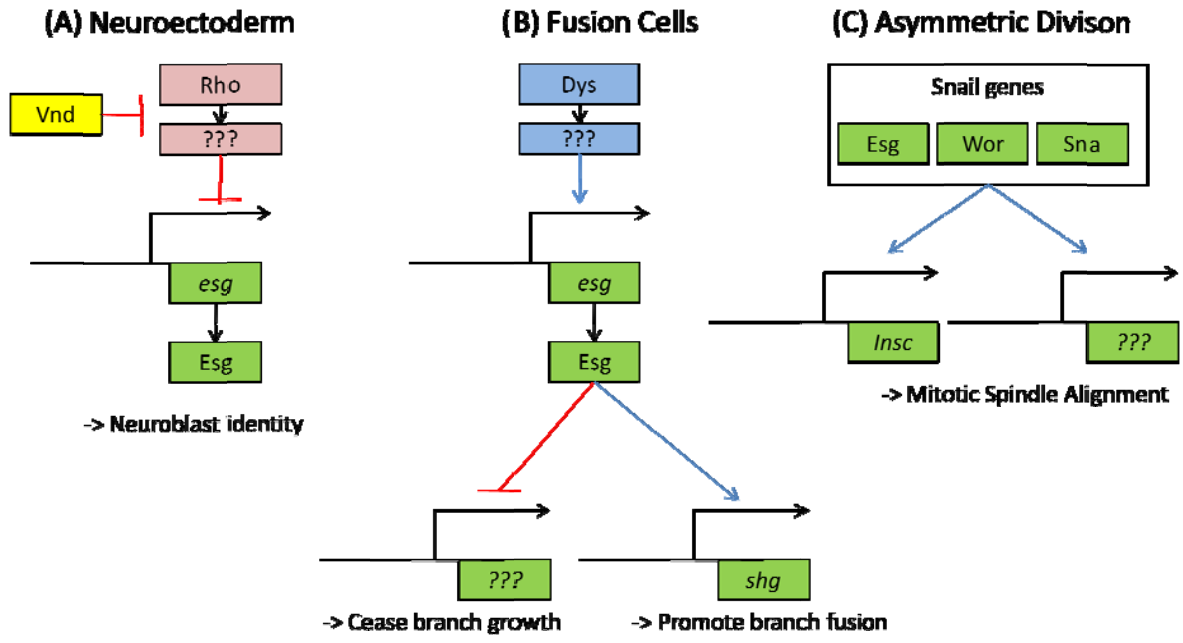


Figure 12 – Models of *escargot* function in various tissues. (A) In the neuroectoderm, esg is normally under repression from *rhomboid* unless it is freed from inhibition by *vnd* in the medial column. (B) In fusion tracheal cells, *escargot* is under control of dysfusion by an unknown mechanism, which upregulates pro-fusion genes and downregulates branching genes to cease growth. (C) *escargot* as part of the snail genes trio upregulates *inscuteable* and another parallel pathway to regulate mitotic spindle alignment during asymmetric divisions.

of the snail genes, including *escargot,* causes downregulation of *inscuteable* (*insc*) at either the transcriptional or translational level and causes disorientation of the mitotic spindle (Figure 12C). *inscuteable* mutants had been previously shown to mislocalise basal complex proteins and disrupt asymmetric division up until telophase rescue, however, mutations of all three snail genes abolish asymmetric spindle division completely including the process of telophase rescue. This implies that *escargot* regulates mitotic spindle alignment through *inscuteable* and also by a parallel pathway with or without its snail partners (Cai et al., 2001; Cai et al., 2003).

From the outset, it appears that *escargot* has completely different targets and functions in the CNS and trachea. For the trachea, fusion events involve changes in cell morphology and *escargot* downregulates branch growth genes and upregulates branch fusion genes such as *shotgun*. The contrasted effects in the CNS are very specific, with cell specification affected in the early neuroectoderm, and the alignment of the mitotic spindle along the apical-basal axis disrupted in the dividing neuroblasts. The differences in the CNS occur through the combined functions of three redundant genes in asymmetric differentiation. However, this does not preclude the ability for *escargot* to cause remodelling of the cell structure during the asymmetric divisions.

