Identification of target genes of the T-box proteins in *Drosophila melanogaster*

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„Eins weiß ich, dass ich nichts weiß“

Sokrates

Contents

1 Introduction

1.1 T-Box Proteins

The family of T-box proteins consists of transcription factors with a transcriptional activator or repressor domain and a DNA-binding T-box domain (Fig.1). The first two proteins discovered were Brachyury and Omb [1], [2], which were found to have a new distinct domain, designated the T-box domain. Other homologous proteins have been discovered in both invertebrate and vertebrate organisms. This family has 17 members in *Homo sapiens*.

Figure 1. The DNA binding domain of the T-box proteins.
Members of the T-box gene family are defined by the presence of a conserved T-domain that is involved in DNA binding and dimerization of the protein. Residues in the C- terminus are involved in transcriptional activation or repression. Residues in the N-terminus may interact with cofactors [3].

A T-box protein is typically 50 - 78 kDa, within which the T-box domain is typically 17 - 26 kDa. The T-box domain comprises approximately 180 amino acid residues [4] and shares approximately 70% homology between T-box proteins. T-box proteins have folds characteristic of the immunoglobin fold. Some residues are conserved throughout the entire T-box family [5]. However, it has been suggested that functions such as mediating protein-protein interactions lie within the T-box [14]. The transcription modulating domains can either activate or repress and are much less conserved. T-box factors are grouped into distinct sub-families based on sequence conservation within the T-domain.

The loci of Tbx genes are dispersed throughout the genomes of metazoan organisms, though a few cases of clustering exist. They contain multiple exons, typically 8, such as Tbx5. There are also cases of alternative splicing for instance TBX3, TBX5, TBX20 [6].

The first T-box DNA binding sequence, TBE (T-box binding element), was defined as the sequence with the highest affinity for Brachyury [7]. Brachyury binds this palindromic sequence as a dimer [8], with each monomer of Brachyury binding half of the sequence, or T-half site (5′-AGGTGTGAAATT-3′). Extensive studies have demonstrated that all T-box proteins tested are capable of binding the T-half site as monomers [9],[10],[11], but some have different optimal target sequences [12] while thermodynamic and kinetic parameters of the binding reaction differ [13]. This suggests that functional specificity is encoded in

part outside of the T-domain [14]. Comparisons between T-box proteins have shown preference for different synthetic combinations of T-half sites in varying orientations, numbers, and spacing [15],[16], a property linked to the promoter specificity of their target genes.

Tbx proteins are important developmental regulators [17]. Expressed in specific organs or in particular cell types, they are involved in early cell fate decisions. In many cases, the level of T-box factor activity is crucial to normal function. In humans, mutation of a TBX gene for instance TBX1, TBX15, TBX19, TBX20 or TBX22 cause DiGeorge syndrome, Cousin syndrome [18], isolated ACTH deficiency, congenital heart disease or X-linked cleft palate and ankyloglossia [19],[20],[21], respectively. Mutations in members of the Tbx2 subfamily cause haploinsufficiency syndromes in which, among others, limb development is affected like the Ulnar-mammary syndrome ($TBX3^{+/-}$), the Small patella syndrome ($TBX4^{+/-}$), or the Holt-Oram syndrome ($TBX5^{+/-}$), [19], [22].

1.2 *optomotor blind (omb)*

optomotor-blind (omb) encodes a *Drosophila* T-box transcription factor [1], [14]. In *Drosophila*, eight T-box genes exist. Based on sequence homology of the T-domain, T-box genes were grouped into five subfamilies [3]. *omb* is the only *Drosophila* member of the Tbx2 subfamily which, in vertebrates, encompasses four paralogs (Tbx2–Tbx5). The T-domain and the transcriptional properties of Omb are more closely related to Tbx2/3 than to Tbx4/5 [23]. Tbx2 and Tbx3 are, therefore, considered orthologous to *omb*.

The development of the limbs, in particular of the wings, is also affected in *Drosophila omb* mutants. *omb* null mutants have severely stunted wing blades, whereas ectopic *omb* expression is sufficient to promote the development of a second pair of wings [24]. This suggests that *omb* fulfills a number of independent roles in wing development.

In the regulatory hypomorph *bifid (bi,* omb^{bi}*),* a small patch of tissue in the wing hinge does not develop [24],[25]. In the anterior wing compartment, *omb* is required to maintain the straightness of the anterior/posterior compartment boundary, while in the posterior compartment *omb* is required to suppress epithelial fold and subsequent rupture along this boundary [26],[25]. *omb* has many additional functions outside the wing, in particular in brain, eye, and abdominal tergite development [27],[28],[29],[30].

The closely *omb*-related genes TBX2 and TBX3 are overexpressed in several human cancers, such as mammary carcinoma, melanoma, as well as in pancreatic, ovarian, renal, and liver cancers [29]. Tbx2/3 can suppress cellular senescence presumably by interfering

with the mitotic cell cycle [31]. TBX3 overexpression promotes the invasiveness of melanoma cells [32]. Omb-overexpressing cells in the *Drosophila* wing imaginal disc exhibit a similar metastasis-like behavior [33]. TBX2 and TBX3 are, therefore, potential targets in cancer therapy [34]. Tbx3 is also required for self-renewal in mouse embryonic stem cells [35].

1.3 *Drosophila melanogaster*

Drosophila melanogaster was among the first organisms used for genetic analysis, and today it is one of the most widely used and genetically best-known of all eukaryotic organisms [36],[37],[38]. All organisms share common genetic systems; therefore, understanding developmental processes in fruit flies helps understanding these processes in other eukaryotes, including humans. The complete *Drosophila melanogaster* genome was sequenced and first published in 2000. Approximately 75% of the identified human disease genes have a detectable match in the genome of fruit flies, and nearly 50% of the fly protein sequences have mammalian homologs [39],[40],[41],[42].

1.3.1 Life cycle and development

Drosophila melanogaster is a combination of the Greek δρόσος-droso= dew, φίλος-phil=friend, μέλας-melano=dark-coloured, γαστήρ-gaster=belly and the Latin feminine ending-a. It is the common fruit fly. Its developmental period, the time required from the fertilized egg to the adult stage, varies with temperature. The shortest developmental time is 7 days, achieved at 28°C. Under ideal conditions, at just about 25°C, the life cycle lasts around 8.5 days (Fig.2).

Gastrulation in the *Drosophila* embryo starts three hours after fertilization [43],[44],[45]. Single layers of cells give rise to all tissues. Endoderm at the anterior and posterior ends forms the midgut [46]. Ectoderm remains on outside. Mesoderm is formed from ventral tissue. Between the mesoderm and the outer ectoderm a layer of neuroblasts is created [47]. In *Drosophila*, in contrast with the dorsally laying vertebrate nerve cord (VNC), the nerve cord lies ventrally [48].

The germband [49], (ventral blastoderm) is the main trunk region. The process of germ band extension pushes the posterior end over the dorsal side.

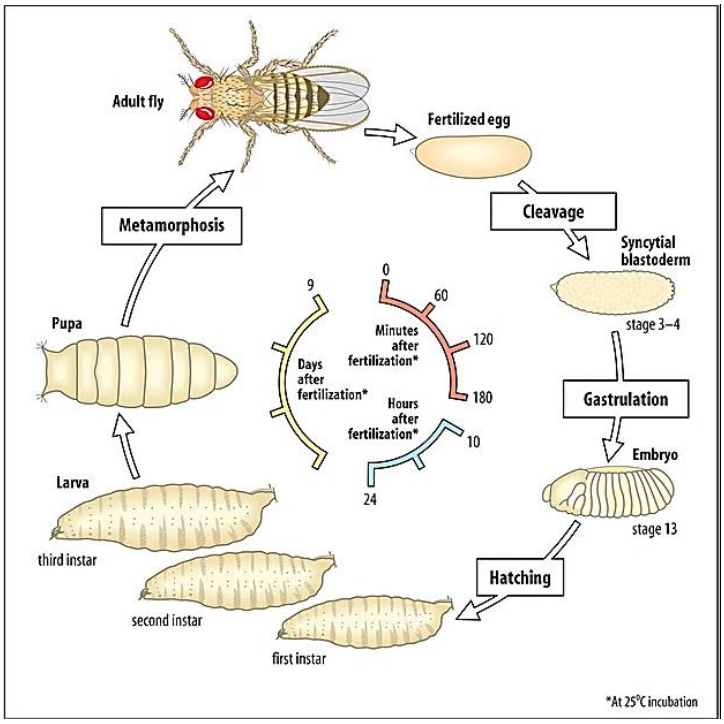


Figure 2. Life cycle of *Drosophila melanogaster* at 25°C.
The larva hatches 1 day after the egg is fertilized. First, second, and third instar are larval stages, each ending with a molt. During pupation most of the larval tissues are destroyed and replaced by adult tissues derived from the imaginal discs that were growing in the larva.

The first signs of segmentation waves outline subdivision of the embryo into a repeating series of body segments (Fig.3). Fourteen segments are created, three mouth, three thorax and eight abdominal [50],[51],[52],[53].

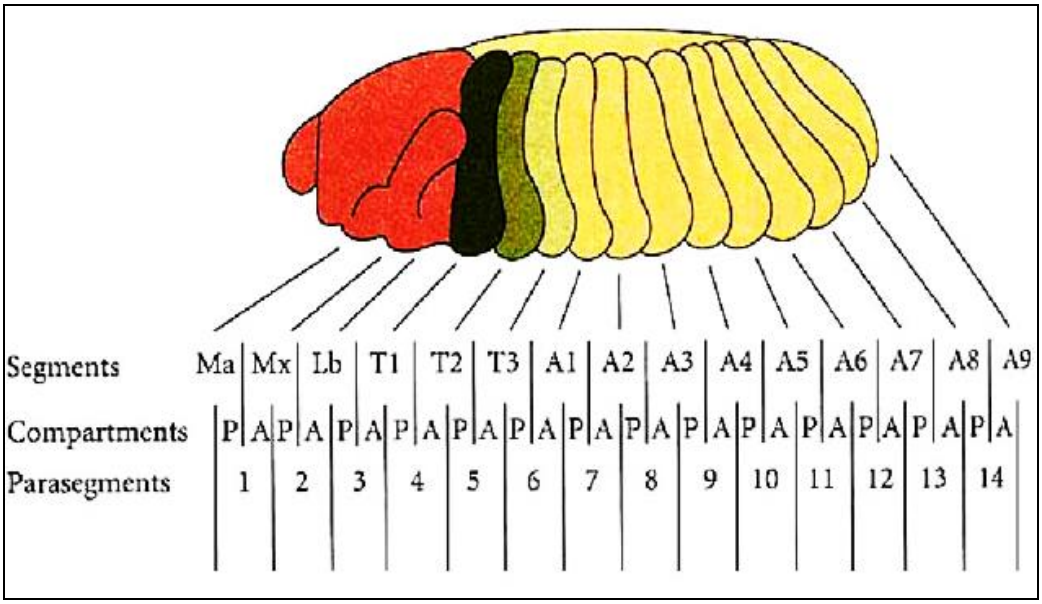


Figure 3. Embryo of *Drosophila* melanogaster at the end of germband extension.
Segments and parasegments. 14 segments: 3 mouth, 3 thorax, 8 abdominal (http://www.zoology.ubc.ca/~bio463/lecture_13.htm).

Drosophila larvae hatch at 24 hours after fertilization. The ventral side of the larvae has denticle belts, alternating patches of denticle hairs and cuticle on each segment, used for locomotion. Three instar stages of larval life are separated by molts. From the third instar larvae the pupa is formed, which subsequently undergoes metamorphosis. The adult tissues arise from imaginal discs and histoblasts.

1.3.2 Embryonic central nervous system

The *Drosophila* central nervous system (CNS) includes the brain and the ventral nerve cord (Fig.4). It develops from around 1,000 neural stem cells, called neuroblasts (NBs). The NBs delaminate from the bilaterally symmetrical neuroectoderm during early embryonic development [54],[55]. The delamination of NBs follows a stereotyped spatial and temporal pattern, making it possible to identify individual NBs based on their positions [56],[57]. After delamination, most NBs undergo asymmetrical self-renewal divisions to generate ganglion mother cells (GMCs), which either directly differentiate into neurons [58],[59] or divide once to produce a pair of post-mitotic cells that can differentiate into neurons, glia, or die prematurely [60],[59],[61],[62]. The molecular mechanism determining direct differentiation against one more round of cell division of the GMCs is not clear, but *numb, brain tumor,* and *prospero* are known to act in the GMCs to promote cell-cycle exit and differentiation [63],[64].

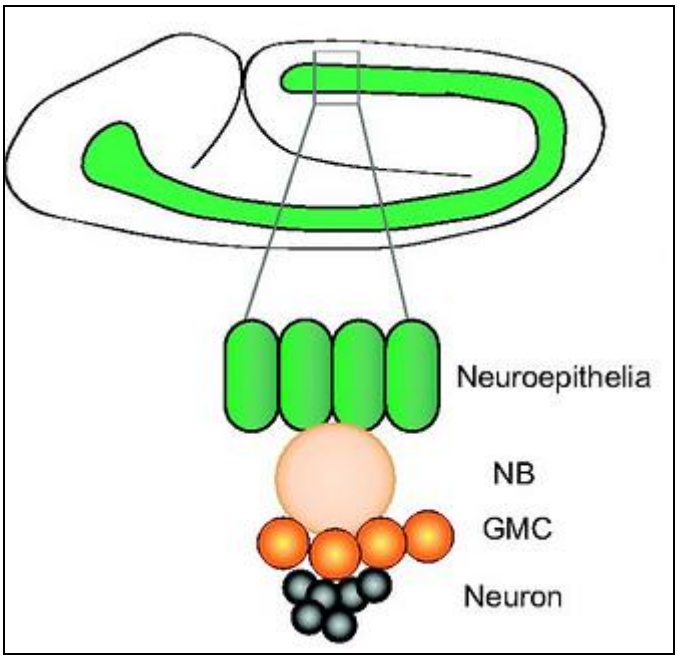


Figure 4.Embryonic ventral nerve cord of *Drosophila melanogaster*
Homem, C. C. and J. A. Knoblich (2012). Development 139(23): 4297-4310.

In addition to the unique position, individual NBs acquire a distinct identity in the neuroectoderm, which is verified by each NB expressing a particular combination of molecular markers, including many genes and enhancer-trap reporters [56],[66]. Moreover, each NB produces a distinct neuronal lineage [67],[68],[69].

Drosophila VNC, as above mentioned, consists of repeated segmental units. Each segment is arranged in a pair of mirrored hemisegments, and each hemisegment encloses just about 30 NBs. On the AP axis, the genetic cascades of gap and pair-rule genes adjust the local specific expression of segment-polarity genes, such as *wingless, hedgehog, patched, gooseberry, engrailed, mirror,* and *invected* [70],[55]. The combination of segment-polarity genes subdivides each hemisegment into four parallel rows [71],[55]. On the DV axis, nuclear factor NF-κB (dorsal), Dpp and epidermal growth factor receptor (EGFR) signaling pathways regulate the expression of columnar genes, such as *ventral nervous system defective (vnd), intermediate neuroblasts defective (ind), muscle segment homeobox (msh),* and *Drosophila* EGF receptor (DER), which divide the neuroectoderm into three longitudinal columns [70] [72],[55]. The combination of the segment-polarity genes and columnar genes creates a Cartesian coordinate map in every hemisegment (Fig. 5).

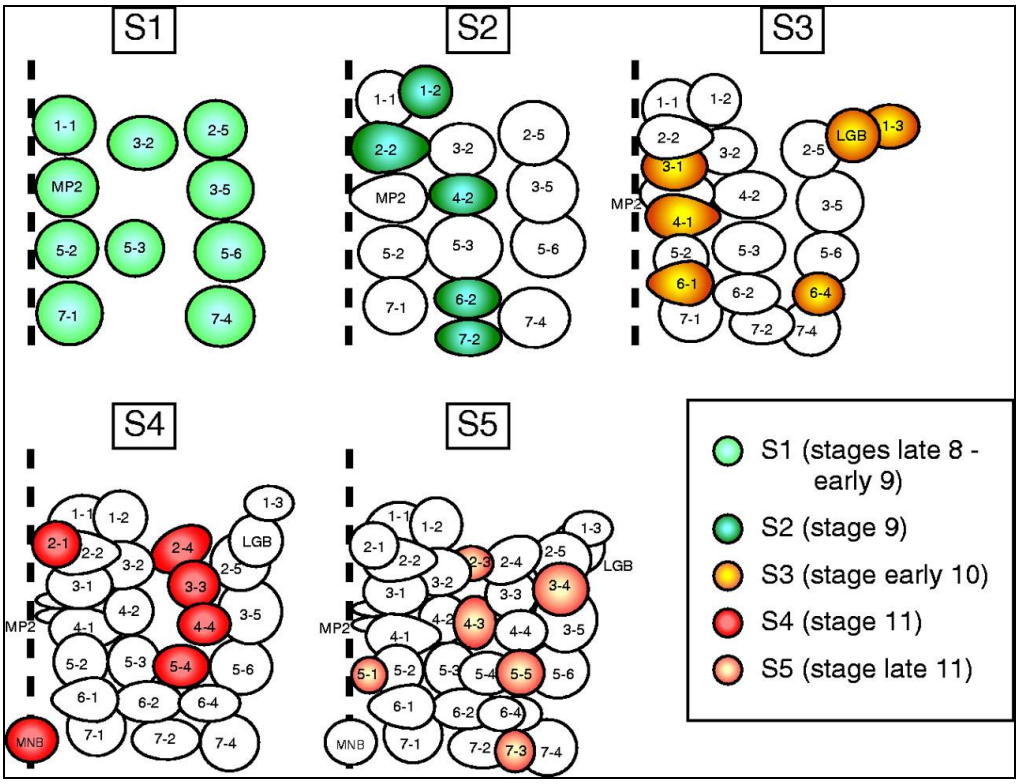


Figure 5. Spatial arrangement and temporal sequence (S1-S5) of segregating neuroblasts.
Each map represents the pattern of one hemisegment (thorax, abdomen) with highlighted those NBs that are added during the respective wave of segregation. Anterior is upwards; ventral midline is marked by broken line.
Bossing, T., G. Udolph, et al. (1996). Dev Biol 179(1): 41-64.

On each coordinate, this specific combination of the segment-polarity and columnar genes, activating a unique set of downstream genes, defines the identity of the NB at that position [71],[55]. Neuroblast formation is stimulated by the proneural genes *achaete, scute,* and *lethal of scute* [74]. Each of these proneural genes is expressed in groups of 4–6 cells at different positions within the neuroectoderm. Neuroblast formation is, furthermore, inhibited by the Notch signaling. The balance between the proneural genes expression and Notch activity results in the formation of a single neuroblast in each group of cells [74].

1.3.3 Larval imaginal discs

The imaginal discs are small sheets of epidermis (~40 cells each of cellular blastoderm) which grow throughout most of larval life. They include, six leg, two wing, two haltere, two eye-antennal, genital and head discs.

They consist of tissue-specific progenitors which are derived from different regions of the embryo, remaining inactive during embryonic and early larval life. During *Drosophila* metamorphosis, most larval cells die. Many adult tissues are shaped from rapidly developing imaginal discs (Fig. 6).

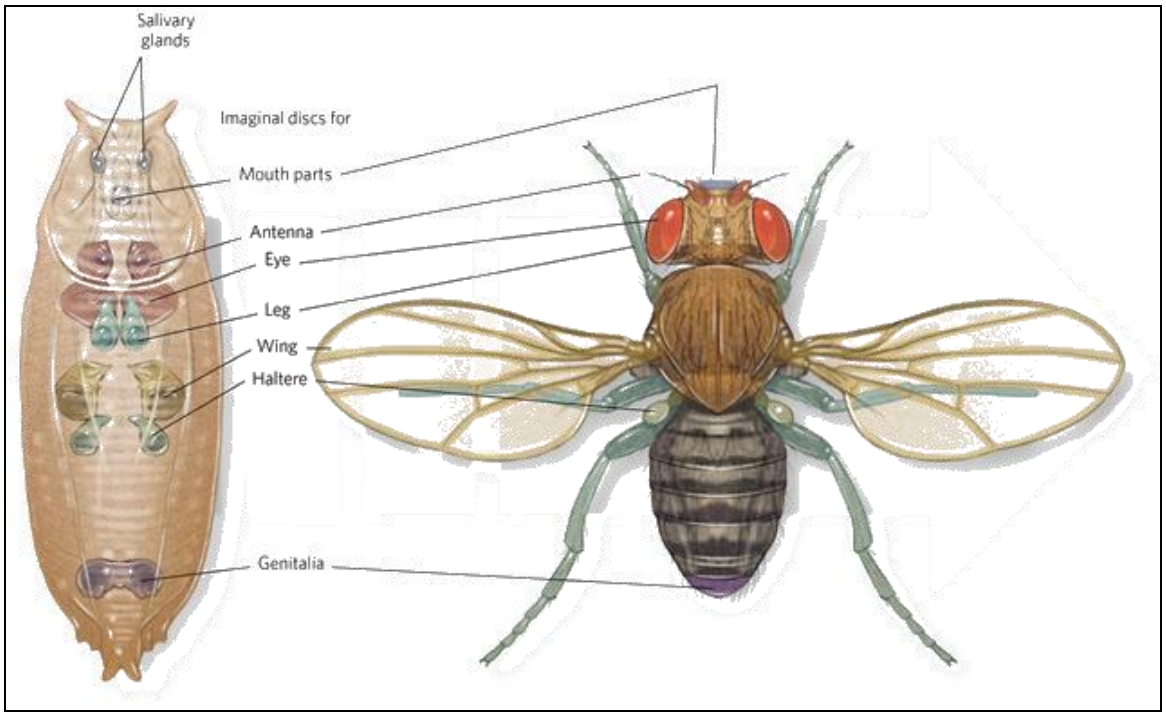


Figure 6. 3rd instar larva imaginal discs.
Locations of imaginal discs in a third instar larva, and the morphology of several major imaginal discs. There are 10 major pairs of imaginal discs, which give rise to adult cuticular structures of the head and the thorax, and a single genital disc, which forms the adult genitalia Instant messaging during development,

communication between organs determines their relative final size. Savraj S. Grewal, Clark H. Smith Brain Tumour Centre, University of Calgary, Alberta. The Scientist, March 1, 2013.