4.3.2. *pointed* directs terminal branching and differentiates glial cells

pointed is a major gene involved in branch migration and terminal branching of tracheogenesis and is detected in the CNS (Egger et al., 2002; Myat et al., 2005). *pointed* involvement in terminal branching upregulates *blistered* (*bs)* and downregulates *escargot*, which is in accordance with the role of the pro-fusion gene *escargot*. The main difference in *pointed* expression during terminal branching is that this is a response to *similar* activated Bnl/Btl signalling, as opposed to *trachealess/ventral veins lacking* activation as seen in primary branching (Centanin et al., 2008; Romero et al., 2007).

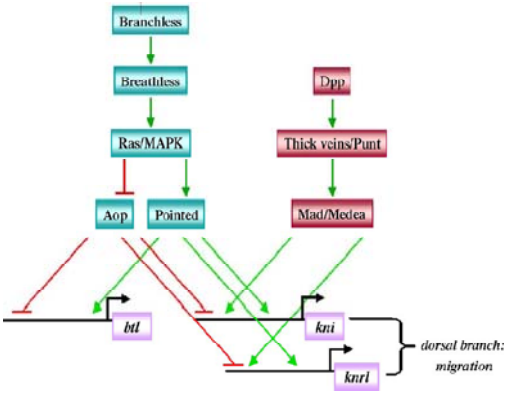


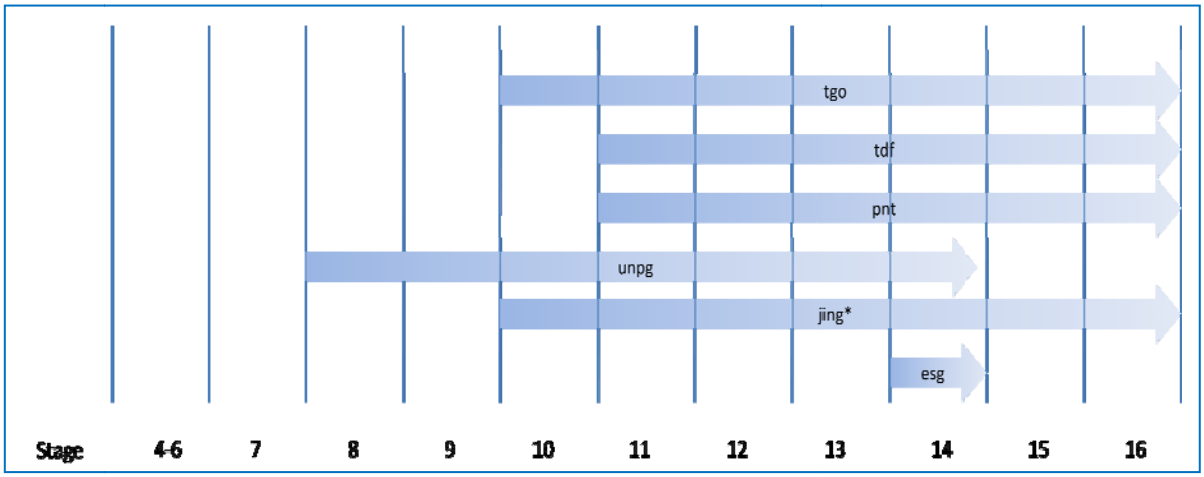
Figure 13 – Model of *pointed* activity. (In blue) Pnt acts downstream of Bnl/Btl signalling (to upregulate *breathless, kni* and *knrl*. Bnl/Btl signalling also antagonises Aop. (In pink) pnt activity in blue is analogous to Mad transcription downstream of Dpp (pink). Image from Myat et al. (2005).

One of the characteristics of secondary branching is the phosphorylation and degradation of the protein product of *anterior open* (*aop*) Aop (a repressing *ets*-domain transcription factor) and the induction of *pointed* expression (an activating *ets*-domain transcription factor). The activation of *pointed* has the following effects: *pointed* maintains expression of *breathless* and induces genes

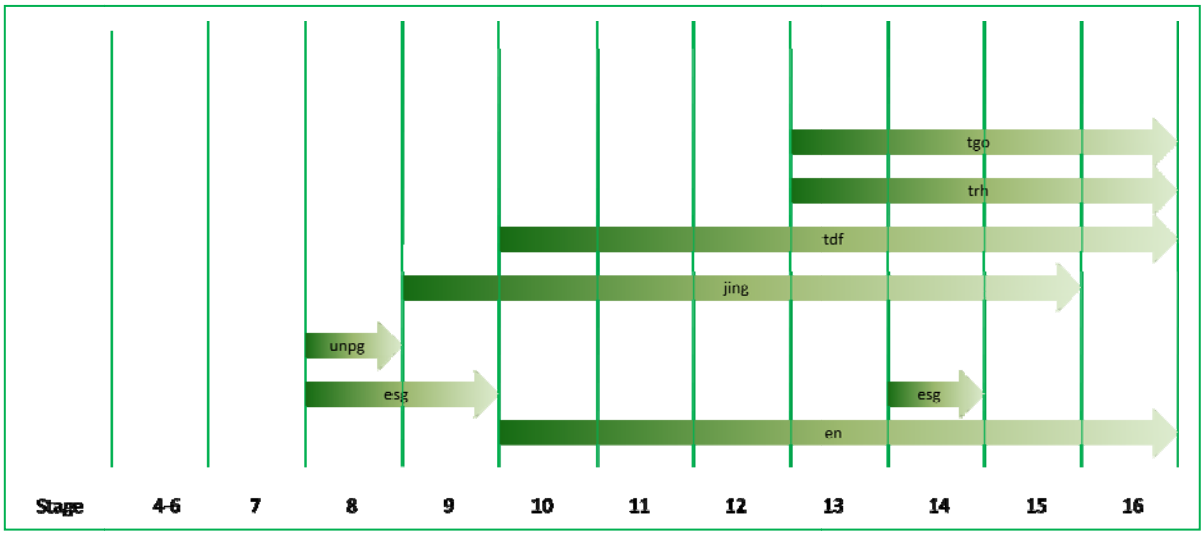
involved in tube formation such as cytoskeletal components and surface proteins, and also genes involved in orientation of secondary branch outgrowth. It also regulates *sprouty* (*sty*), which is needed for the negative feedback pathway to restrict *breathless* signalling. Mutants of *pointed* cause migration defects and the role of wildtype *pointed* is postulated at activating *knirps* and *knirps-related* similar to Dpp/Mad signalling and the opposite of *aop*'s effects (Figure 13)(Myat et al., 2005).

pointed is also involved in differentiation of glial cells, specifically the longitudinal glia of the VNC and VML glia. *pointed* mutants result in no glia in the VML and the misplacement of longitudinal glia. This affects subsequent axon guidance with deformations in the formation of the longitudinal and commissural tracts (Brody, 1999).