Each disc everts and elongates, with the central portion of the disc becoming the distal part of whichever appendage it will develop. During the larval stage, the cells in the growing disc appear undifferentiated, but their developmental fate is already determined [75],[76],[77].

Wing disc

The adult *Drosophila* wing develops from the larval wing imaginal disc, a single-layered sac of polarized epithelial cells. The wing disc consists of two epithelial cell types: the squamous epithelium or peripodial membrane and the columnar epithelium that gives rise to the adult epidermis. The adepithial layer contains myoblasts, which convert into the flight muscles of the thorax, tracheal cells of the larval and future adult airways (Fig. 7).

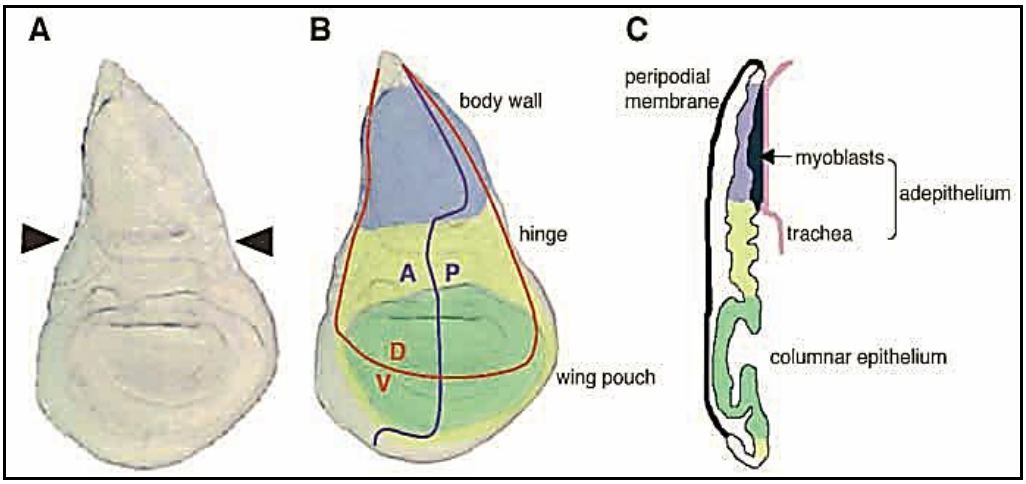


Figure 7. *Drosophila* wing disc and fate maps.
(A) Third instar wing disc. (hinge region, arrowheads). (B) Fate map of the wing disc showing the anterior-posterior (AP) and dorsal-ventral (DV) compartment boundaries and major regions in the disc. In the adult, the wing pouch (green) gives rise to the wing blade, the hinge (yellow) gets thinner to form a connection to the body wall (blue) or mesonotum of the fly. (C) Cell layers of the wing disc. There are three cell layers: the squamous epithelium or peripodial membrane, the columnar epithelium that gives rise to the adult epidermis, and the adepithial layer comprised of myoblasts, which develop into the flight muscles of the thorax, and tracheal cells of the larval and future adult airways. Butler, M. J., T. L. Jacobsen, et al. (2003). Development 130(4): 659-670.

The wing disc is subdivided into anterior (A) and posterior (P) compartments along the A/P axis, and is further subdivided into dorsal (D) and ventral (V) compartments along the D/V axis (Fig. 7). Only the central part of the imaginal disc develops into the adult wing blade. The D/V border of the imaginal disc develops into the margin of the adult wing.

The identity of cells in the P compartment is driven by the expression of the gene *engrailed* [79],[80]. Under the control of En, cells of the posterior compartment synthesize Hh, which

is secreted into the A compartment [79]. There, Hh induces several target genes, including *patched, en* and *dpp*, and patterns the central domain of the wing. Dpp is expressed along the border between the A and P compartments, and induces several target genes including *sal* and *omb (bifid), omb* being expressed in a broader domain than *sal* [81] (Fig. 8).

Both patterning and growth of the larval wing depend on Hh and Dpp morphogens. Ectopic Hh induces a mirror duplication of the A compartment, whereas the duplication caused by an ectopic expression of Dpp lacks the central domain [82].

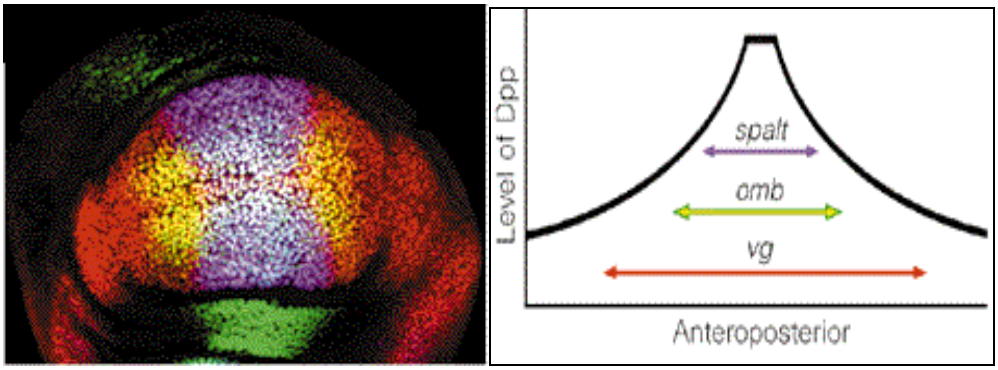


Figure 8. Wing patterning genes activated from Dpp.

Triple-labelled third-instar wing disc with nested domains of Spalt (purple and light blue) and Omb (green, and yellow where coexpressed with Vg) expression within the broad Vg (red) domain. These wing patterning genes are activated over different distances from the Dpp source [81].

Eye disc

The *Drosophila* compound eye is a highly organized structure that establishes an excellent developmental system to address the molecular and cellular mechanisms of pattern formation [83],[84],[85]. The retina is composed of 750–800 identical units called ommatidia in a hexagonal arrangement. Each ommatidial unit contains eight photoreceptors (R1–R8), four cone cells and two primary pigment cells surrounded by secondary and tertiary pigment cells. The photoreceptors project axons into the optic lobes of the brain, where they develop retinotopic projections in two ganglia, the lamina and the medulla [86].

Pattern formation and ommatidial differentiation begin in the third larval instar with the appearance of a groove known as morphogenetic furrow (MF) at the posterior margin of the eye disc [84], (Fig. 9). This groove in the epithelium, moves progressively across the eye disc from posterior to anterior over a two-day period. Cells anterior to the MF are undifferentiated, proliferating asynchronously, whereas cells posterior to the MF are

organized into columns of persistent cell groups within which photoreceptor differentiation occurs in a defined sequence [87],[88]. One critical signal driving the initiation and progression of the MF is the secreted protein Hedgehog (Hh) [89].

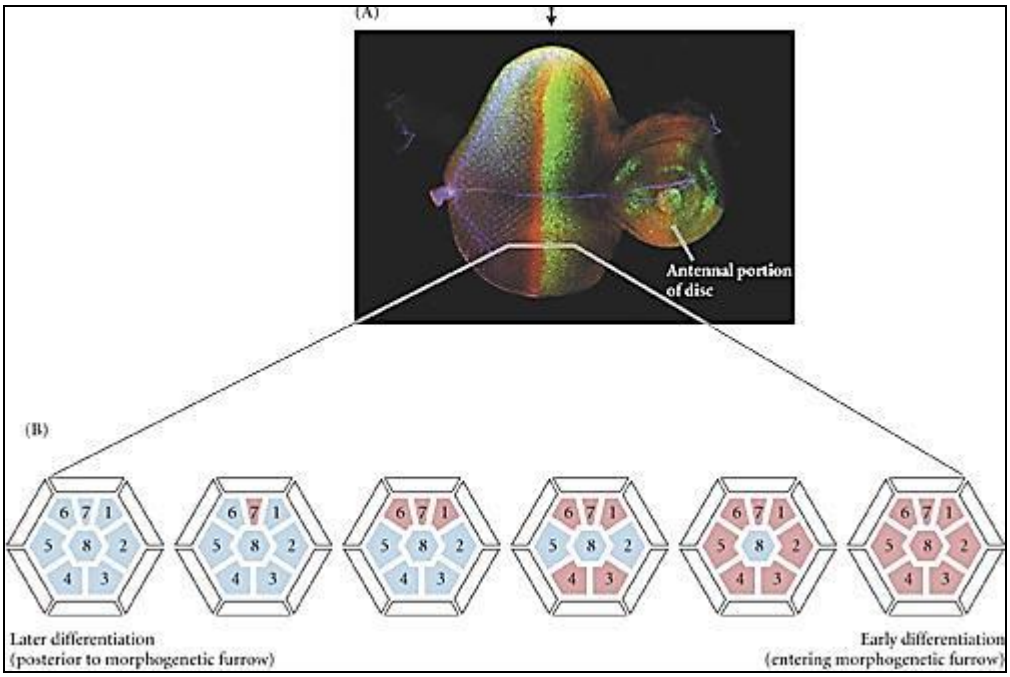


Figure 9. Differentiation of photoreceptors in the *Drosophila* compound eye.
The morphogenetic furrow (black arrow) crosses the disc from posterior (left) to anterior (right). (A) Confocal micrograph of a triple-labeled late larval eye/antennal imaginal disc, showing *hairy* expression in green ahead of the morphogenetic furrow (arrow). Within the furrow, the Ci protein (red) is expressed, as a consequence of the Hedgehog signal, which activates subsequently *decapentaplegic*). The neural specific protein, 22C10, is stained blue in the differentiating photoreceptors behind the morphogenetic furrow. The blue horizontal line of staining is Bolweg's nerve. (B) Behind the furrow, the photoreceptor cells differentiate in a defined sequence. The first photoreceptor cell to differentiate (blue) is R8. R8 induces the differentiation of R2 and R5, and a cascade of induction continues until the R7 photoreceptor is differentiated [90].

1.3.4 Morphogens

Morphogens are identified in most developmental systems as secreted signaling proteins. These proteins bind to the extracellular domains of transmembrane receptor proteins, which use a compound process of signal transduction to convey the level of the morphogen to the nucleus. There, the activity of transcription factors is regulated, correspondingly to the level of the morphogen received at the cell surface. The expression of target genes is controlled by enhancers to which transcription factors bind. Once bound, the transcription factor enhances or inhibits the transcription of the corresponding gene thereby controlling the level of expression of the gene product. Distinct target genes respond to different thresholds of morphogen activity. 'Low-threshold' target genes require only low levels of morphogen activity to be regulated. 'High-threshold' target genes have

relatively fewer binding sites or low-affinity binding sites that require much higher levels of transcription factors to be regulated [91],[92].

The roles of morphogens during the development of *Drosophila* appendages have been extensively studied. These include (Fig. 10) the Hedgehog family, gene Hh [93], the Wnt family member Wingless (Wg) [94],[95], and members of the TGFβ family, including Decapentaplegic (Dpp). All of them have a role during *Drosophila* development.

In this thesis, emphasis is set on their role during appendage development, in the imaginal discs [96],[97].

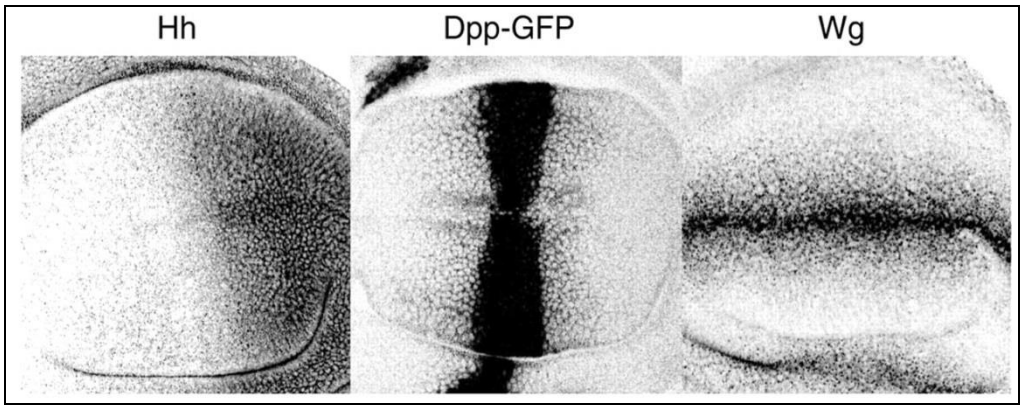


Figure 10. Distribution of Hh, Dpp-GFP and Wg in the wing imaginal disc.
Remarkable the graded distribution away from the expressing domain. Tabata, T. and Y. Takei (2004). Development 131(4): 703-712.

Hh in the wing imaginal disc

The Hh gene was first discovered and further characterized in *Drosophila melanogaster*. Hh signaling is required for the proper patterning of the embryo and many adult structures [99].

In the wing disc P cells, Hh is synthesized as a precursor that undergoes lipid modifications. The lipid-modified Hh protein is able to reach the Patched (Ptc) receptor in anterior compartment cells (Fig. 11), [100]. In response to the Hh short-range activity gradient, the expression of a number of target genes is dose-dependently activated, including *engrailed (en), ptc, collier(col), decapentaplegic (dpp), and iroquois (iro)* [101],[79],[82].

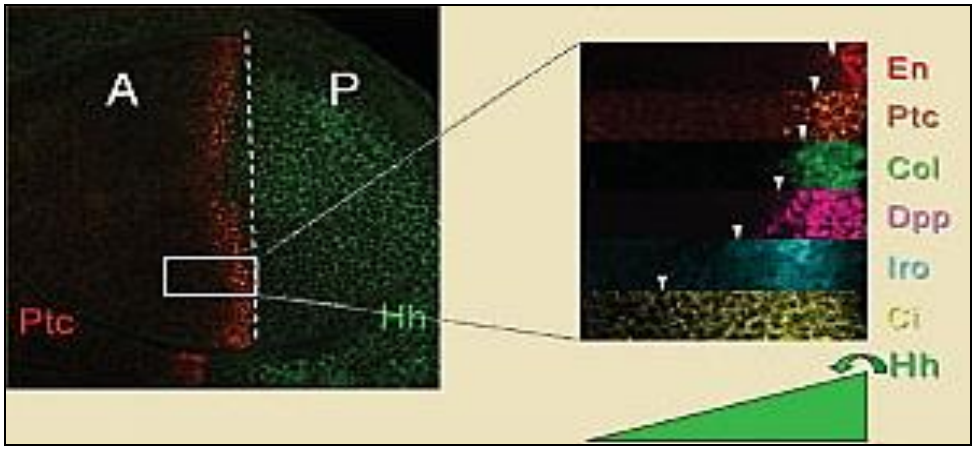


Figure 11. Hh signaling in the wing imaginal disc of *Drosophila*.
Hh and Ptc antibody staining in a WT wing pouch. Dashed line represents the A/P border and the white frame shows the area enlarged in the right figure, where gene expression patterns in response to the Hh gradient are shown. Engrailed (En, red) and Ptc (brown) respond only to high levels of Hh; Collier (Col, green) and Decapentaplegic (Dpp, purple) to medium levels; and Iroquois (Iro, blue) to low levels. Ci protein stabilization (yellow) is also dependent on the Hh gradient**.** Torroja, C., N. Gorfinkiel, et al. (2005). J Neurobiol 64(4): 334-356.

Dpp in the wing imaginal disc

The morphogen Decapentaplegic (Dpp) is essential for the wing patterning [103]. It is expressed in a narrow stripe of anterior cells along the anterior/posterior compartment boundary, establishing a precise concentration gradient along the A/P axis of the wing disc [104].

Once Dpp is secreted, it binds and activates its receptor complex, type I (Tkv or Sax) and type II (Put) serine/threonine kinase receptors, leading to the activation of the pathway in a broad stripe of anterior and posterior cells occupying most of the presumptive central region of the wing. The domain of Dpp signalling accumulates phosphorylated Mad (PMad), a direct target of the Tkv/Put kinase, which, once phosphorylated, forms a complex with Medea (Med) that enters the nucleus and regulates the expression of target genes [103], such as *sal* and *omb*. Activated Tkv directly upregulates *sal* expression while *omb* is activated by repression of its repressor *brk* [105].

Unlike its role in patterning of the wing, which is well-defined, the influence of Dpp gradient in the induction of uniform wing disc proliferation remains unspecified [103].

Wg in the wing imaginal disc

Wnt proteins comprise a conserved family of secreted signaling molecules with key functions during embryonic development and adult homeostasis [106]. They act as

morphogens [107], affecting tissue organization by providing spatial information in the form of a concentration gradient. In the developing *Drosophila* wing imaginal disc, Wingless (Wg) is secreted by a narrow stripe of cells at the dorsoventral boundary (Fig. 12).

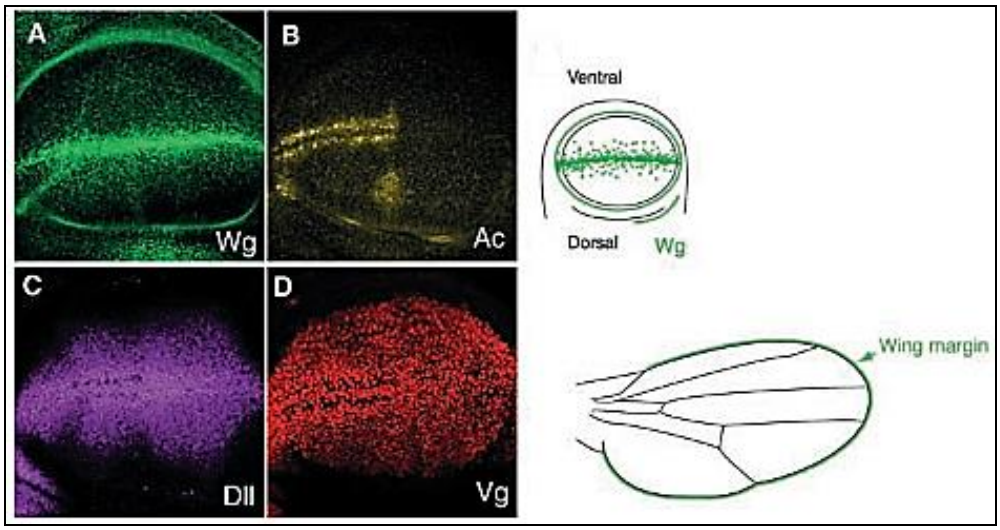


Figure 12. Wg and its target genes in the wing disc.
(A-D) Wg (A, green) is produced along the DV border and induces the expression of target genes, such as Ac (B, yellow, expressed only in the A compartment), Dll (C, purple) and Vg (D,red), at high, middle and low thresholds, respectively. Anterior is to the left. On the right: Schematics showing the domains of Wg expression. Tabata, T. and Y. Takei (2004). Development 131(4): 703-712.

The protein Apterous acts on cells in the dorsal compartment of the wing disc [108], inducing expression of the gene *fringe* [109], which results in activation of the Notch receptor pathway at the DV border [110]. Activated Notch induces Wg synthesis at the DV border [111], where Wg functions as a morphogen to induce the expression of target genes, such as *achaete (ac) Distalless (Dll) and vestigial (vg)*, influencing the patterning of the wing (Fig. 12).

1.4 Tbx proteins in development

T-box genes are implicated in a variety of developmental processes in chordates, including the formation of mesoderm [112], development of the tail and the notochord [113], patterning of the appendages [114], development of sense organs and pharyngeal arches [115] and the subdivision of the brain [116].

1.4.1 Tbx proteins in the embryonic nervous system

Members of the T-box transcription factor family are associated with several human syndromes caused by embryogenesis defects. Cell migration is a crucial feature of

organogenesis and tissue formation during embryogenesis. Likewise, in the developing brain, many classes of newly formed neurons migrate in regular patterns in order to establish the proper cytoarchitecture of the nervous system [117].

Members of the T-box transcription factor family have been associated with the proper development of migrating cells [118]. During gastrulation, Brachyury and Tbx2 control the cellular reorganization of the embryo that drives notochord formation [119]. Similarly, Tbx5 and Tbx20 are implicated in the cardiac cell migration related to the formation of heart chambers [120], Tbx1 in neural crest migration and proper middle/inner ear development [121] and Tbx20 is expressed by migratory branchiomotor (BM) and visceromotor (VM) neurons in the hindbrain [122].

Tbx20 is closely related to the *Drosophila* segment polarity gene H15 which is required both for neuroblast formation and the specification of neuroblast identity [123]. Tbx20 is also expressed in the cranial motor neurons and developing cardiovascular structures in zebrafish [124]. The TBX2 protein was also found important for both motoneuron and sensory neuron differentiation in *Caenorhabditis elegans* [125].

1.4.2 Tbx proteins in *Drosophila* embryonic CNS

In *Drosophila melanogaster*, eight T-box genes from five subfamilies have been identified; these are *Dorsocross-1 (Doc1), Doc2, Doc3*, *optomotor blind (omb), optomotor-blind-related-gene-1 (org-1), brachyenteron (byn), H15 (H15*, also known as *neuromancer 1*), and *midline (mid*, also known as *neuromancer 2*).