(A) Tracheal system



(B) VNC



(C) VML

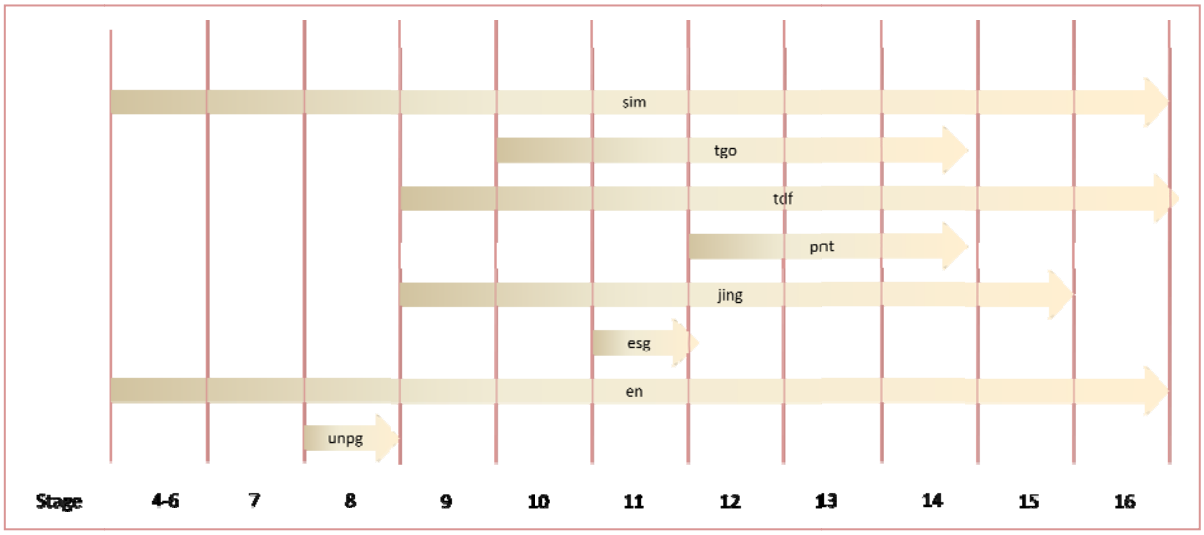


Figure 14 – Co-expression profiles of known transcription factors for each organ. (A) The tracheal system, (B) VNC, and (C) VML.

5. Discussion

The tracheal system and the VNC of the CNS are two distinct organ systems arising from the ectoderm which coincidentally share a number of transcription factors in their developments. The VML of the CNS arising from a specialised region called the mesectoderm also shares some transcription factors with the tracheal system during development. Approximately sixty transcription factors were linked to tracheogenesis in analyses of databases and the sparse number of reviews available. Of these sixty transcription factors sixteen were linked to the CNS, either in the VML or VNC and several more with loose ties have been documented in Appendix 7.1.

5.1. Co-expression patterns and functional groups

Co-expression patterns were not systematically analysed in this instance due to the wide variety of developmental stage time points used in literature, the low spatial and temporal resolution of BDGP images, and the lack of entries in Flybase. Figures 14 details an attempt to visualise the temporal aspects of each transcription factor where information was available but these figures are limited by spatial resolution. In the ideal circumstance every transcription factor would be catalogued and systematically analysed in a particular suborgan or cell type for every time point.

Some transcription factors were the sole transcription factor in their functional modules however some were linked by a common element. *hedgehog* signalling is one such common link during segmentation processes and is directly involved with *cubitus interruptus*, *engrailed* and *invected*. *hedgehog* is expressed in a gradient originating from posterior compartment cells of a segment towards the anterior segment. Ci is the effector of *hedgehog* signalling through an indirect effect. This effect of *hedgehog* on Ci is the shifting of Ci-155 to Ci-75 ratios which will presumably affect the number of genes up- and down- regulated. *engrailed* appears to act upstream of *hedgehog* by causing upregulation and secretion of the Hh protein to achieve its effect. During later stages of neuroectoderm development *engrailed* again utilises this paracrine Hh signalling mechanism on an adjacent neuroectodermal row to specify the developmental fates of neuroblasts contained in that row (Merabet et al., 2005; za-Blanc and Kornberg, 1999). Although the function of early specification in the segments is relatively generic for both the CNS and tracheal system, the same basic mechanism is used to express different genes. Later in development during neuroectodermal compartmentalisation, the re-usage of the mechanism shows that the functional unit also has a temporal aspect in controlling gene expression.

5.2. Shared and unshared functions and mechanisms

Transcription factors seem to have a few shared roles in the CNS and tracheal system with cell migration and cell specification being the main roles shared. Similarities were harder to identify at the molecular level but not completely non-existent. With respect to the molecular mechanisms of the transcription factors in both tissue organs, most seem to have differential effects in the two organ systems but again was not completely non-existent.

Cell migration is an importance aspect of tracheal branching given that cells no longer divide and must move and change morphology to penetrate tissues requiring oxygen. Every cell must undergo migration during tracheogenesis making it of fundamental importance. Cell migration in the CNS is important for a number of reasons, such as neuroblasts delaminating and differentiating and especially in glia who have important roles in axogenesis. In particular the VNC relies on the migration of midline glia for separating axon tracts and disruptions to their ability to move will cause deformations in CNS structure. The transcription factor *ventral veins lacking* was found to be an important regulator of genes involved in migration by interacting with the *breathless* receptor in both tissues (Anderson et al., 1995).