The segment polarity gene *midline*, encodes a T-box protein expressed, among others, in the *Drosophila* CNS. *mid* mutants show cuticle defects, suggesting that Mid protein is required for ectodermal patterning [126], as well as defects in the lateral chordotonal axons, with shorter and defasciculated dorsally routed axons in the peripheral nervous system [127]. Moreover, *midline* contributes to the specification of anterior neuroblast identity by negatively regulating the expression of Wingless and positively regulating the expression of Mirror. In the posterior-most part of the segment, *midline* and its paralog, *H15*, have redundant functions in the regulation of the NB marker Eagle [123].

Midline acts in the NB4 lineage, NB4-2→GMC-1→RP2/sib, [123]. In a screen for mutants that affect the RP2/sib lineage, a mutation was identified, characterized by an RP2-like extra neuron in each hemisegment in the periphery of the nerve cord {P. Wai, unpublished}. This was allelic to the previously identified mutation mid [126] or lost in space (los) [127]. Whereas some researchers suggested that the extra neuron present at

the periphery of the nerve cord in *mid* mutants is not an RP2 neuron [123], others claimed that it is indeed an RP2 neuron [128]. In *mid* mutants a GMC from the NB row 5 adopts the GMC fate of the RP2/sib lineage without affecting the expression pattern of key genes at the parent NB or NE level. Expression of Mid in the M-GMC in mid mutants rescues the fate change, indicating that Mid specifies neurons at the GMC level [128].

Mid/H15 co-expressing neurons are interneurons, while a single Eve-positive U/CQ motor neuron weakly co-expresses Mid. Lineage studies map a subset of Mid/H15 positive neurons to neuroblast lineages 2-2, 6-1, and 6-2 while genetic studies reveal that mid collaborates with nkx6 to regulate eve expression in the CNS [129].

The expression of *omb* in the embryonic CNS is described below.

1.4.3 Tbx genes in the development of vertebrate appendages

The T-box genes Tbx2, Tbx3, Tbx4 and Tbx5, are linked in the mouse genome, as gene pairs. They are expressed in the developing mouse limbs, either at the time of limb field specification, during bud outgrowth, or both [114]. Interestingly, Tbx2 and Tbx3 are expressed in similar spatiotemporal patterns in both limbs, whereas Tbx4 and Tbx5 expression is primarily restricted to the developing hindlimb and forelimb buds, respectively, being the earliest factors required to initiate forelimb and hind limb outgrowth [130].

1.4.4 Tbx genes in the development of *Drosophila* appendages

The *Drosophila* Tbx6-related gene, *Dorsocross*, *Doc* 1, 2 und 3, shows a segmented embryonic expression starting from stage 9, correlating with that of Dpp. Expression of all *Doc* genes is during the embryogenesis identical. In the thoracic segments *Doc* pattern corresponds to the imaginal disc primordia, having a role in their specification [131]. Its expression appears restricted, by the Vestigial and Homothorax, in two narrow Doc stripes where the wing epithelial folds evolve. There *Dorsocross* promotes the progression of folds which separate the hinge and blade regions of the wing disc. *Doc* mutant clones prevent the lateral development of these folds at the larval stage and suspend therefore the wing disc evagination in the early pupal stage [132].

H15 and, its paralogous gene, *midline* are members of the Tbx20 class of T-box transcription factors. They retain a significant role to the specification of the dorsoventral axis in the *Drosophila* legs, a conserved function shared with *omb* [133]. *midline* and *H15*

are restricted to identical ventral domains of expression through activation by Wingless and repression by the dorsal signal Decapentaplegic [134]. They function redundantly and cell autonomously in the formation of ventral-specific structures [42].

The function of *omb* in the development of *Drosophila* appendages is described below.

1.4.5 TBX2 and TBX3 genes in carcinogenesis

optomotor-blind vertebrate orthologues, TBX2 and TBX3, are members of the Tbx2 transcription factor subfamily [135]. They are important developmental regulators, often found overexpressed in various human cancers like melanoma [136], breast, bladder, liver, and pancreas carcinoma [137]. TBX2 and TBX3 stimulate tumor genesis by interacting with various cellular mechanisms [138]. They enhance proliferation [139], suppress senescence [140] and anoikis [141], while promoting epithelial-mesenchymal transition (EMT) and invasive-metastatic cell behavior in melanoma and breast cancer cells [136],[137].

1.5 Omb in *Drosophila*

In *Drosophila*, the only orthologue of TBX2 and TBX3, is Optomotor-blind. Omb is known to be active in several developmental processes, in particular in those of wing, eye, abdominal tergites, and optic lobes.

1.5.1 Omb in the *Drosophila* nervous system

omb is known to be expressed in neurons and glia of medulla and lamina, where the initial steps of visual-motion analysis are performed [30].

Furthermore*, omb* was recently shown to be regulated by Prospero in the developing ventral nervous system. *omb* displayed decreased levels in a *prospero* mutant background, indicating direct or indirect repression by Prospero [63].

1.5.2 Omb in the *Drosophila* imaginal discs

bifid [142] is a hypomorphic allele of *omb* affecting predominantly wing development. In wild-type wings, longitudinal veins 1-5 fuse at the base of the wing into two separate stalks (1-3, 4-5). In *bi*, all five longitudinal veins fuse into one stalk due to the lack of a small wing territory that normally divides these stalks [24]. This phenotype is sexually dimorphic and

temperature dependent, being more strongly expressed in males and at higher temperature.

In the third larval instar wing imaginal disc, Omb expression extends in the wing pouch, declining towards the lateral periphery. *omb* expression in the pouch is under combined regulation of the diffusible proteins, Decapentaplegic and Wingless [105]. *omb* is required for the development of the entire wing blade. This is obvious from the undeveloped wings of *omb* null mutant adults. In these winglets, only the most proximal structures, costa and alula, can be distinguished [24].

In the wing imaginal disc, the anterior/posterior compartment boundary functions as an organizer of wing development [143]. In the anterior cells *omb* is essential to establish the anterior fate [26]. In the posterior compartment, *omb* seems to be required to inhibit the formation of an apical fold along the A/P boundary [25].

Apart from its growth-repressive role in the ventral wing disc (pleura) [144], Omb has been reported to act downstream of Dpp for the establishment of regional proliferation in the wing disc, medially and laterally. Dpp, enhances proliferation in the lateral wing region and represses proliferation in the medial region of the wing disc. Omb, on the other hand, affects proliferation rate through transcriptional regulation of the microRNA gene *bantam.* It enhances *bantam* in the lateral wing region while suppressing it in the medial wing disc [145].

omb was shown to regulate the expression of several important wing developmental genes such as *spalt, vestigial, thickveins, and master of thickveins* [146], but it is still unknown if this is through direct regulation by Omb or not. *omb* expression in the wing disc overlaps, as well, partly, with *brinker* expression. Cells expressing *brinker* and *omb* induce the development of the longitudinal vein L5 in the posterior compartment [147].

1.6 The objective of this thesis: Omb target gene identification

The Omb transcription factor can be expected to regulate several genes during the development of *Drosophila*. Therefore, the identification of potential Omb target genes constitutes a main research goal.

Given the importance of TBX2 and TBX3, a better comprehension of the function of their ortholog Omb in *Drosophila melanogaster* is worth the effort.

The initial approach was by bioinformatic analysis {Diplom thesis, University of Mainz, Institute of Genetics, H. Bimbas, 2009}. The *Drosophila melanogaster* genome was

screened for potential TBEs. A positional weight matrix (PWM) was built from 40 *bona fide* Tbx target sequences, including sequences obtained from an Omb in vitro selection procedure {Pflugfelder, unpublished}. The scores in this training set ranged from 4.4 to 10.9. Some 800 genes, known to be involved in wing development, were screened with this PWM using an arbitrary threshold of 8.0. Target sequences were considered potential Tbx targets when the average score of the homologous sequences from the 12 published *Drosophilidae* genomes also was above 8.0 (phylogenetic foot printing criterion) [148]. Using these criteria, around 160 genes were considered to contain potential TBEs. Since only four *Drosophila* Tbx genes are known, so far, to have a role in wing development (*omb* and the three highly related *Doc* genes) [132], this selection was likely to contain some Omb target genes.

From this 'Top Gene List' lines with enhancer trap/reporter lacZ insertions were ordered from the *Drosophila* stock centers. These lines were characterized by imaginal disc X-gal stainings in wt and *omb* mutant background. The aim was to achieve a first inventory of the expression of these genes in the imaginal discs and, simultaneously, obtain information on *omb* mutant effects on their expression pattern. Some of the investigated potential target genes subsequently appeared promising and therefore transgenic fly lines have been constructed and further characterized.

Furthermore, *Drosophila* is an excellent model system for the study of neural development mechanisms. Much is known about the lineages, patterns, and identities of glia and neurons, and about the projections and pathways taken by axons in the developing CNS and PNS. Neurons and glia are arranged in a stereotypical pattern repeated in each segment. They are easily identified by position, and by a large array of markers [54],[70],[55],[74],[73]. Taking advantage of these features, an analysis of Omb expression in the *Drosophila* embryonic central nervous system was attempted.

The results presented below provide a contribution to an understanding of TBX2/3 function in development.

2 Materials and Methods

Here it is accessible the detailed list of the methods and materials used during this research. This part is organized in 2 (2.1 Materials - 2.2 Methods) subsections.

2.1 Materials

2.1.1 Chemicals

Chemicals from AppliChem, Roth, and Sigma-Aldrich were employed for this study.

2.1.1.1 Enzymes

2.1.1.1.1 Restriction endonucleases

Restriction endonucleases from NEB were put to use.

Enzyme	Company	Recognition Sequence-
BstBI	NEB	5'-TT*CGAA-3'
DraI	NEB	5'...TTTAA*GTGC...3'
EcoRI	NEB	5'-G*AATTC-3'
NdeI	NEB	5'---CA*TATG---3'
PstI	NEB	5'—CTGCA*G--3'
StuI	NEB	5'—AGG*CCT--3'

Table 1. Restriction endonucleases used during this study.

2.1.1.1.2 Other enzymes

Moreover the following enzymes were utilized:

RNase A (AppliChem), Phusion Polymerase (NEB), Pfu Ultra DNA Polymerase (Stratagene), Taq Polymerase (QIAGEN), Gateway LR Clonase Enzyme Mix (Invitrogen).

2.1.1.2 Solutions and Buffers

Solutions employed for this study are below classified according to the protocol to which they were implemented.

2.1.1.2.1 Ethanol precipitation

Sodium acetate (3 M, pH 7.0); Ethanol (100% and 70%); ddH_2O (double distillated, Roth).

2.1.1.2.2 Phenol extraction

Sodium acetate (3 M, pH 7.0); Chloroform/Isopentanol (24:1); Ethanol (100% and 70%); ddH_2O (double distillated, Roth), TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

2.1.1.2.3 Plasmid Minipreparation

P1 Buffer (QIAGEN); P2 Buffer (QIAGEN); P3 Buffer (QIAGEN); EB Buffer (10 mM Tris-Cl, pH 8.5).

2.1.1.2.4 Gel electrophoresis

5X Stop Buffer-loading buffer (15 % Ficoll 400, 5 % SDS, 0,05 % Bromophenol blue, 0,05 % Xylencyanol FF-AppliChem); 10X TBE-running-buffer (121,1 g Tris, 61,8 g Boric acid, 19,6 g Na_2EDTA, up to 2L in dH_2O); Agarose gels (0,8% in 1X TBE); EtBr staining solution (8-10 drops aqueous 1 mg/ml Ethidium bromide in 500 ml dH_2O); Mass Ruler DNA Ladder Mix (Fermentas).

2.1.1.2.5 Media

Drosophila standard medium (flour/ semolina with antibiotic); LB Medium (25 g/L in autoclaved dH_2O); Agar-plates medium (15 g Agar in LB Medium); S.O.C. medium (Invitrogen);

2.1.1.2.6 Enzymatic (X-Gal) staining

10X PBS (1,3 M NaCl, 0,07 M Na_2HPO_4, 0,03 M Na_2HPO_4); Fixative (0,1 M Sodium cacodylate buffer, pH 7,3),0,75 % Glutaraldehyd); Staining solution (1,5 ml 50 mM $K_4(FeII(CN)_6)$, 1,5 ml 50 mM $K_3(FeIII(CN)_6$, 5 ml 10X PBS, 50 µl 1M $MgCl_2$, 42 ml ddH_2O); X-Gal Dye Solution (1/30Vol. Staining solution+ X-Gal Dye (8%ig)), Glycerol (50% und 70% in 1X PBS).

2.1.1.2.7 Antibiotics

Carbenicillin (50 µg/ml); Kanamycin (50 µg/ml); Spectinomycin (100 µg/ml).

2.1.1.2.8 Immunostaining (fluorescence)

1X PBT (1X PBS, 1% BSA, 0,3% TritonX); Fixative (425µl PBS, 75µl 40% formaldehyde and 500µl n-heptane);

Antibodies

Primary antibodies		
Antibody	Organism	Concentration used
a-Omb	Rb	1:1000

a-GFP	Ch	1:500
a-Eve	M	1:50
a-bGal	Ch	1:1000
a-bGal	M	1:375
a- Dpn	GP	1:500
a- Pros	Rb	1:1000
a-Repo	GP	1:100
a-Mid	Rb	1:500
a-Eg	M	1:50
Secondary antibodies		
Antibody	Organism	Concentration used
FITC (488)- 493nm	Ch	1:250
Cy3 (alexa546)- 550nm	Rb	1:500
Cy5 (alexa 647)- 650nm	M	1:500
Cy3- 550nm	GP	1:250
488 (alexa 488)- 495nm	Rb	1:500
Cy3 (alexa 568)	M	1:500

Table 2. Antibodies used during this study.

2.1.2 Kits

Furthermore the following kits were used:

QIAprep Spin Miniprep Kit (QIAGEN); Hi Speed Plasmid Maxi Kit (QIAGEN); QIAquick PCR Purification and Gel Extraction Kit (QIAGEN);

High Pure PCR Product Purification Kit (Roche); Quick Change II XL Site Directed Mutagenesis Kit (Stratagene); pCR8/GW/TopoTA Cloning Kit (Invitrogen); pCR XL Topo Cloning Kit (Invitrogen).

2.1.3 Oligonucleotides

Oligos synthetized from MWG Biotech were used.

Subcloned Fragment	Oligos used	l	Tm	PE	Oligo sequence

hh [3.1Kb]- TBE3	1014	22	69,5	420	TGGGAACTGATCGACG
	1296	22	78	506	TGGGTGGGCCTGGTC
hh [2.4Kb]- TBE5	1297	22	78,6	463	GTTAATAGCCAGACGC
	1015	22	68,3	457	TGCGCACTTGAATTAT
vvl1+2 [1Kb]	1333	21	72.2	471	TAATTGCGTCCGAAGC
	1334	21	67.8	467	GGAGAGGGCCAGCAT
vg BE [1.1Kb]	1159	21	75,7	519	AATTTGTTTGAACGGG
	1160	21	65,2	449	TATTAGCTGCAGGCGA
vg BE [1.1Kb] with TBE mutagenesis	1161	32			CGTCCGCCCTTTCGAA
	1162	32			GCTGGCTGGCCAAAGT
tkv A [5.2Kb]	970	22	63.8	471	CTAATATGCCCCCAAT
	971	22	70.1	468	TATGAGGATGCCGACA
tkv C [3.9Kb]	974	22	75,7	449	CCGAAGGCGTGTGAAT
	975	22	72,0	473	CCGAAGGCGTGTGAAT
tkv D [3.4Kb]	976	22	73,8	447	GAGACTCGCGAAGACT
	977	22	72,7	498	GCAGCGCGTGTAACGA
inv [2Kb]	1414	21	62,0	415	GATTTAACATGCCATA
	1415	21	62.4	437	CTCTATCAGTGCGGTG
inv [2Kb] with TBE mutagenesis	1418	41	89.6	415	CCATAGAAGCGTAAAA
	1419	41		586	CCTTTGTGCAATTGAG
inv [2Kb] with Ci binding site mutagenesis	1416	38	92	570	CGCTGATCTCGTGGGA
	1417	38			CGATACCTTTCGTTTC

Table 3. Oligonucleotides used during this study.

2.1.4 Laboratory devices

Additionally, the below mentioned devices were essential for the accomplishment of this study.

2.1.4.1 Microscopes

Confocal Laser Microscope TCS SP5 (Leica); Binocular MZ 10F (Leica); Microscope Axioplan 2 imaging (Zeiss).

2.1.4.2 PCR device

Mastercycler Gradient (Eppendorf)

2.1.4.3 Gel Electrophoresis device

Sub Cell GT (Bio-Rad)

2.1.4.4 Gel Documentation system

Gel Jet Imager (Intas)

2.1.4.5 Pipets

Pipetman to 1000 µl; Pipetman to 100 µl; Pipetman to 10 µl (Gilson)

2.1.4.6 Centrifuges

Centrifuge 5424; Centrifuge 5415 C; Centrifuge 5417 R (Eppendorf)

2.1.4.7 Other laboratory devices

Microwaves oven (Home Electric); Phase-Lock-Tubes (PLG- Qiagen).

2.1.5 Living material

Drosophila melanogaster flies and bacteria strains are the living organisms used during this study.

2.1.5.1 Bacteria strains

One Shot TOP10 Chemically Competent E. coli (Invitrogen); XL10-Gold Ultracompetent Cells (Stratagene)

2.1.5.2 Fly lines

The fly lines derived from Bloomington stock or from Prof. Pflugfelder collection or from transgenic constructs that have been induced during the corresponding work.

PZ Insertion	Chromo-somal location	Sequence location	Stock	Genotype
Dmel\P{PZ}ap^{rK568}	2R (41F8)		Bloomington 6374	P{ry[+t7.2]=PZ}ap[rK568]/CyO
Dmel\P{lwB}aos^{W11}	3L (73A2)		Bloomington 2513	w[8]; P{w[+mW.hs]=lwB}argos[W11]/TM3,

Dmel\P{PZ}aos^{05845}	3L (73A2)	3L:16,476,667..16,476,667 [+]	Bloomington 11674	
Dmel\P{lwB}ab^{1D}	2L (32E1-32E2)		Bloomington 9586	w[*]; P{w[+mW.hs]=lwB}ab[1D]/CyO
D298.1= y w hs-flp; Sp/CyO; BM14/TM6B	X		GOP66	y w hs-flp; Sp/CyO; BM14/TM6B
Dmel\P{PZ}bun^{00255}	2L (33E5-33E9)		Bloomington 10936	P{ry[+t7.2]=PZ}bun[00255] cn[1]/CyO; ry[506]
Dmel\P{PZ}dac^{P}	2L (36A1)	2L:16,485,982..16,485,982 [+]	Bloomington 12047	P{ry[+t7.2]=PZ}dac[P]/CyO; ry[506]
Dmel\P{PZ}Dll^{01092}	2R (60E3)	2R:20,739,179..20,739,179 [-]	Bloomington 10981	P{ry[+t7.2]=PZ}Dll[01092] cn[1]/CyO; ry[506]
Dmel\P{PZ}ds^{05142}	2L (21E2)	2L:701,895..701,895 [+]	Bloomington 11394	P{ry[+t7.2]=PZ}ds[05142] cn[1]/CyO; ry[506]
Dmel\P{PZ}dve^{01738}	2R (58D2)	2R:18,158,428..18,158,435 [+]	Bloomington 11073	cn[1] P{ry[+t7.2]=PZ}dve[01738]/CyO; ry[506]
y1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP'}ZH-22A			Bloomington 24481	y[1] M{vas-int.Dm}ZH-2A w[*]; M{3xP3-
w; grn-hinge #8/TM6B				w; grn-hinge #8/TM6B
Dmel\P{PZ}hth^{05745}	3R (86C2)	3R:6,355,185..6,359,632 [-]	Bloomington 11670	
Dmel\P{lArB}neur^{A101}	3R (85C2-85C3)		Bloomington 4369	P{ry[+t7.2]=lArB}neur[A101] ry[506]/TM3, ry[RK] Sb[1] Ser[1]

Dmel\P{PZ}retn^{02535}	2R (59F5)	2R:19,520,111..19,520,111 [+]	Bloomington 11200	cn[1] P{ry[+t7.2]=PZ}retn[02535]/CyO; ry[506]
Dmel\P{PZ}Sdc^{10608}	2R (57E5)	2R:17,364,231..17,364,231 [+]		P{ry[+t7.2]=PZ}Sdc[10608] cn[1]/CyO; ry[506]
w; slp-lacZ; UAS:omb/TM3, Sb			M Porsch, 2002 doctoral	w; slp2-lacZ; UAS-omb/TM3, Sb
Dmel\P{PZ}Stat92E^{06346}	3R (92F1)	3R:16,375,625..16,375,625 [+]	Bloomington 11681	ry[506] P{ry[+t7.2]=PZ}Stat92E[06346]/TM3,
wg-lacZ (CyO)/l(2)			Koni Basler, Zürich	wg-lacZ (CyO)/l(2)
Dmel\P{lacW}tkv^{k16713}	2L (25D1)	2L:5,237,595..5,237,595 [+]	Bloomington 11191	y[1] w[67c23]; P{w[+mC]=lacW}tkv[k16713]/CyO

Table 4. Fly strains from Bloomington used during this study.

PZ Insertion	Stock	Genotype
sal[10.2S/C]	GOP 522	P{salm10.2S/C-lacZ}
sal PS18	GOP 980	SphI – PvuII, insertion 18
sal PS19	GOP 981	SphI – PvuII, insertion 19
salm-lacZ	Bloomington 11340	P{ry[+t7.2]=PZ}salm[03602] cn[1]/CyO; ry[506]
vgBE	GOP 314	multiple inserions

Table 5. Additional fly strains used during this study.

PZ Insertion	Stock	Genotype
vgBE [1.1Kb]	Line did not survive	58A insertion
vgBE [1.1Kb] with TBE mutagenesis	Line did not survive	58A insertion

vvl1+2 [1Kb]	Line did not survive	58A insertion
tkvA [5.2Kb]	Line did not survive	58A insertion
inv [2Kb]	Line did not survive	58A insertion
inv [2Kb] with TBE mutagenesis	Line did not survive	58A insertion
inv [2Kb] with Ci binding site mutagenesis	Line in GOP stock	58A insertion

Table 6. Fly strains generated and used during this study.