Cell specification is an early embryonic event which determines future identities of cells and is a result of patterning of gene combinations. The process is an obvious necessity in all tissues because early specification determines late identity such as pre-determining which placode cells grow into which branches and which neuroblasts will undergo gliogenesis or neurogenesis. *Stat92E, jing* and *tango* all have roles in establishment of early cell identity in both tissues where *Stat92E* is highest in the cascade followed by *tango* followed by *jing* (Ghabrial et al., 2003; Li et al., 2003; Sonnenfeld et al., 2004). Regulatory mechanisms for generating tissue specific effects but with the same process of specification may exist at *jing* because of its possession of a *D* binding site and other regulatory modules. *cubitus interruptus* also plays a part in identity specification due to its effects as downstream activator or repressor of Hh paracrine signalling along the segment. In addition *hedgehog* is under control of *engrailed* which adds another layer of transcriptional regulation of identity specification (Bossing and Brand, 2006; Brody, 1999; Merabet et al., 2005).

As an example of similar molecular mechanism with a shared function is that of *ventral veins lacking* in migration of MGM cells of the VML and tracheal cells. Both tissues also express *breathless* which is the main receptor used during tracheal branching and is also present in midline cells. *breathless* is the common factor which acts downstream of *ventral veins lacking* and is the receptor for *branchless* signalling from nearby tissues. However, as an example of a similar molecular mechanism with an unshared function is that of *Mothers against dpp*. *Mothers against dpp* in the tracheal system upregulates *knirps* in response to *decapentaplegic* signalling via phosphorylation by

thickveins to specify a DB fate in the placode. In the NMJ *Mothers against dpp* is also activated by phosphorylation by *thickveins* for synaptogenesis purposes. The common phosphorylation by Tkv protein is the only linking factor (McCabe et al., 2004).

As an example of a mostly dissimilar function, *grainyhead* was found to exert differential effects in CNS and tracheal tissue because of its ability to generate splice variants. The splice variants generated have different binding partners where the tracheal system variant forms homodimers during tube size determination and the CNS variant forms heterodimers for asymmetric differentiation. Although they are both recipients of phosphorylation, that is where the similarity ends because the splicing variation alters their binding specificities for differential gene regulation (Cenci and Gould, 2005; Uv et al., 1997).

On a more abstract level, one can surmise more similar functions such as the role of *pointed* which is not characterised in the CNS but is involved in apical-basal orientation in the tracheal system. This points to the obviously analogous event of asymmetric division, which also requires an apical-basal orientation, and the subsequent search for cognate genes (Centanin et al., 2008; Romero et al., 2007).

5.3. Conclusion

There are a lot of complex developmental processes in the *Drosophila* embryo which remarkably have its base in a small number of common transcription factors. However it is the differential combination of these transcription factors coupled with regulatory mechanisms expressed in space and time which generate the diversity seen in a eukaryotic organism. At this current moment a lot of further research still needs to be conducted to determine the exact molecular mechanisms involved during transcriptional regulation in an effort to characterise all pathways and interactions. In addition the fourth dimension of temporal regulation is not as fully investigated as the spatial aspect and is definitely an area which should be expanded on in future revisions.

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7. Appendix

7.1. Other transcription factors for possible follow-up

Spalt-related – discovered in the CNS by a thesis detailing systematic analysis of tracheal genes

araucan – discovered in the CNS by a thesis detailing systematic analysis of tracheal genes

hunchback – technically this is found in the mesoderm bridge cell, however, it signals dorsal trunk fusion

invected – no papers deal with this directly in the tracheal system and its role is inferred by interactions with *engrailed.* This one is current speculation.

CG11696 – no information at all, but is detected in both tissues

No ocelli – omitted due to technically being in the ocellar structures and brain only

Ultrabithorax – was initially included but removed due to being technically present only in the larval stages of tracheal development.

CrebA – fairly ubiquitous but needs to be verified