2.2 Methods

The following section encompasses an annotation of the methods acquired during this research. It is subdivided in 4 parts: molecular biology methods, histological methods, immunohistochemical methods, enzymatic reactions and Fly work.

2.2.1 Molecular biology methods

2.2.1.1 Agarose gel electrophoresis

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size. Agarose gel electrophoresis can be used for the separation of DNA fragments ranging from 50 base pair to several megabases using specialized apparatus. The distance between DNA bands of a given length is determined by the percentage of agarose in the gel.

An agarose gel was prepared (0.8% for all PCR amplified products and fragments below 2 Kb) in 1 X TBE. DNA samples were loaded directly with the appropriate amount of 5X loading buffer. A size marker (ladder) was also loaded. The gel ran at 100 volts for approximately two hours. DNA was visualized under UV on a transilluminator and photographed with a Polaroid camera.

2.2.1.2 Gel extraction-purification

Gel extraction or gel isolation is a technique used to isolate a desired fragment of intact DNA from an agarose gel following agarose gel electrophoresis. After DNA samples ran on an agarose gel, extraction involves four basic steps: identifying the fragments of interest, isolating the corresponding bands, isolating the DNA from those bands, and removing the accompanying salts and stain.

The DNA fragment was excised from the agarose gel with a clean scalpel. The gel slice was weighed in a 1.5 ml Eppendorf tube. The gel slice was then purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Recovery was tested by gel electrophoresis (section 2.3.1).

2.2.1.3 Phenol extraction, Ethanol precipitation

Phenol extraction is a commonly used method for removing proteins from a DNA sample. DNA is a polar molecule due to its negative charged phosphate backbone, so when the water, DNA, protein and phenol are mixed in the protocol, the DNA does not dissolve in the phenol, but remains in the water phase. On the other side, proteins are permanently denatured by the new solvent environment provided by the phenol and become more soluble in phenol than in water.

Oh the other side, ethanol precipitation is a widely used technique to purify or concentrate nucleic acids. This is accomplished by adding salt and ethanol to a solution containing DNA or RNA. In the presence of salt ethanol precipitates efficiently nucleic acids. The purified precipitate can be then collected by centrifugation, and suspended in a volume of choice.

The two methods can be implied separately or in combination in the laboratory routine. Their combination was preferred during this study. The corresponding protocol is described below.

The following components were added to a Phase-Lock-Tube (Qiagen) in the order they appear: the corresponding DNA sample volume V, 1/10 V of 3M sodium acetate (pH 5.2); 1 V phenol; 1 V chlorophorm: isopentanol (24:1). They were all mixed by inverting several times and centrifuged at maximum speed for 5min at 25ºC. Then the upper solution was transferred to a new PLG. 1 V chlorophorm: isopentanol (24:1) was added to the new PLG and centrifuged at maximum speed for 5min at 25ºC. The upper solution was transferred to a new Eppedorf tube.

2.5 V of 100% ethanol were further added, mixed and precipitated for 30min at -20°C. Spinning followed at full speed in a standard microcentrifuge at 4 °C for 20 minutes. The supernatant was carefully removed. The pellet was washed with 500µl 70% ethanol and centrifuged at maximum speed for 1min at 25ºC. The supernatant was again carefully removed. The pellet finally was air dried (tubes open, ~15 min) and resuspended in TE buffer.

2.2.1.4 Transformation of bacteria

The purpose of this technique is to introduce a foreign plasmid into bacteria and to use those bacteria to amplify the plasmid in order to obtain large quantities of it. This is based on the natural function of a plasmid: to transfer genetic information vital to the survival of the bacteria.

The transformation protocol depends on the type of the used bacteria strains. In the corresponding study the One Shot Top10 Chemically competent *E.coli* (Invitrogen) and the XL10-Gold Ultra competent Cells (Stratagene) were used. The protocol provided by the company was each time implied.

2.2.1.5 Culture of bacteria

Once a single colony of bacteria is obtained, the next step is the growth of a large amount of the bacteria containing the desired plasmid. Luria broth (LB) is a nutrient-rich media used to culture bacteria in the laboratory. LB agar plates are frequently used to isolate individual colonies of bacteria carrying the specific plasmid. However, a liquid culture is capable of supporting a higher density of bacteria and is used to develop sufficient numbers of bacteria necessary to isolate enough plasmid DNA for experimental use.

In every cloning procedure during this study, several times, liquid bacterial cultures were essential. First, liquid LB was added to a tube along with the appropriate antibiotic to the correct concentration (according to each cloning protocol). For mini-preps the cultures were as little as 2ml LB in a falcon tube, while for maxi preps 200ml of LB in a 1l flask were used. Using a sterile pipette a single colony was selected from the LB agar plate. The tip was dropped into the liquid LB with the antibiotic. The culture was loosely covered with a cap that is not air tight. The bacterial culture was each time incubated at 37°C for 12-18hr in a shaking incubator.

2.2.1.6 Plasmid preparation

For plasmid mini and maxi preparation the appropriate protocols were used each time from the corresponding QIAGEN kits (QIAprep Spin Miniprep Kit, Hi Speed Plasmid Maxi Kit).

2.2.1.7 Sequencing

Sequencing during the construction of plasmids in the terms of this study was performed from the company Starseq.

2.2.1.8 Polymerase Chain Reaction (PCR)

2.2.1.8.1 Standard PCR

PCR (Polymerase Chain Reaction) is the revolutionary method based on the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to define a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons). The PCR reaction requires the DNA template (contains the target sequence), the DNA polymerase (the enzyme that synthesizes new strands of DNA complementary to the target sequence), primers (short pieces of single-stranded DNA that are complementary to the target sequence), dNTPs (single units of the bases A, T, G, and C).

Regarding the PCR conditions, in each PCR performed during this study, the protocol of the corresponding restriction endonuclease was used, taking additionally into account the primer characteristics.

2.2.1.8.2 Site-Directed Mutagenesis PCR

In order to induce successfully mutagenesis in specific sites of a sequence the Quick Change II XL Site Directed Mutagenesis Kit of Stratagene was implied. The specific protocol for the corresponding PCR is provided with the kit.

2.2.1.9 Production of enhancer-reporter constructs for transgenesis

For the germ line transformation of *Drosophila* an enhancer reporter vector system was introduced. The pGWattBlacZ, a modified form of the Gateway destination vector pBPGUw

was used. This vector contains, instead of the Gal4 reporter gene cassette, a lacZ cassette.

Recombination takes place between recognition sequences of the vector (attB) and the ones for provided fly strain (attR line). The transgenic constructs induced during the corresponding study are shortly illustrated in the following table.

Gene locus	Construct	Status
hh	hh [3.1Kb]- TBE3 hh [2.4Kb]- TBE5	Both in the destination vector, not injected
vvl	vvl1+2 [1Kb]	injected
inv	inv [2Kb]	unsuccessful injection
	inv [2Kb] with TBE mutagenesis inv [2Kb] with Ci binding site	injected
vg	vg BE [1.1Kb]	injected
tkv	tkv A [5.2Kb]	injected
	tkv C [3.9Kb] tkv D [3.4Kb]	in the destination vector, not injected

Table 7. Transgenic constructs induced during this study.

2.2.2 Histological methods

2.2.2.1 Preparation of L3 larvae imaginal discs

3rd instar larvae were cut off and inverted in PBS buffer. Inversion was best achieved by orienting the dissected head with the mouth hooks upwards and gently pushing the mouth straight down with one forcep while holding tight the entire head with the other forcep. In this manner, the cuticle was on the inside and the imaginal discs, brain, fat, etc. on the outside. Unnecessary tissue such as digestive tract, fat, and salivary glands, were gently dissected away leaving the imaginal disc complex attached to the mouth area and the wings, halteres, and metathoraxic legs attached to the cuticle. Dissections were done in black glass wells filled with PBS 1X.

2.2.2.2 Flat preparation of embryonic central nervous system

The immunostained embryos were transferred on a microscope slide in a drop of glycerol-based VectaShield medium. They were staged [149] and then, with a needle, the embryos were cut open along the body-axis and then attached to the coverslip; the gut and fat body were removed by gentle force and the embryos were flattened. The trachea and the hindgut were also removed. This way, the body wall was opened and the CNS was exposed.

Specimens were analyzed and documented on a confocal laser microscope. Confocal microscopy was performed on a Leica SP5 using 20x water immersion objectives. In some cases, overlays or stack-projections were generated and processed with Leica AF Lite or Adobe Photoshop software.

2.2.3 Immunohistochemical methods

2.2.3.1 Immunostaining of *Drosophila* embryos (fluorescence)

Flies were on yeast diet for 3 days before they were placed in vials containing agar-apple juice for collection of embryos. The vials were incubated at 25^{o}C for approximately 12h. Embryos were harvested from agar plates and attached to laying pots. The eggs were exposed to bleach from a stock solution for about one to three minutes and washed several times in a cage with tap water in order to remove the chorion. Then the embryos were transferred into an 1.5 ml Eppendorf vial with fixative and kept under rotation for approximately 25 minutes. Subsequently, the lower phase was removed and 500 µl methanol was added to the embryos. They were vortexed for one minute in order to remove the vitelline membranes and washed in methanol for 3 times. Finally, the embryos were stored at -20^{o}C in 100% ethanol.

Otherwise, if the immunostaining was planned on the same day the above washing steps were followed by three more with PBT 1X for 15 minutes. Then they were incubated with the primary antibody (in PBT 1X) overnight at 4ºC. After this, the antibody solution was removed and the specimens were rinsed in PBT 1X three to five times, 10 minutes each. Specimens were afterwards incubated with the secondary antibody for two hours at room temperature and then rinsed in PBT 1X three to five times. Finally specimens were stored in glycerol-based VectaShield (Vector Laboratories) medium at 4^{o}C.

2.2.4 Enzymatic reactions

Like in nature, enzyme reactions acquire a key role. Thus they could not be missing from the corresponding study.

2.2.4.1 X-Gal staining of L3 imaginal discs

Once the discs were prepared to be stained, they were removed from the PBS filled well, and immersed into the fixative. For each single staining event, 300 µl of staining solution to which the X-gal has been added was prepared (incubation in 37^{o}C; centrifugation at top speed for 5 min). After 15-20 minutes, the fixative was dismissed and the discs were rinsed with 1x PBS. The PBS was then discarded from the discs and the already prepared staining solution was provided to them. The discs then were allowed to develop in dark, at 25^{o}C for several hours- overnight. The reaction was stopped by rinsing the discs with PBS 1X. The discs were stored at 4^{o}C in 70% glycerol: 1x PBS. The dissection occurred in a droplet of 70% glycerol: 1x PBS on microscope slides. The discs were finally covered with a coverslip and the coverslip was sealed with nail polish. The samples were each time documented within a few days.

2.2.4.2 Restriction digestion

Restriction digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called restriction endonucleases which recognize a specific sequence in the DNA molecule wherever that sequence occurs.

2µl 10x Buffer, 15.5µl dH_2O, 2µl DNA, 0.5µl enzyme in total volume of 20µl were combined in an eppedorf tube. The 10x buffer was always provided with the restriction enzyme. Incubation followed for 1.5- 2 hours at 37°C in a waterbath. Sometimes DNA was digested with more than one enzyme. In these cases it was confirmed that the buffer was compatible with all the enzymes. Recovery was tested by gel electrophoresis (section 2.2.1.1).

2.2.4.3 A-Tailing of PCR products

This protocol was utilized for adding a 3' terminal 'A' overhang onto PCR products that were amplified using a blunt-end enzyme. The product of this protocol was then suitable for PCR-based cloning into vectors such as TA topo vector. First, the PCR product was

purified (Roche High Pure PCR Product Purification Kit) to remove the blunt-end enzyme from the reaction. Then the purified product was resuspended in 30 µl of EB buffer. To a new tube 4 µl of purified product, 1 µl 10X Taq PCR buffer, 1µl 25mM $MgCl_2$, 2µl 1mM dATP, 1 µl Taq polymerase were added. The reaction was incubated at 72°C for 15 minutes, put shortly on ice and then used immediately for the topo cloning protocol.

2.2.5 Fly work

2.2.5.1 *Drosophila* maintenance

The first step in preparing culture vials is adding food media. In the Institute of Genetics the fly food is cooked. The food media is added to a plastic vial to about 1/5 to 2/5 of the total volume. The vials are covered with air-permeable lids. Cooked media can be stored in a refrigerator for several weeks. The optimum rearing condition to grow flies is a temperature of 25°C and 60% humidity. In these conditions generation time is 9-10 days from egg to adult. The flies should be transferred to new vials every 10 to 14 days using carbon dioxide anesthetizing or quick hands.

2.2.5.2 Transgenesis

The production of transformant flies by microinjection procedure was performed of the company Rainbow Transgenic Flies or intern in the Institute of Genetics at the University of Mainz.

Germline transformation requires the stable integration of the DNA of interest into the germ cells of the recipient embryo. This is accomplished by the physical delivery of the desired DNA at the posterior pole of syncytial blastoderm in *Drosophila* embryos where the precursors of the germ cells form. On cellularization the DNA will then, in theory, be incorporated into the so-called pole cells and integrated into their genome. Deposition of the DNA of interest is fulfilled by penetrating the preblastoderm of the embryos with a suitable injection capillary and application of an injection mix including the transformation vector [150].

2.2.5.3 Identification of transgenic flies

After each injection 60 adults emerged on average. G0 crosses were set up with single males and females.

1 G0 female x 3 w^- males

1 G0 male x 3 w^- females

The crosses are kept at 25°C. Transformants were identified from the orange pigmented eye color. Eclosing transformant males and females of F1 were collected as virgins. Up to three individual transformed flies could be used for the subsequent balancing/homozygosis crosses. Then the balancer crosses were set up with w; CyO/Sco. One transformed fly and 3-4 balancer animals were used for each cross. The crosses were held at 25°C for about ten days until about 20 animals have eclosed. This generally allowed to decide on which chromosome the insertion is according to the cross scheme (2.2.5.4).

2.2.5.4 Crosses

The crosses that were implemented in the corresponding work could be classified in two main crosses schemes. On the one hand, the crosses employed to balance the new created transgenic lines. On the other hand, the cross of each enhancer trap/ reporter line with an *omb* mutant line, which implies the use of a balancer cross prior to the cross with the *omb* mutant line.

2.2.5.4.1 Crosses to balance transgenic lines

Two types of balancing crosses are below presented, the one used in case of P-element insertion in the 2nd (II) chromosome and the one used in case of P-element insertion in the 3rd (III) chromosome.

P-Element on the II chromosome

w/w ; Cyo/Sco X w/y ; P/II

w/w or w/y ; Cyo/P (orange Cy) | w/w od w/y ; Sco/P (orange Sco) | w/w od w/y ; Cyo/II (white Cy) | w/w od w/y ; Sco/II (white Sco)

→ After the F1 Generation is the distribution of the orange Cy, orange Sco und white Cy, white Sco of the crosses of the chromosome II und III the same, thus new crosses were essential:

w/w ; Cyo/P (orange Cy) X w/y ; Sco/P (orange Sco)

w/w od w/y ; Cyo/Sco (white Cyo/Sco) | w/w oder w/y ; Cyo/P (orange Cyo) | w/w od w/y ; Sco/P (orange) | w/w oder w/y ; P/P (deep orange wt)

→ In the FII generation, the flies, that have no Cy oder Sco mutation have deep orange eyes

P-Element on the III chromosome

w/w; Cyo/Sco ; III/III X w/y ; II/II ; P/III

w/w od w/y ; Cyo/II ; P/III orange Cy
w/w od w/y ; Cyo/II ; III/III white Cy
w/w od w/y ; Sco/II ; P/III orange Sco
w/w oder w/y ; Sco/II ; III/III white Sco

→ After the F1 Generation is the distribution of the orange Cy, orange Sco und white Cy, white Sco of the crosses of the chromosome II und III the same, thus new crosses were essential:

w/w ; Cyo/II ; P/III (orange Cy) X w/y ; Sco/II ; P/III (orange Sco)

w/w od w/y ; Cyo/Sco ; P/P deep orange Cyo/Sco
w/w od w/y ; Cyo/Sco ; P/III orange Cyo/Sco
w/w od w/y ; Cyo/Sco ; III/III white Cyo/Sco

w/w od w/y ; Cyo/II ; P/P deep orange Cyo
w/w od w/y ; Cyo/II ; P/III orange Cy
w/w od w/y ; Cyo/II ; III/III white Cy

w/w od w/y ; II/Sco ; P/P deep orange Sco
w/w od w/y ; II/Sco ; P/III orange Sco
w/w od w/y ; II/Sco ; III/III white Sco

w/w od w/y ; II/II ; P/P deep orange wildtyp
w/w od w/y ; II/II ; P/III orange wildtyp
w/w od w/y ; II/II ; III/III white wildtyp

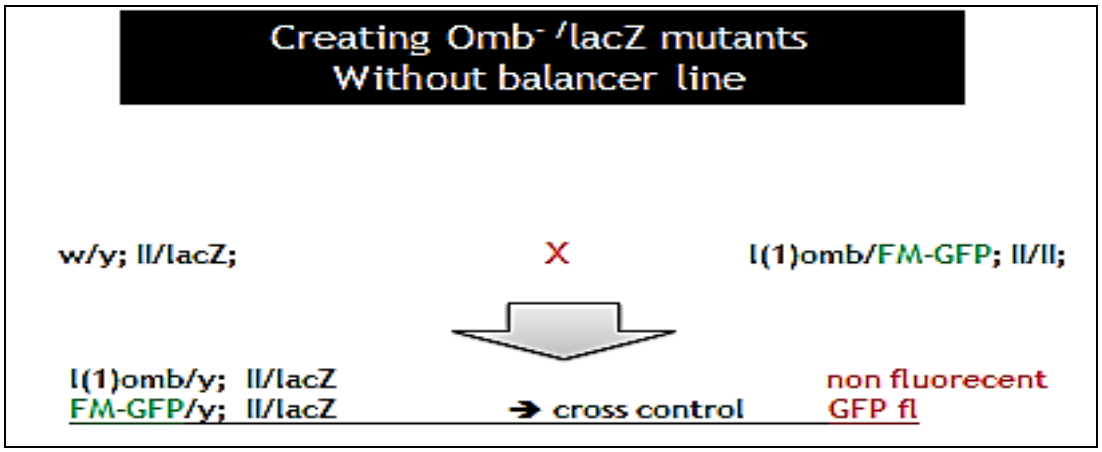
→ In the FII generation, the flies, all the mutations are present in combination with all the eye colors

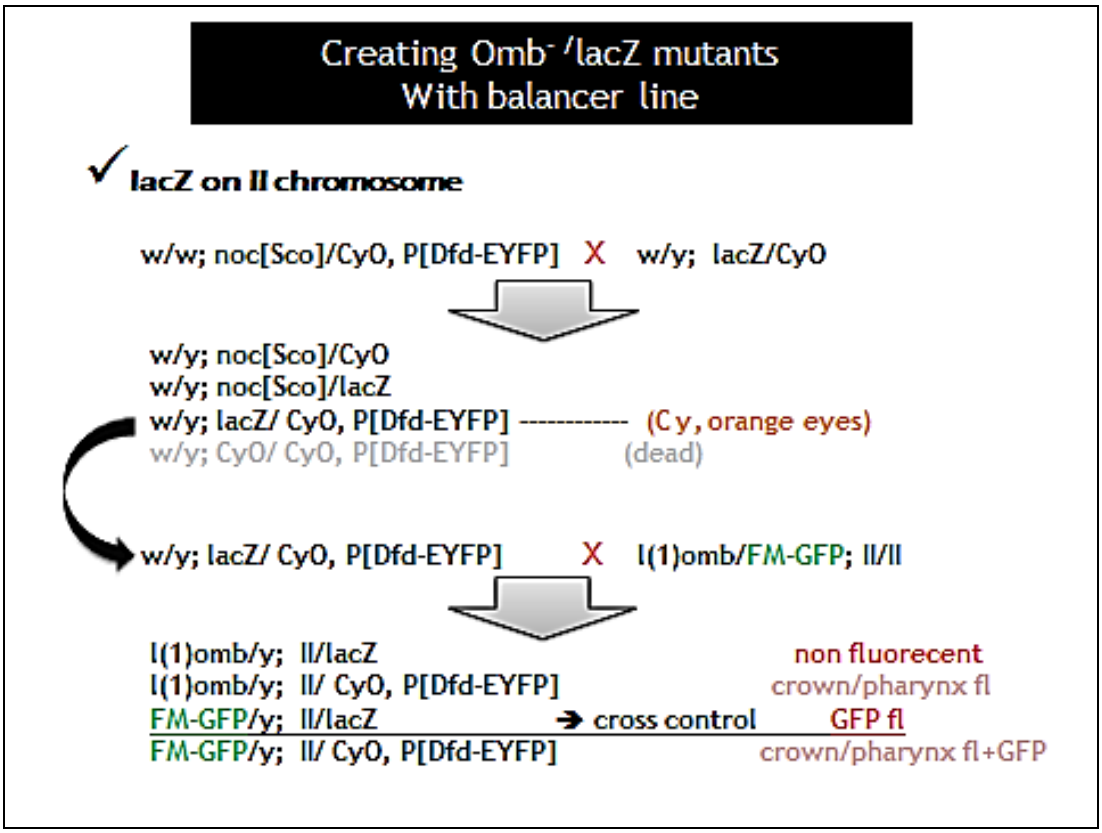
Figure 13. Transgenic lines balancing crosses.

2.2.5.4.2 Crosses with *omb* mutant lines

Several enhancer trap or reporter lines are balanced. If a line was balanced, before the cross with an *omb* mutant line occurred, another balancer line was required to intervene. Otherwise, if the line was homozygous to the lacZ insertion, the cross to virgins of the *omb* mutant line could be preceded directly.

The cross scheme for each case, is described below. Balancer chromosomes come with some sort of physical marker. This marker can serve as a double check in case of genotypic selection.





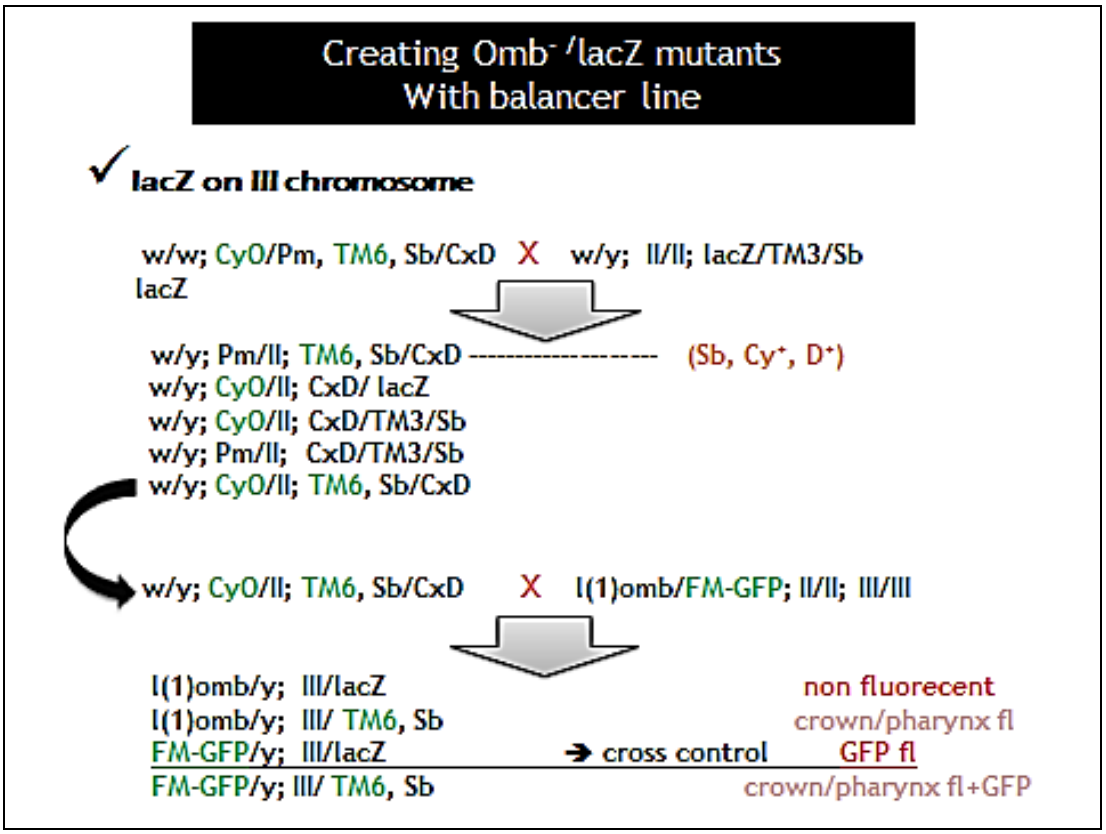


Figure group 14. Crosses with *omb* mutant lines.

2.2.6 Used Computer programs

MS-Office 2010, Adobe Ilustrator CS4, Adobe Photoshop CS4, FinchTV, NCBI/Blast, BCM Search Launcher, NEB Cutter 2.0, LAS AF Lite, EndNote X4.

3 Results and Discussion

The following section consists of a description of the results obtained through this research along with discussion on them. It is structured in three separate subsections:

(3.1) Part I- Candidate Omb target genes,

(3.2) Part II- Potential Omb target genes and

(3.3) Omb in the *Drosophila* embryonic central nervous system.

3.1 Part I- Candidate Omb target genes

The 'Top Gene List' contained several genes involved in wing imaginal disc development. Corresponding lines with enhancer trap/reporter lacZ insertions were ordered from the *Drosophila* stock centers. These lines were characterized by imaginal disc X-gal stainings in *wt* and *l(1)omb* mutant background.

The initial project (Part I) was to achieve a first inventory of the expression of these genes in the imaginal discs and, additionally, obtain information on *l(1)omb* mutant effects on their expression pattern. A subset of these results is presented below alphabetically.

3.1.1 *abrupt (ab)*

abrupt is an ubiquitously expressed muscle regulatory gene encoding a nuclear Zn-finger protein [151] with a conserved BTB domain. The BTB domain defines a gene family with 40 estimated members in *Drosophila*. This domain is found primarily at the N terminus of zinc finger proteins and is evolutionarily conserved from *Drosophila* to mammals [152].

The *abrupt* gene is required for efficient cell migration in the *Drosophila* ovary [153], as well as for the embryonic formation of neuromuscular junctions (NMJs) between subsets of motoneurons and muscles [154],[155].

abrupt mutations reveal its role in establishing and maintaining muscle attachments in formation of adult sensory cells and in the morphogenesis of adult appendages. Mutations in the ab^{1} allele are characterized by the failure of longitudinal wing vein L5 to extend to the wing margin. Ab^{1D} represents a null mutation, and shows a severe wing vein development defect with subsequent the complete absence of L5 between the PCV and margin. The ab^{1D} excision mutation of P^{94} contains an 0.7-kb deletion, removing exon 1, with the P-element [151] being reinserted in the opposite orientation to P^{94}.

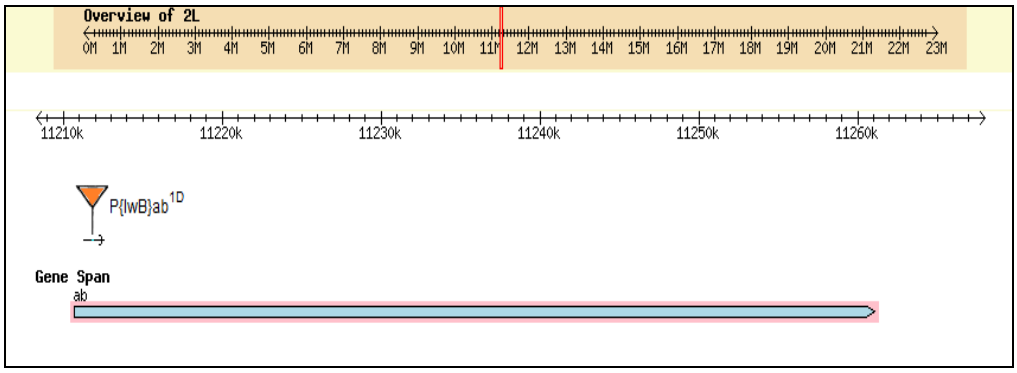
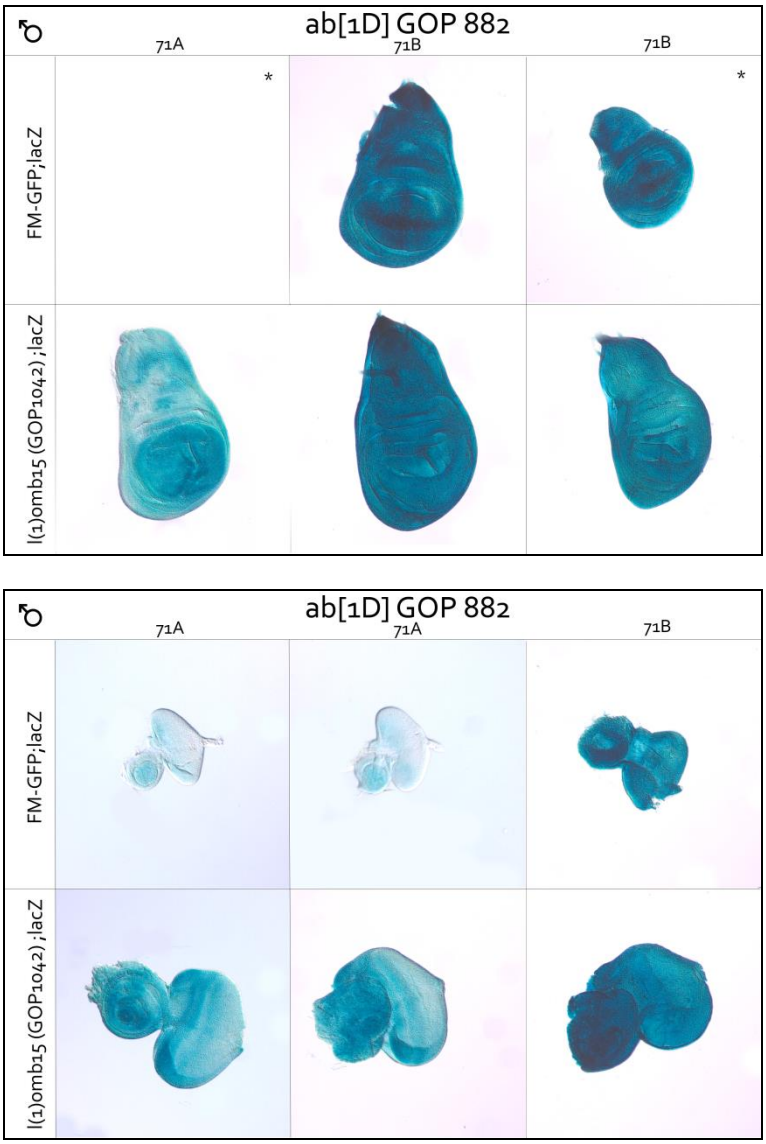


Figure 15. The P-element insertion 1D (orange) in the *ab* locus.



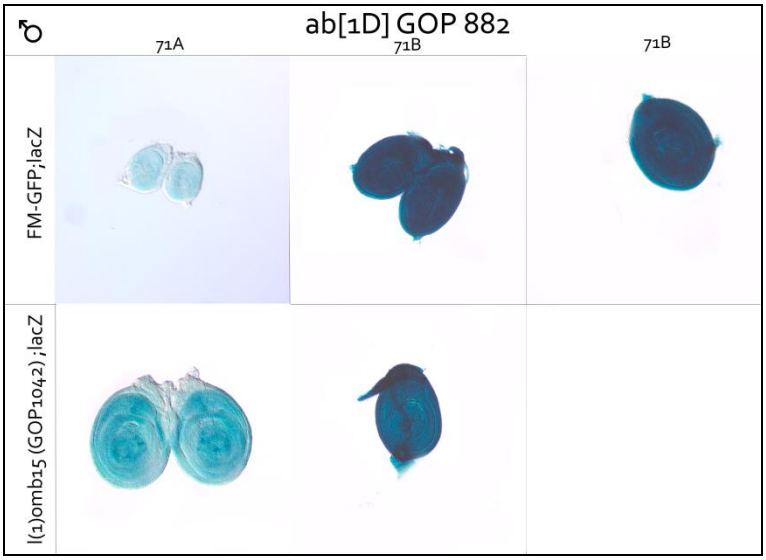


Figure 16. *ab-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain ab^{1D}, after cross with $l(1)omb^{15}$ mutant line (cross 71A, 71B, appendix). 1st row: *FMGFP/Y;* ab^{1D}*-lacZ/II.* 2nd row: $l(1)omb^{15}/Y$; ab^{1D} *-lacZ/II.* Expression appears ubiquitous. In all discs, anterior is to the left and dorsal is to the top.

The *ab-lacZ* wing disc expression occurred ubiquitously with local enhancements. In FM-GFP/Y it was increased along the D/V and A/P boundaries. The enhancer trap expression, thus, has only a poor correspondence to the previously reported antibody staining pattern [147]. The enhanced expression on the D/V boundary was absent in *l(1)omb* discs. In the eye and leg *disc ab-lacZ* was also ubiquitously expressed, without any alteration in *l(1)omb* discs.

ab is already known as a vein-organizing gene for the L5 vein. L5 development is dependent on *omb* and *brk* [147]. The L5 primordium forms within the *omb* domain adjacent to cells expressing high levels of *brk*. This combination of signals results in the expression of Ab along the future L5 vein.

Omb seems to have an impact on *ab* expression in the wing discs. However, there are no well conserved, high-score TBEs in or within 10 kb boundary of this large gene locus, indicating indirect regulation. Whether *omb* maintains a role on *abrupt* expression, remains to be shown by further studies.

3.1.2 *apterous (ap)*

Activity of the LIM-homeodomain protein Apterous, which is expressed in the dorsal wing disc cells, subdivides the *Drosophila* wing primordium into a dorsal and a ventral compartment. Apterous-dependent expression of Serrate and Fringe in dorsal cells leads

to the restricted activation of Notch at the DV boundary. Notch is required for the maintenance of the compartment boundary and the growth of the wing primordium [156].

apterous is also required for proximal-distal leg development [157]. During embryogenesis, *ap* is expressed in a small subset of mesodermal precursors that give rise to 6 muscles in each abdominal hemisegment and in 5 neurons within each corresponding CNS hemisegment [158].

In severe *ap* mutants reductions in the wing are caused, or worse, the wings are absent, but the notum and scutellum are essentially normal. The P-insertion allele rK568 exhibits the above mentioned strong hypomorphic phenotype. The insertion lies upstream of the *apterous* locus [159].

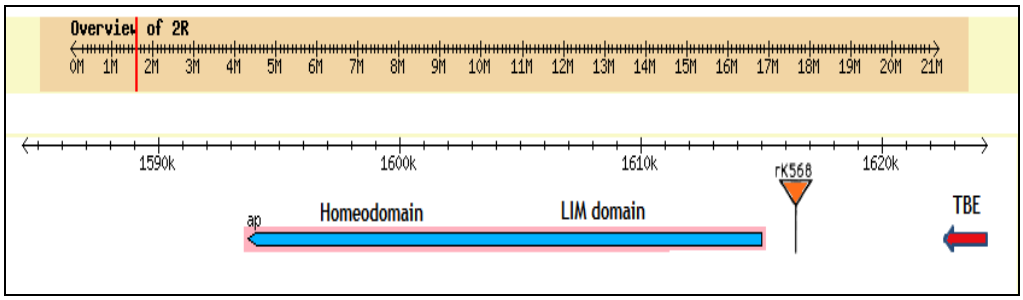
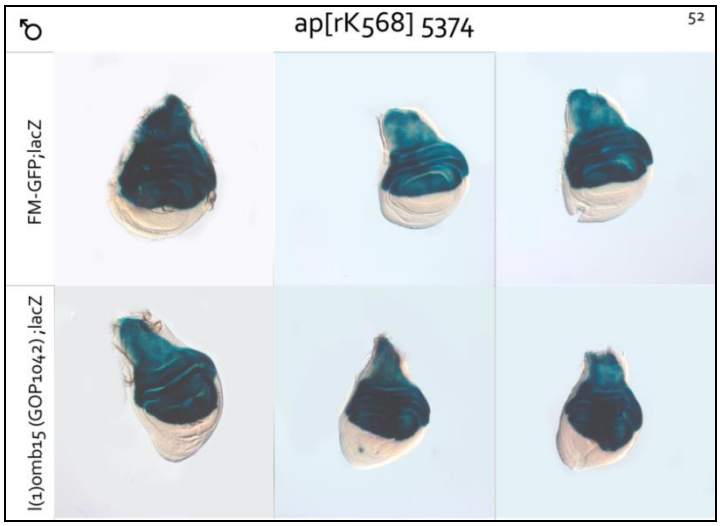


Figure 17. The potential TBE site (red horizontal arrow) and the P-element insertion rk568 (orange) upstream of the *apterous* locus.



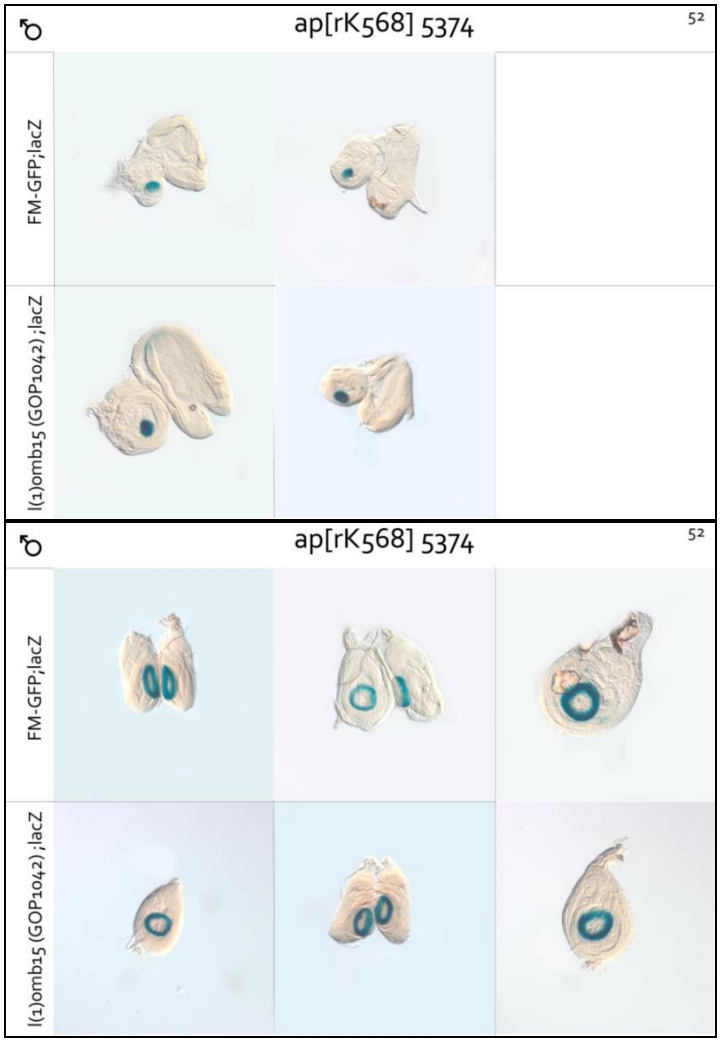


Figure 18. *ap-lacZ* expression.
X-Gal staining of L3 imaginal discs of the fly strain ap^{rk568}, after cross with $l(1)omb^{15}$ mutant line (cross 52, appendix). 1st row: *FMGFP/Y*; ap^{rk568}*-lacZ/II.* 2nd row: $l(1)omb^{15}/Y$; ap^{rk568} *-lacZ/II*. No significant change in the expression was detected.

The pattern of *ap* expression in the imaginal discs as visualized by X-Gal staining in the insertion line rK568 was indistinguishable from the pattern of *ap* transcript expression previously reported for these tissues [159]. *l(1)omb15* mutant background seemed to have no effect on ap^{rk568} *-lacZ* expression in the imaginal discs, regardless of the highly conserved TBE located upstream of the *apterous* locus.

Previous studies examined the expression of ap^{rk568} *-lacZ* in $l(1)omb^{3198}$ and l(1)omb^{D4} discs and detected that *ap* is misexpressed in the ventral compartment of the wing [146]. omb^{3198} and omb^{D4} are lack-of-function alleles produced by a point mutation that introduces a stop codon and generates a truncated protein [24], [160].

The *omb* allele used in this study was $l(1)omb^{15}$. $l(1)omb^{15}$ *is a* point mutation that introduces a stop codon 83 residues downstream of the T-box domain. $l(1)omb^{15}/bi^{1}$ larvae

exhibit a variable wing phenotype and increased lethality [161]. Even though *omb*^{15} is a lethal allele it may not to be complete null mutation. *omb*^{15} larvae recover at a higher rate than *omb*^{3198} or *omb*^{D4} larvae, which was the reason that *omb*^{15} was used in most of my crosses.

It is recognized that different alleles can result in different observable phenotypic traits {Lawrence, Eleanor (2005) Henderson's Dictionary of Biology. Pearson, Prentice Hall. ISBN 0-13-127384-1}. The use of a different *omb* allele may possibly be responsible for the difference between previously published and my results.

3.1.3 *argos (aos)*

The EGF receptor plays important roles in cell proliferation, differentiation, and survival. Activation of the receptor and its downstream signals is required for cells to grow and function normally. In *Drosophila,* the epidermal growth factor (EGF) receptor, DER, is activated by multiple ligands, including Spitz, Gurken, and Vein [162]. Binding of these ligands to DER triggers the activation of the Ras1/MAPK pathway.

Argos is a secreted protein that contains an EGF-like domain comprising a structure similar to these activating ligands [163],[164]. Argos inhibits EGFR activation [165]. *argos* transcription is induced by the *Drosophila* EGF-receptor pathway forming an inhibitory feedback loop [166].

In loss-of-function *argos* mutants cellular differentiation in various tissues is triggered by DER activation [167]. On the other hand, overexpression of *argos* inhibits cellular differentiation and induces programmed cell death [168]. *argos*^{05845} and *argos*^{W11} adults exhibit extensive blistering along the posterior edge of the eye [169],[163]. In *argos*^{W11} flies the shape of the wing is affected [170], where an ectopic wing vein phenotype occurs [171].

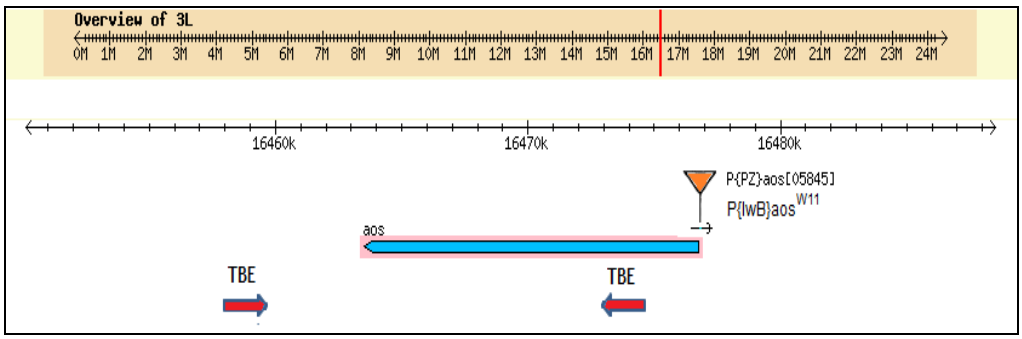


Figure 19. The two potential TBE sites (red horizontal arrows) and the P-element insertions 05845 and w11 (orange) in the *argos* locus.

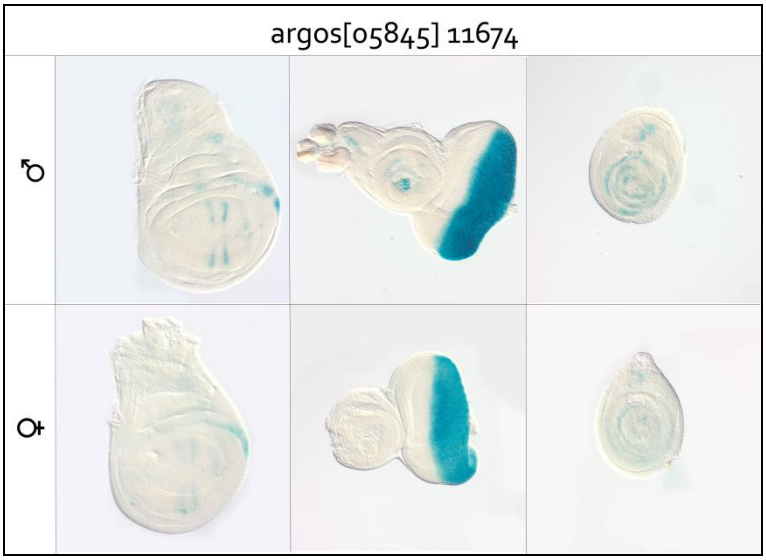


Figure 20. *argos-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain *aos*^{05845}. 1st row: *aos*^{05845}*-lacZ*, males. 2nd row: *aos*^{05845}*-lacZ*, females. In the wing disc, a significant staining is apparent in the L3 and L4 veins region in this homozygote *argos* line. A very weak L5 staining is also distinct along with a small stripe in the posterior periphery of the wing disc. Furthermore, a strong staining is apparent in the posterior part of the eye disc (posterior to MF) and a concentric staining in the leg disc.

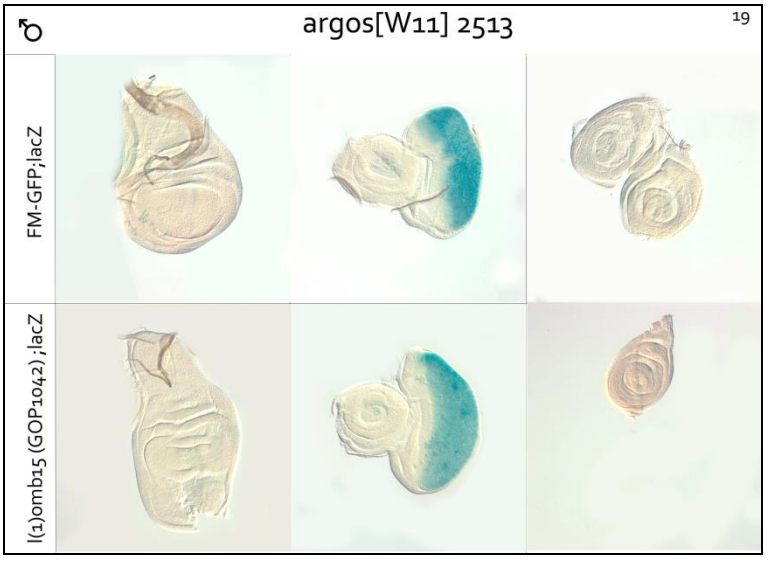


Figure 21. *argos-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain *aos*^{W11}, after cross with *l(1)omb*^{15} mutant line (cross 19, appendix). 1st row: *FMGFP/Y*; *aos*^{W11}*-lacZ/III*. 2nd row: *l(1)omb*^{15}*/Y; aos*^{W11}*-lacZ/III*. The above seen wing disc L3/L4/L5 staining appears faint in the heterozygote FMGFP wing discs and invisible in the *l(1)omb* wing discs. No staining in the leg discs as well as no significant change in the eye expression is detected in both of the genetic backgrounds.

The expression of the homozygous *aos*^{05845}*-lacZ* or the heterozygous *aos*^{W11}*-lacZ* in *wt* and *l(1)omb* mutant background was strong in the posterior part of the eye disc, posterior to the MF, displaying no significant change in the different genetic backgrounds.

In the wing disc, the homozygous strain *aos*^{05845}*-lacZ* exhibited a narrow-stripe staining in the L3 and L4 veins region plus a very weak L5 stripe staining along with a small stripe in the posterior periphery of the disc. The expression is formerly identified [172] and characteristic for a gene involved in initiating wing vein development in third larval instar wing discs [173].

LacZ expression in the wing disc of heterozygous larvae was faint and was not enhanced in the *l(1)omb* mutant background. In *l(1)omb*^{15}, the morphology of the wing disc was altered, apparently due to ventral over proliferation. This was observed, to a variable extent, in other crosses, too.

3.1.4 *brinker (brk)*

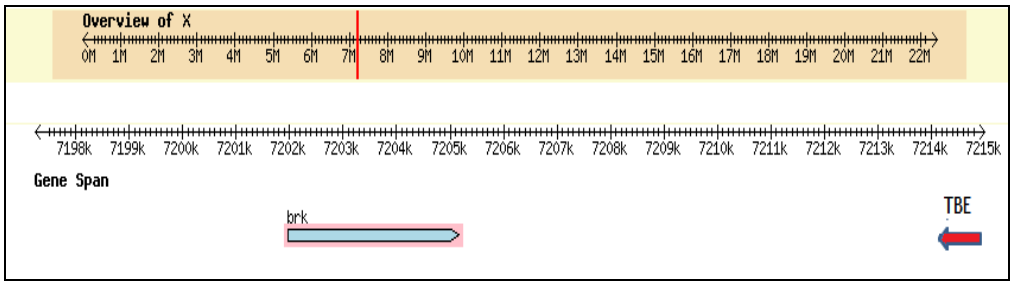


Figure 22. The potential TBE site (red horizontal arrow) downstream of the *brk* locus.

brinker is a key Dpp target gene which is repressed in a graded manner by Dpp signaling in the central region of the wing disc [174]. Brk encodes a transcriptional repressor [175] that acts in a dosage-dependent manner to establish the centrally congregated expression of the Dpp target transcription factors encoded by *omb, sal* and *vg* [176],[105]. The opposing and complementary activities of Dpp and Brk along the AP axis of the wing disc lead to differential activation of target genes in a way that the more responsive a gene is to BMP signaling and the less sensitive it is to Brk repression, the broader its expression domain will be.

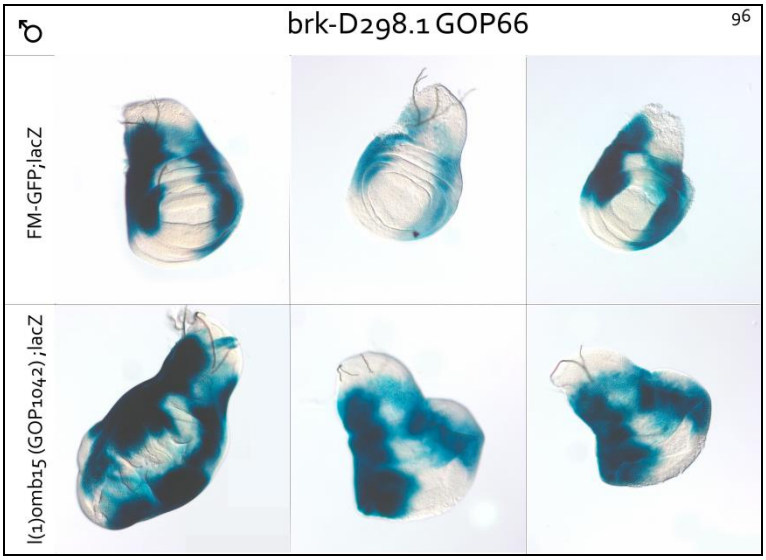


Figure 23. *brk-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain $brk^{298.1}$ after cross with $l(1)omb^{15}$ mutant line (cross 96, appendix). 1st row : *FMGFP/Y;* $brk^{298.1}$ *-lacZ/III*. 2nd row: $l(1)omb^{15}$*/Y;* $brk^{298.1}$ *-lacZ/III*. Detection of the expected peripheral *brk* in *FMGFP,* lateral domains invading the disc center without total coverage of the central territory in $l(1)omb^{15}$.

The reporter line $brk^{298.1}$ *–lacZ* [176] seemed to partially reproduce the expected peripheral *brk* wing pattern [175], in the *FMGFP* background*. brk-lacZ* expression in $l(1)omb^{15}$ invaded the disc center without totally covering of the central territory of the wing. This effect was previously described in $l(1)omb^{3198}$ discs, where the two lateral domains of *brk* expression expanded towards the A/P compartment boundary but did not cover the central region of the disc [146].

It is well known that the broad *omb* expression pattern in the center of the wing disc is complementary to that of *brk*, in the lateral regions of the disc, where *brk* represses *omb* expression [105],[177]. It has been previously shown that a boundary between *omb* and *brk* expression domains is necessary and sufficient for inducing L5 wing vein development in the posterior regions. *brk*-expressing cells produce a short-range signal which can induce vein formation in adjacent *omb*-expressing cells [147].

The expansion of *brk* expression towards the center of the wing disc in case of *omb* deficit is a sign of Omb influence on *brk* expression. Thus, a reciprocal negative regulation is indicated between the two transcription factors. Whether Omb directly represses *brk* or whether loss of *omb* attenuates Dpp signaling needs to be further established.

It is known that *brk* promoter regulatory region contains multiple compact modules that can independently drive *brk*-like expression patterns and inputs from multiple signaling pathways are integrated to generate the final *brk* pattern [147].

3.1.5 *bunched (bun)*

bun is required throughout development. During embryogenesis, *bun* regulates peripheral nervous system development and segmental patterning [178]. It regulates photoreceptor patterning during eye development, where it interacts with Dpp, EGF and Hh pathway genes [179].

In the follicle cells, *bun* is regulated by opposing Dpp and EGF signals and is required to establish a boundary between two dorsal–anterior fates, the dorsal appendage and the operculum [180]. In the eye imaginal discs *bun* is required for photoreceptor differentiation, optic lobe morphogenesis, and in the wing disc for patterning and notum formation [181].

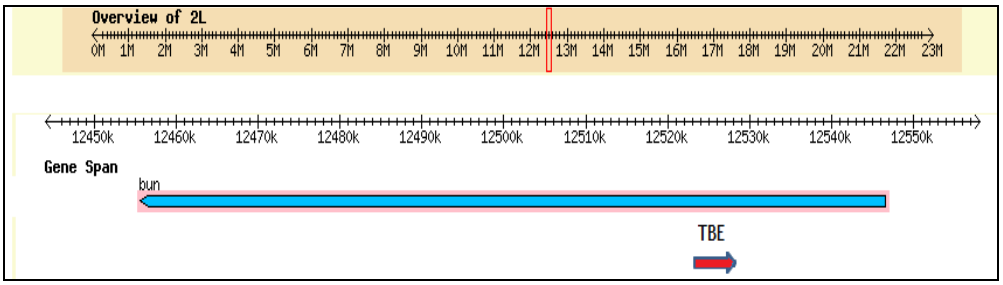


Figure 24. The potential TBE site (red horizontal arrow) in the *bun* locus.

The *bun* sequence encodes a leucine zipper and DNA binding domain that is nearly identical to mammalian members of the TSC-22/DIP/BUN protein family [182], the members of which are shown to regulate transcription [183].

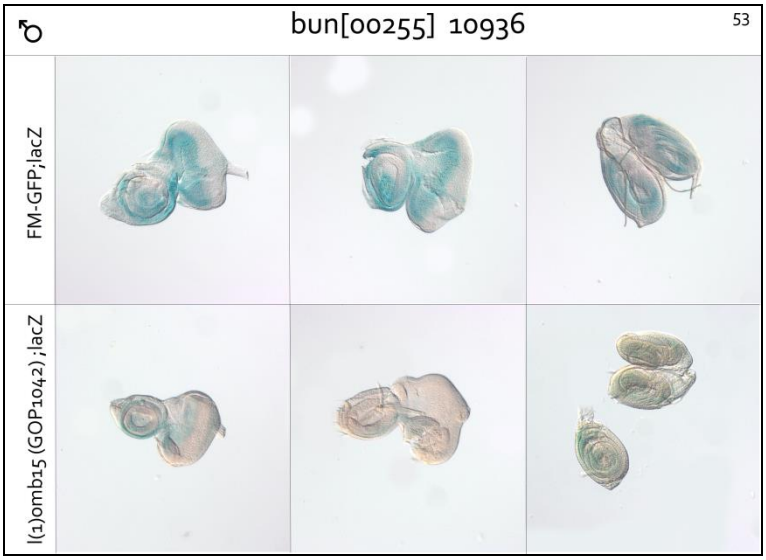


Figure 25. *bun-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain *bun*^{00255} after cross with *l(1)omb*^{15} mutant line (cross 53, appendix). 1st row : *FMGFP/Y; bun*^{00255}*-lacZ/II*. 2nd row: *l(1)omb*^{15}*/Y; bun*^{00255}*-lacZ/II.* In *FMGFP/Y* wing disc, detection of a circular hinge staining along with a spot staining in the proximal notum. Also, in *FMGFP,* observation of *bun-lacZ* expression anterior to MF in the eye discs and a concentric pattern in antenna and leg discs. In *l(1)omb*^{15} staining was reduced in all discs.

In *FMGFP* genetic background, *bun-lacZ* expression was detected around the wing hinge, as a spot in the proximal wing notum, in the eye discs anterior to the MF and concentric in the antenna and leg discs. In *l(1)omb*^{15}*,* the lacZ expression appeared reduced in all discs, including in areas in which omb is not expressed suggesting a difference in staining conditions.

BunA (the long *bun* isoform) was identified as a positive growth regulator [184],[185]. Omb can promote proliferation in the lateral wing disc [145]. An interaction between these two growth promoting factors is conceivable and needs to be further investigated.

3.1.6 *dachshund (dac)*

dachshund encodes a novel nuclear protein required for normal cell-fate determination of imaginal disc cells. *dac* has an effect on cells at the posterior margin of the eye disc which in *dac* absence fail to follow a retinal differentiation pathway and appear to acquire a cuticle fate instead. *dac* is also necessary for proper differentiation of a subset of segments in the developing leg. Null mutations of *dac* result in flies with no eyes and shortened legs [186].

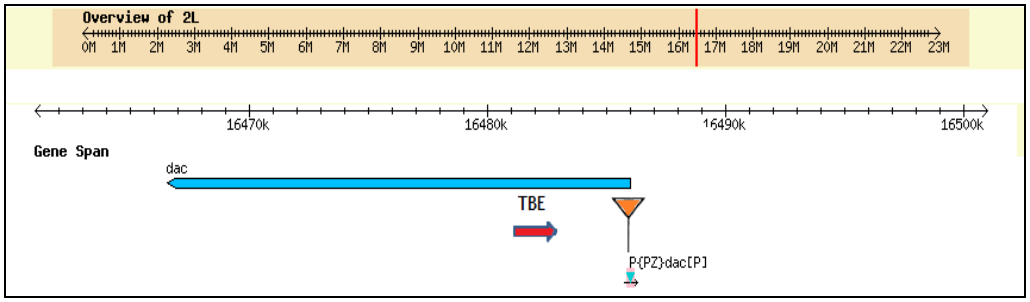
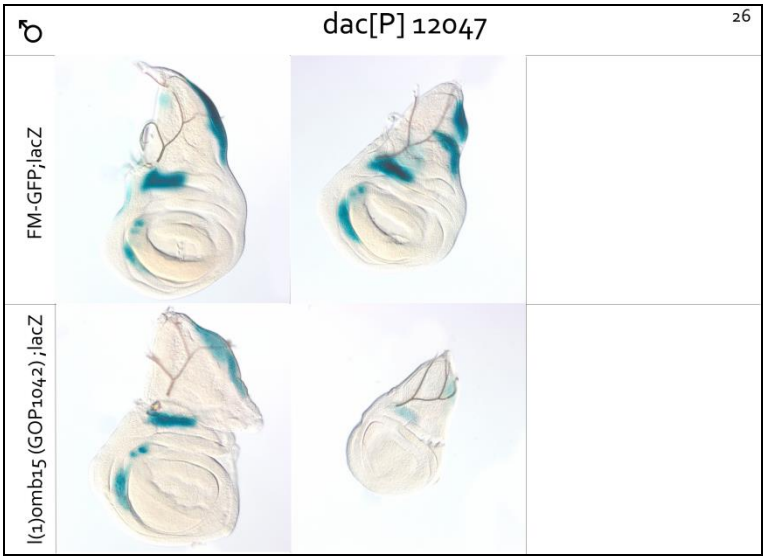


Figure 26**.** The potential TBE site (red horizontal arrow) and the P-element insertion P (orange) upstream on the *dac* locus.

Targeted expression of *dac* is sufficient to direct ectopic retinal development in a variety of tissues, including the adult head, thorax and legs [187]. It has been suggested that *dac* may function downstream of *dpp* signaling [188]. Likewise, *dac* is expressed in a sex-specific manner, having an important role in the development of both male and female genitalia [189].

dac plays a key role in metazoan development, regulating ocular, limb, brain, and gonadal development [190]. Recent studies have demonstrated an important role for human *DACHSHUND* [191] in tumorigenesis, in particular, breast, prostate and ovarian cancer [192],[193],[194].



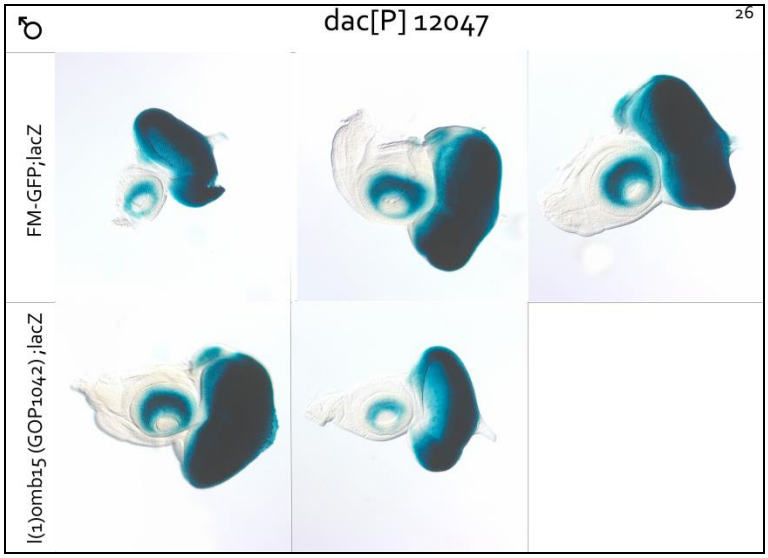


Figure 27. *dac-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain dac^{12047} after cross with $l(1)omb^{15}$ mutant line (cross 26, appendix). 1st row : *FMGFP/Y*; dac^{12047} *-lacZ/II*. 2nd row: $l(1)omb^{15}$ */Y;* dac^{12047} *-lacZ/II*. In *FMGFP* wing disc, detection of a posterior notum staining along with two (one dorsal and one ventral) anterior hinge stripes. Also, in *FMGFP,* observation of very strong *dac-lacZ* expression in the eye discs and rather strong concentric in the antenna discs. In $l(1)omb^{15}$ no significant change in *dac-lacZ* expression is detected.

In the *FMGFP/Y* genetic background, *dac-lacZ* expression was detected posteriorly in the wing notum along with two (one dorsal and one ventral) anterior wing hinge stripes. The expression was also very strong in the eye discs and rather strong concentric in the antenna discs. In $l(1)omb^{15}$ discs, no significant change in *dac-lacZ* expression was observed.

3.1.7 *dachsous (ds)*

In Drosophila five cadherins have been isolated: *DE-cadherin/shotgun* [195], *DN-cadherin/Cadherin-N* [196], *flamingo/starry night* [197], *fat (ft)* [198] and *dachsous (ds)* [199].

ds is the only known cadherin gene in *Drosophila* with a restricted spatial pattern of expression in imaginal discs from early stages of larval development [200]. Dachsous is involved in the non-canonical Wnt signaling pathway that controls the establishment of planar cell polarity (PCP) [201],[202].

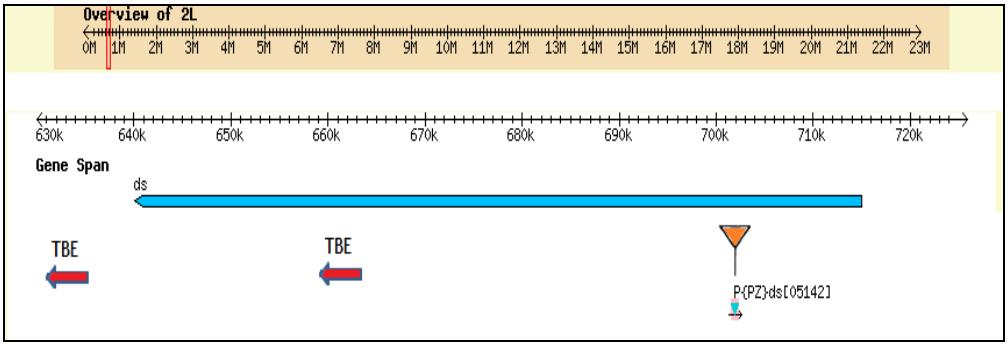
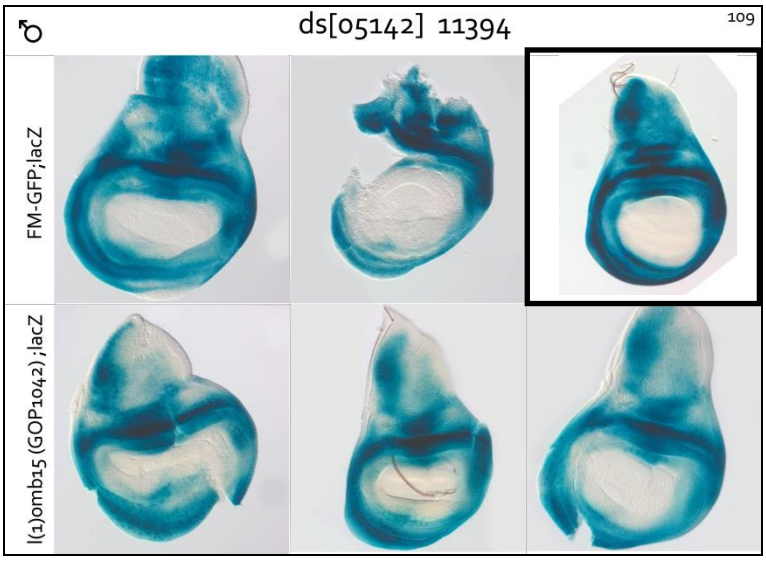


Figure **28.** The two potential TBE sites (red horizontal arrows) and the P-element insertion 05142 (orange) in the *ds* locus.

In the wing disc, *ds* is first expressed distally, and later is restricted to the hinge and lateral regions of the notum [203]. Flies homozygous for strong *ds* hypomorphic alleles display a smaller hinge territory and an ectopic notum reminiscent of phenotypes caused by reduction of Wingless during early wing disc development, which are then rescued by an increase in Wg activity [200].

In the eye disc, it is shown that the gradient of Ds expression, along with Fj, provides partially redundant positional information essential for specifying the polarization axis [204]. Moreover, Ds/Ft signaling pathway is essential for leg regeneration, controlling growth along the PD axis of the regenerating leg [205].



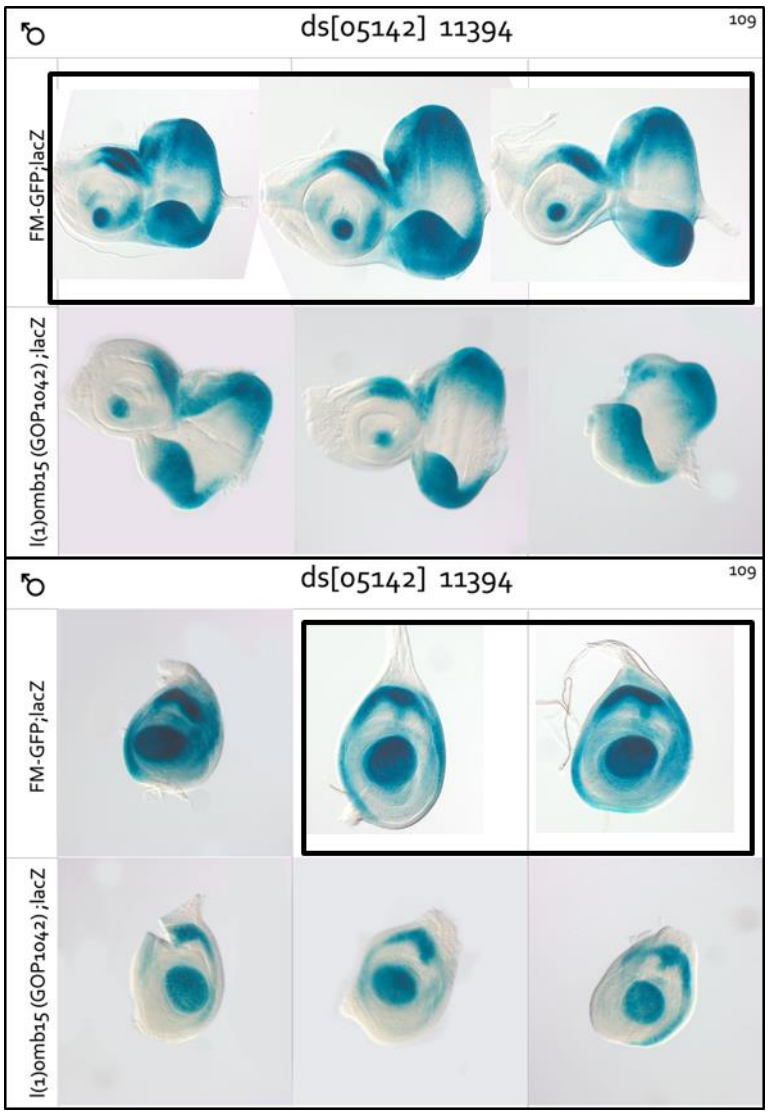


Figure 29. *ds-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain ds^{05142} after cross with $l(1)omb^{15}$ mutant line (cross 109, appendix). Pictures with frame: ds^{05142} -lacZ from Fred Eichinger (homozygous). 1st row: *FMGFP/Y*; ds^{05142} *-lacZ /II*. 2nd row*:* $l(1)omb^{15}$ */Y*; ds^{05142} *-lacZ /II*. Wing disc expression: circular expression domain in *FMGFP* which in *l(1)omb15* appears unaffected. Eye disc: polar expression in $l(1)omb^{15}$. Antenna disc: central dot expression. Leg disc: weaker expression in the mutant background.

Fat/Dachsous signaling is, in conjunction with the Frizzled (Fz) pathway, one of the two signaling pathways which appear to control *Drosophila* wing PCP [206]. The proximal expression of Ds and distal expression of Four-jointed (Fj) are proposed to generate opposing activity gradients along the proximal-distal wing axis [207]. On the other hand, studies in the *Drosophila* abdomen have led to an alternative model in which the Ft/Ds and Fz pathways function independently to organize PCP [208].

Moreover, the protocadherins Ft/Ds pathway has been shown to be critical for PCP in vertebrate development [209] and is implicated in human disease [210],[211].

In the *FMGFP/Y* genetic background, *ds-lacZ* expression was detected anteriorly in the notum and peripherally of the wing pouch, excluding the central part of the pouch. The expression appeared polar in the eye discs and in a central dot-like domain in the antenna discs. In the leg discs, *ds-lacZ* was observed in a central pattern surrounded by a peripheral ring expression. In the *l(1)omb*^{15} discs the wing and eye-antennal expression domains appeared unaffected whereas the leg disc expression was weaker.

3.1.8 *Distalless (Dll)*

The homeobox *Dll* protein is already present at the embryonic stage 11 in clusters of cells in the positions where leg primordia will be later distinguishable [212]. *Dll* expression is required early for the development of the larval leg discs, the Keilin's organs [213] and later for the development of the distal structures of the leg disc [212].

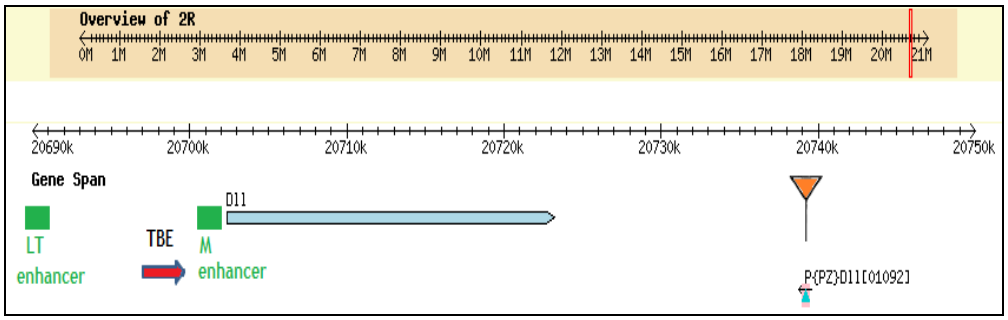


Figure 30**.** The potential TBE site (red horizontal arrow), the P-element insertion 01092(orange) and the *Dll* enhancers [214], (green rectangles) near the *Dll* locus.

Expression in the leg primordia initially depends on the positional signals that generate the DV and AP patterns of the larval epidermis, like Wg, Dpp and DER [215] but later *Dll* expression seems to become independent of them [216].

Dll expression in the leg discs is mediated at least in part by two separable *cis*-regulatory elements (LT:late acting or leg trigger, and M: Maintenance) [217],[214]. Wg and Dpp pathways are required for LT activity [214]. LT activity also requires *Sp1*, a ventral selector gene that ensures that Wg and Dpp only activate *Dll* in ventral discs, but not dorsal such as the wing [214]. In the late third instar leg discs *Distalless* is expressed in concentric circles [218]. *Dll* mutants lack specific proximal-distal PD regions of the adult leg.

In the *FMGFP/Y* genetic background, *Dll-lacZ* expression was detected in a distinct D/V stripe in the wing disc pouch and the expected concentric patterns in the antenna and leg disc. In *l(1)omb*^{282} eye and leg discs no significant change in *dll-lacZ* expression was

detected, whereas in the wing discs the *Dll-lacZ* D/V pouch stripe appeared attenuated (Fig. 29).

The *Dll* DV stripe expression was previously described as Wg-dependent [95]. It is also published that Nemo, the founding member of an evolutionarily conserved family of proline-directed serine/threonine protein kinases, is an antagonist of Wg during larval wing disc development and can negatively influence Wg-dependent gene expression. Work in our lab has shown, that *nmo-lacZ* is upregulated in *omb* mutant wing discs. The influence of *l(1)omb*^{282} on *dll-lacZ* expression in the wing discs could be downstream of *nemo* [219],[220].

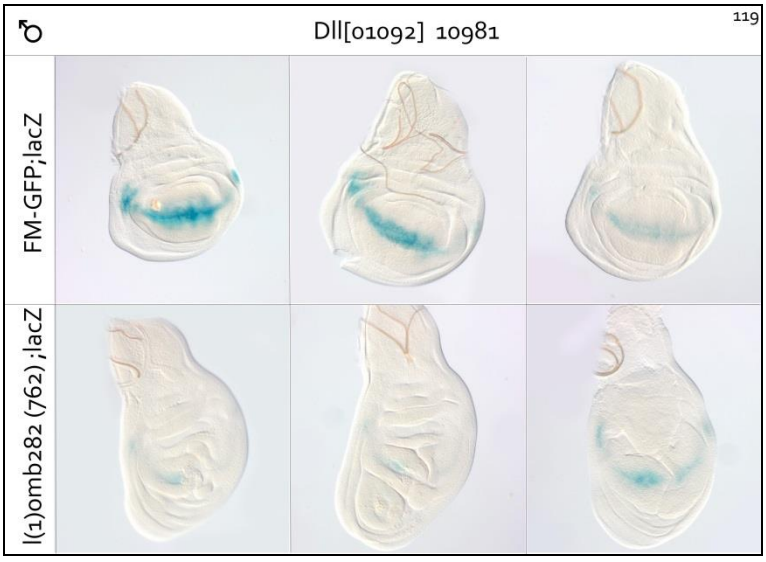


Figure 31. *Dll-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain Dll^{01092} after cross with $l(1)omb^{15}$ mutant line (cross 119, appendix). 1st row : *FMGFP/Y;* Dll^{01092} *-lacZ/II*. 2nd row: $l(1)omb^{15}$ */Y;* Dll^{01092} *-lacZ/II*. Expression of *Dll-lacZ* in *FMGFP:* in a distinct D/V stripe in the wing disc and concentric patterns in antenna and leg disc. $l(1)omb^{282}$*: Dll-lacZ* D/V stripe appears attenuated in the wing disc. No significant change in *Dll-lacZ* expression in eye and leg disc is detected.

3.1.9 *defective proventriculus (dve)*

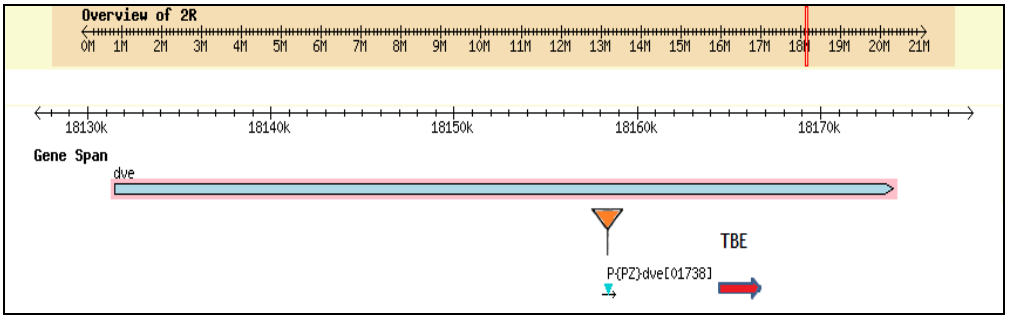


Figure **32.** The potential TBE site (red horizontal arrow) and the P-element insertion 01738 (orange) in the *dve* locus.

dve encodes a type of homeobox protein, which was originally shown to be required for the proper development of the larval proventriculus [221]. In wing discs, Dve is expressed in the prospective wing region, being subsequently repressed along the dorsal–ventral compartment boundary through Notch-mediated signaling[222]. *dve* is required for a region of the PD axis encompassing the distal region of the proximal wing and a small part of the adjacent wing pouch. Loss-of-function of *dve* results in the deletion of this region and, consequently, shortening of the PD axis [223]. Dve is induced by Vg, in a non-autonomous manner, in a domain that is larger than that of Vg and then suppressed at the DV boundary

by the combined activity of Nub and Wg [222]. In leg discs, Dve is expressed in a pattern of concentric rings and is temporally regulated in the Notch-activated region [224].

The enhancer trap line seemed to partially reproduce the expected peripheral pouch *dve* wing pattern [223], ocellar field eye pattern [225], and circular leg pattern [224], (Fig. 33). In *l(1)omb*^{15} wing disc, *dve-lacZ* expression showed an apparently changed pattern which may be attributed to ventral overproliferation of the disc. In *l(1)omb*^{15} eye disc no expression change was observed (Fig. 33). The results have to be verified with more discs in order to reach a conclusion whether *dve* could be an Omb target gene.

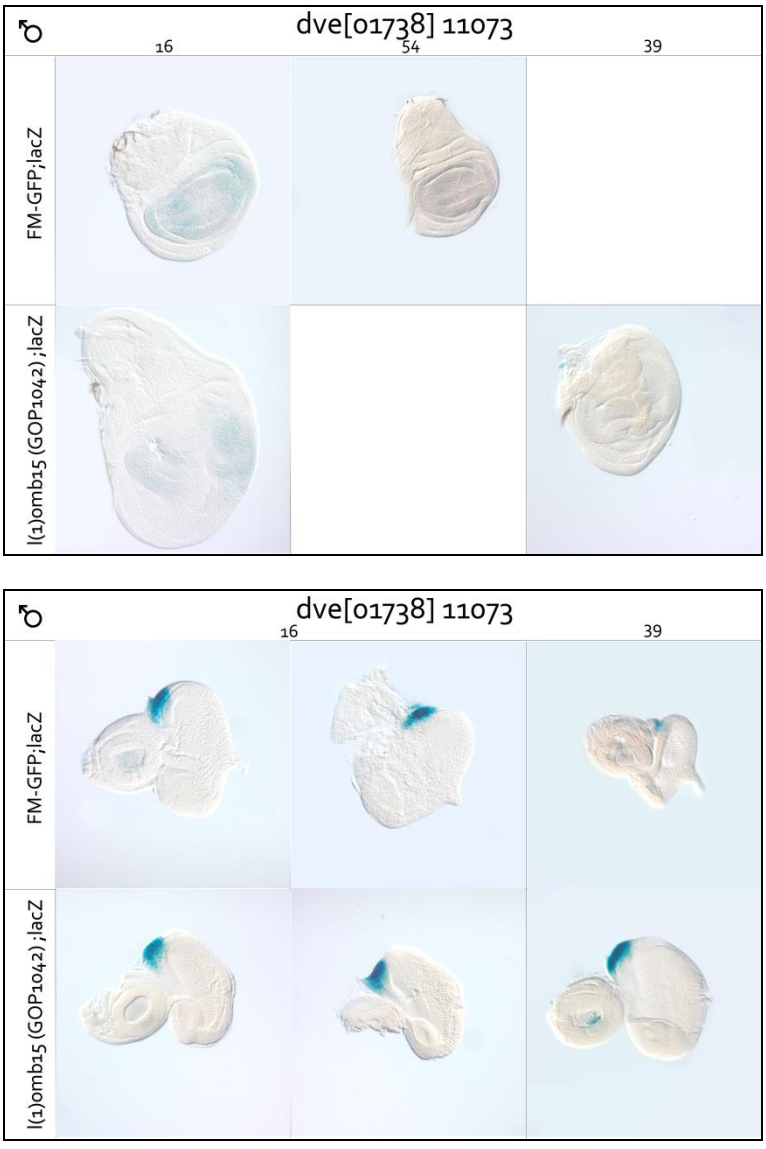


Figure 33. *dve-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain *dve*^{01738} after cross with *l(1)omb15* mutant line (crosses 16,39,54, appendix). 1st row : *FMGFP/Y*; *dve*^{01738} *-lacZ/II*. 2nd row: *l(1)omb*^{15} */Y; dve*^{01738} *-lacZ /II*.

3.1.10 *grain (grn)*

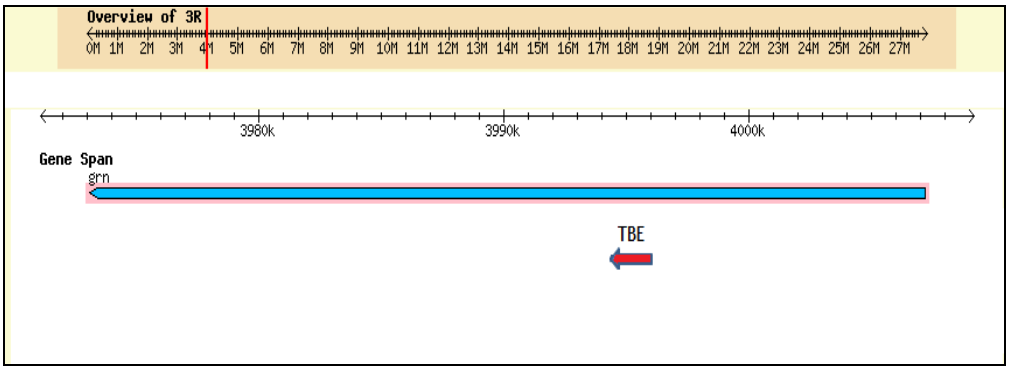


Figure 34. The potential TBE site (red horizontal arrow) in the *grn* locus.

grain encodes a GATA transcription factor that is required during development for epithelial morphogenesis in the embryo and imaginal discs of Drosophila. Loss of *grn* function affects the shape of the *Drosophila* adult legs and the larval posterior spiracles. *grain* affects organ shape by locally controlling cell rearrangement. Mutant legs are short and wide rather than long and thin, while the spiracles are flat instead of dome-shaped [226]. In the embryo, *grn* is specifically expressed within the ISN motoneuron subclass and plays a crucial role for ISN axon projections [227].

Two enhancer reporter constructs from the *grn* locus were tested for imaginal disc expression and *omb* dependence: *grn-1.1*-lacZ and *grn-hinge*-lacZ. Both were obtained from J. Castelli Gair-Hombria.

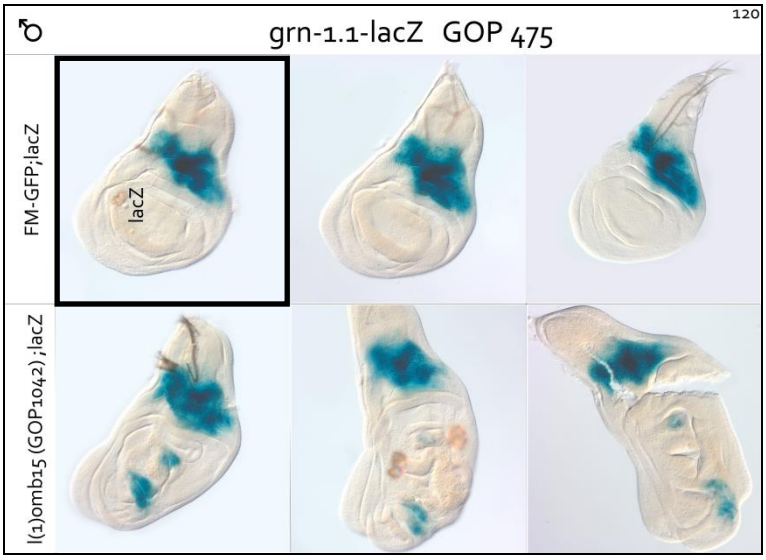
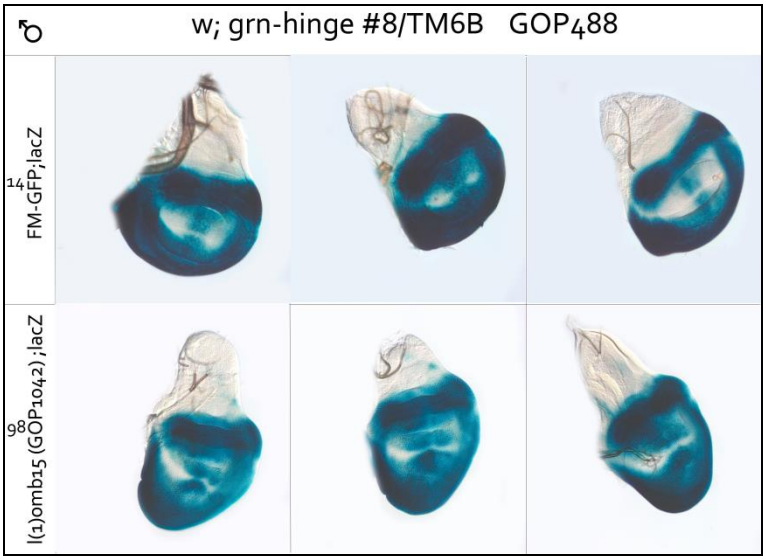


Figure 35. *grn-1.1-lacZ*expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain *grn1-1* after cross with *l(1)omb*^{15} mutant line (cross 120, appendix). Picture with frame: *grn-1.1-lacZ (*homozygous*).* 1st row: *FMGFP/Y*; *grn-1.1-lacZ/III*. 2nd row: *l(1)omb*^{15} */Y; grn-1.1-lacZ/III*. Wing disc expression: dorsal hinge expression domain in *FMGFP/Y* which in *l(1)omb*^{15} appeared unaffected but the appearance of a double dot staining pattern was detected in the malformed pouch region. Eye-antenna, leg disc: no staining observed in both genetic backgrounds (data not shown).

The *grn-1.1-lacZ* enhancer is a 1.7 kb fragment (chr3R:3,956,500 – 3,958,250, Castelli Gair-Hombría, pers. com.). It contains several well conserved potential STAT and Tbx binding sites. In *FMGFP* genetic background, *grn-1.1-lacZ* expression was detected dorsally to the wing pouch. In *l(1)omb*^{15} wisc discs, irregular expression spots were detected in the malformed pouch region. In eye antennal and leg discs, no *grn-1.1-lacZ* staining was observed in both the genetic backgrounds.



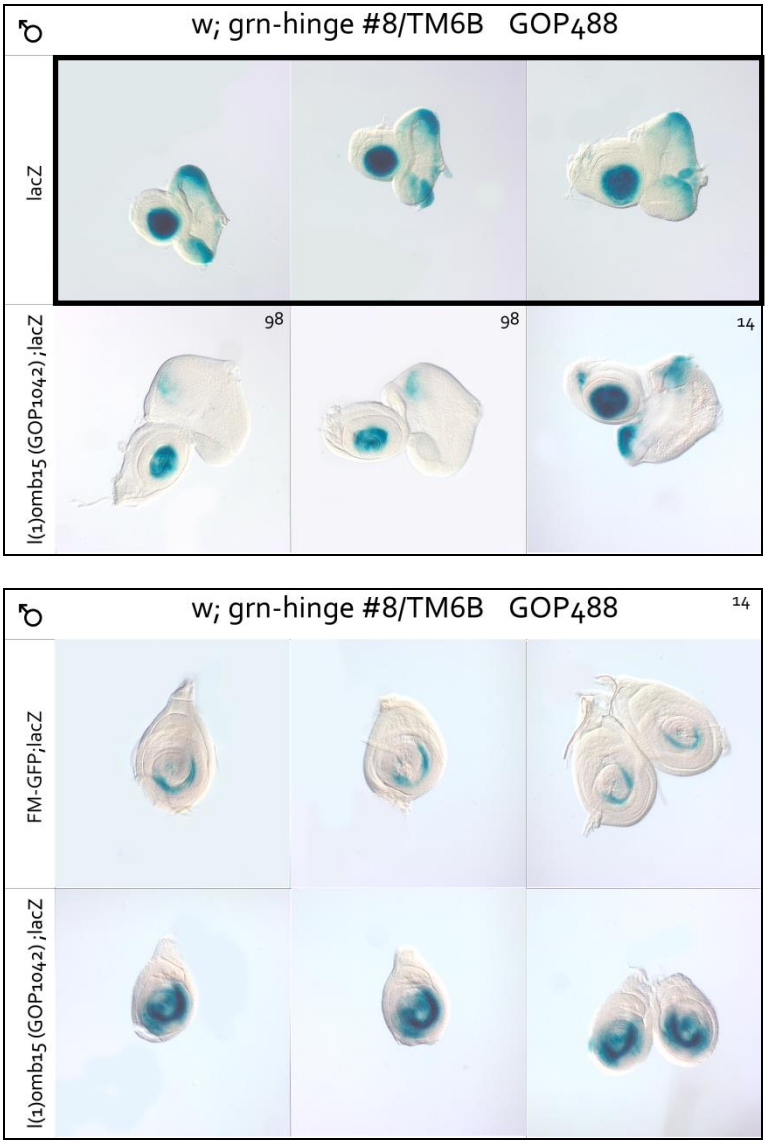


Figure 36. *grn-hinge-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain *grn-hinge* after cross with *l(1)omb*^{15} mutant line (crosses 14,98, appendix). Picture with frame: *grn-hinge-lacZ (*homozygous*)*. 1st row: *FMGFP/Y*; *grn-hinge-lacZ/III*. 2nd row: *l(1)omb*^{15} */Y; grn-hinge -lacZ/III*. Strong circumferential *FMGFP* wing disc expression seen in hinge and pleura region. Lateral margin *FMGFP* eye disc expression is reduced in *l(1)omb*^{15} discs, especially in the ventral margin. Concentric leg staining was observed in both genetic backgrounds.

The irregular pattern of *grn-1.1-lacZ* ectopic expression in *l(1)omb* suggests that the regulation is indirect. Possibly, *omb* loss in the pouch causes a fate change to dorsal hinge which stimulates *grn-1.1-lacZ* expression.

The *grn-hinge* enhancer is contained in a 646 bp fragment (chr3R:4,009,869-4,010,514, Castelli Gair-Hombría, pers. com.). In this fragment, the TBE positional weight matrix only identified a weak and poorly conserved potential TBE (score 5.59). In the wing disc it was active in an intense circumferential pattern. In FM-GFP discs it was outlined along the hinge and pleura region. The wing pattern was slightly altered in *l(1)omb* discs, which may

be due to changes in disc morphology. In the eye disc, *grn-hinge-lacZ* had strongest expression at the lateral margins towards the posterior part of the disc. In *l(1)omb* discs, the ventral aspect of the expression domain appeared reduced. Concentric leg staining was observed in both genetic backgrounds, The enhanced expression in the *l(1)omb*^{15} leg discs was not confirmed in an independent experiment.

3.1.11 *homothorax (hth)*

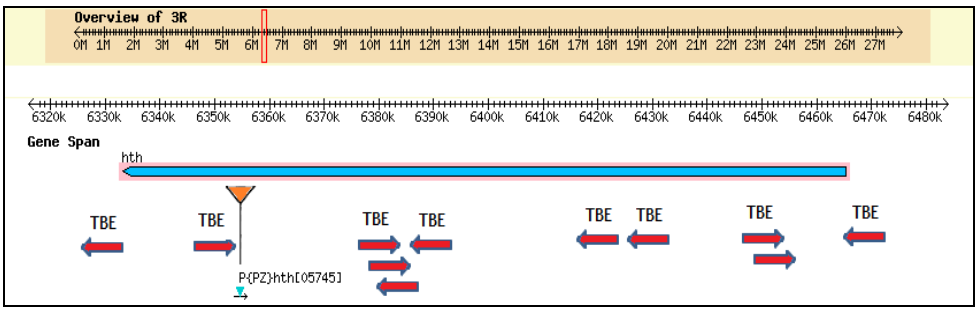


Figure 37**.** The P-element insertion 05745 (orange) and potential TBE sites (red horizontal arrows) in the *hth* locus.

homothorax encodes a conserved homeobox transcription factor [228] which is required for executing Hox functions. Hth plays a role in many important developmental processes like the patterning of the embryonic CNS [229], the eye [230],[231], the wing [232],[233], the leg [234] and the imaginal tracheal cells [235].

Homothorax, together with Extradenticle (Exd), forms a heterodimeric cofactor of the Hox proteins, conferring a higher degree of DNA binding specificity [236] thus, allowing them to recognize structural features of the DNA that cannot otherwise be read [237]. This sort of binding event occurs for example in cells that will give rise to proximal structures [238]. In imaginal discs, *hth* is associated with the regulation of proximo-distal axis development [239]. Hth is expressed in the proximal regions of the wing disc, including the presumptive hinge and notum, but not in the pouch region [230].

Vertebrate homologs of Hth are the Meis family proteins Meis1, Meis2 (Mrg1) and Meis3 (Mrg2). Meis proteins were first identified as co-factors of other homeodomain-containing proteins and play multiple roles in development and disease [240],[241].

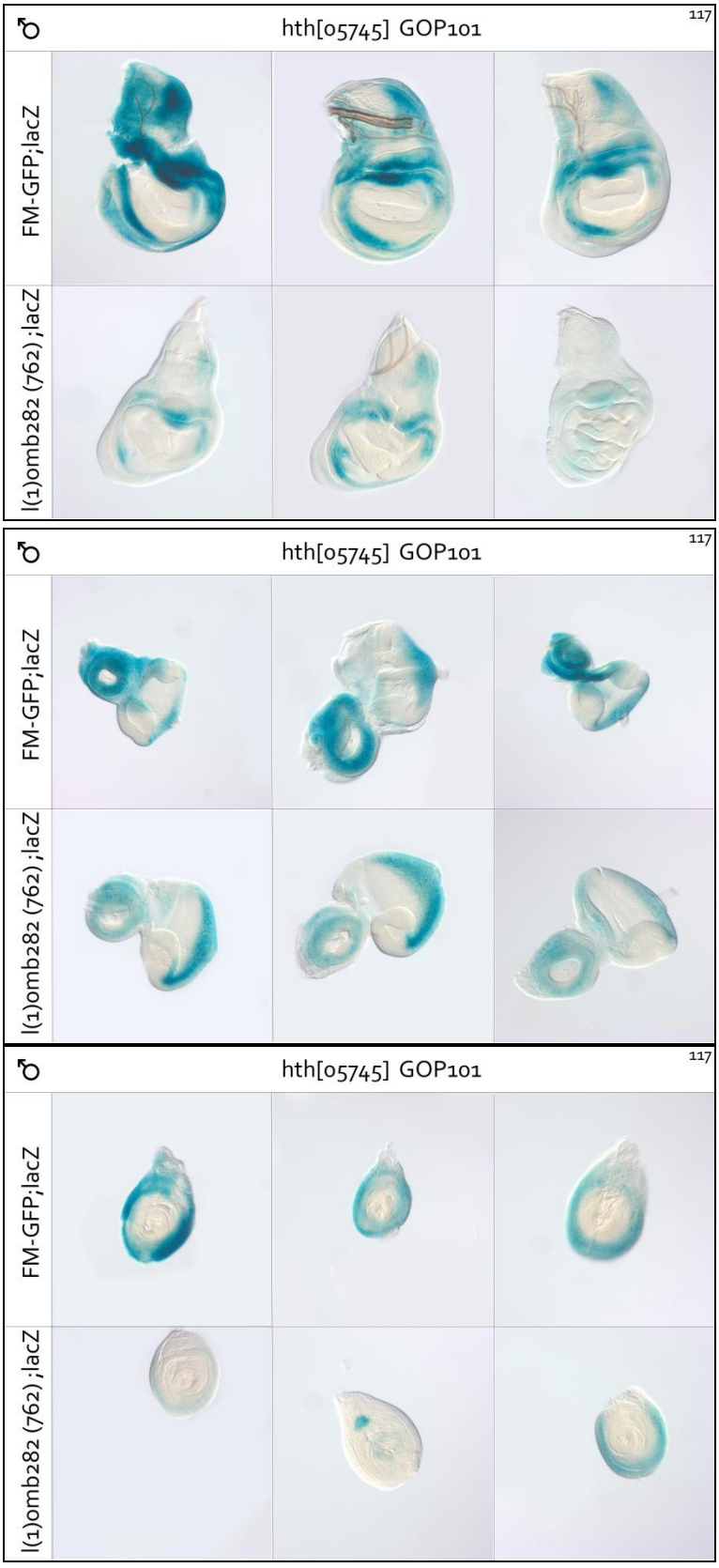


Figure 38. *l(1)omb* mutant effects on *hth*^{05745}*-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain *hth*^{05745} after cross with *l(1)omb*^{282} mutant line (cross 117, appendix). 1st row: *FMGFP/Y*; *hth*^{05745} *-lacZ/III*. 2nd row: *l(1)omb*^{282}*/Y; hth*^{05745} *-lacZ/III*.

In *FMGFP/Y*; *hth*^{05745} *-lacZ* wing disc expression occurredcircumferentially around the wing pouch. This wing pattern was slightly reduced in *l(1)omb* discs, including the notum in which *omb* is not expressed, suggesting different staining efficacies. In the eye disc, *hth*^{05745} *-lacZ* was expressed posterior to the morphogenetic furrow. In *l(1)omb* discs, that expression post MF expanded along the ventral margin. In the FMGFP leg discs a peripheral ring expression appeared which was not observed in a fraction of *l(1)omb* mutant discs.

In the eye disc, *hth*^{05745} *-lacZ* did not well reflect the endogenous *hth* expression pattern which covers the entire eye disc, excepting the morphogenetic furrow. Anterior to the morphogenetic furrow, Hth serves to promote proliferation and to repress retinal differentiation [242]. In *l(1)omb* mutants, the eye disc shows ventral overproliferation {Prof. Pflugfelder, unpublished data}. The ectopic *hth* expression along the ventral margin in *l(1)omb* may mediate this effect.

3.1.12 *neuralized (neur)*

neuralized is a transcription factor with a homeodomain and a C_3HC_4 zinc finger DNA-binding domain [244]. It is a member of the Notch pathway; an E3 ubiquitin ligase that regulates the endocytosis of the Notch ligands Delta and Serrate by ubiquitination [245],[246]. Mindbomb1, its functional homologue [247],[248] seems to have the same role in different cellular and developmental contexts [249].

Neur plays an important role in all three germ layers during embryonic development [250]. In addition, Neur is also required for the development of the adult central and peripheral nervous system [251],[252], being expressed in embryonic neural tissue and in the region of larval imaginal discs that will give rise to adult sensory organs [243].

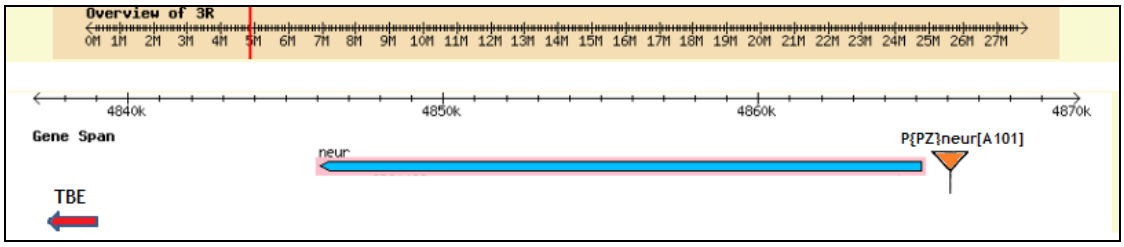


Figure 39**.** The potential TBE site (red horizontal arrow) and the P-element insertion A101 (orange, approximate location [243]) in the proximity of the *neur* locus.

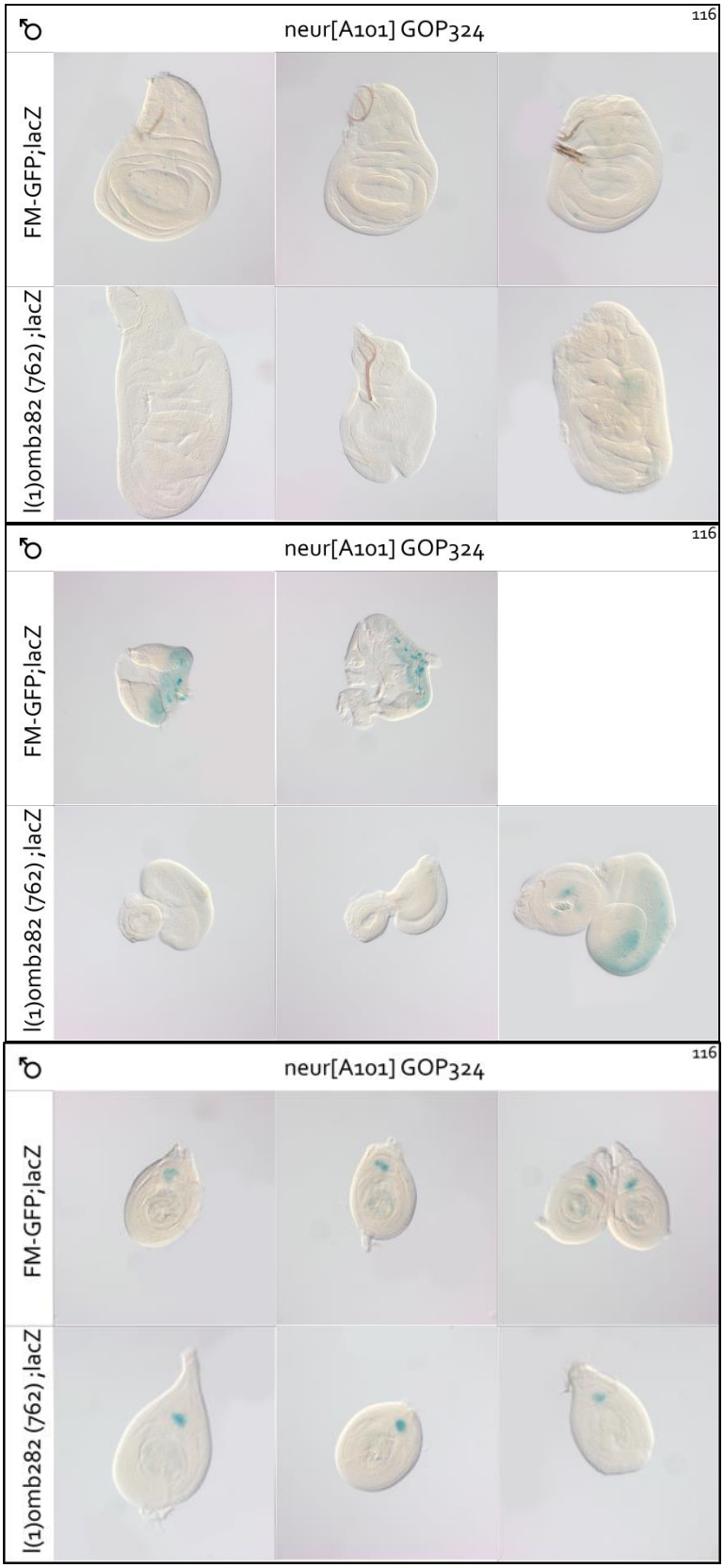


Figure 40. *neur-lacZ* imaginal disc expression.
X-Gal staining of L3 imaginal discs of the fly strain *neur*^{A101}, after cross with *l(1)omb*^{282} mutant line (cross 116, appendix). 1st row: *FMGFP/Y*; *neur*^{A101}*-lacZ/III*. 2nd row: *l(1)omb*^{282} */Y*; *neur*^{A101} *-lacZ/III*.

The presence of Neur in the signal-sending cell appears to be required for ligand endocytosis [249].. Apart from its role in Notch signaling, Neur was also recently shown to regulate epithelial cell polarity in the embryo [253].

Hardly any *neur-lacZ* expression was visible in the wing discs. In FM-GFP eye-antennal discs, $neur^{A101}$ eye pattern was faintly outlined posteriorly to the MF in presumptive glial cells. The latter expression appeared to be missing in $l(1)omb^{282}$ discs. In the leg disc, *neur-lacZ* was detected in a distinct disc region, presumably corresponding to the chordotonal organ. In *l(1)omb* discs, that leg expression domain appeared unaffected.

The same results were observed with the transgenic line $neur^{j6B12}$ (data not shown).

3.1.13 *retained / dead ringer (dri / retn)*

retained encodes a nuclear protein with a conserved DNA-binding domain [254] called the ARID (AT-rich interaction domain),[255].

During *Drosophila* embryogenesis, *retn* is initially involved in the dorsal/ventral and anterior/posterior axis formation, acting either as a repressor or an activator depending on the developmental context [256],[257]. *retn* is required for the formation of a functional CNS. Its expression in a subset of glial cells is part of the cascade of transcriptional regulation that occurs during glial cell differentiation. Post-embryonically, *retn* is expressed in a limited set of neurons in the CNS and eyes. *retn* mutant cells show pathfinding errors in photoreceptor and suboesophageal neurons [258]. *retn* is also essential for myogenesis and hindgut development [259].

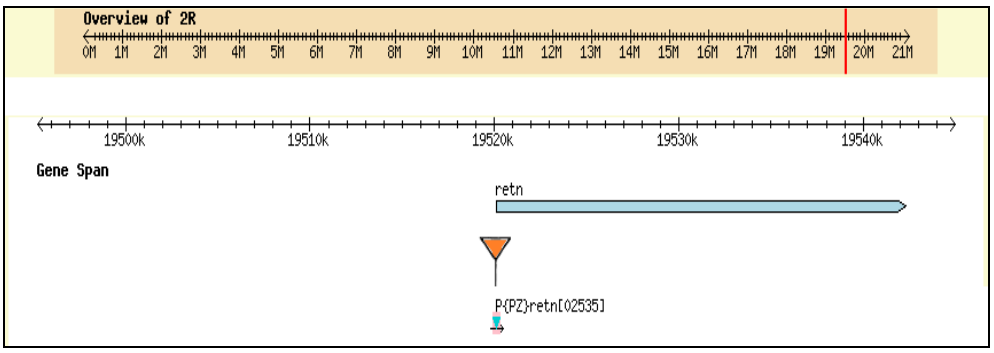


Figure 41. The P-element insertion 02535 (orange) upstream of the *retn* locus.

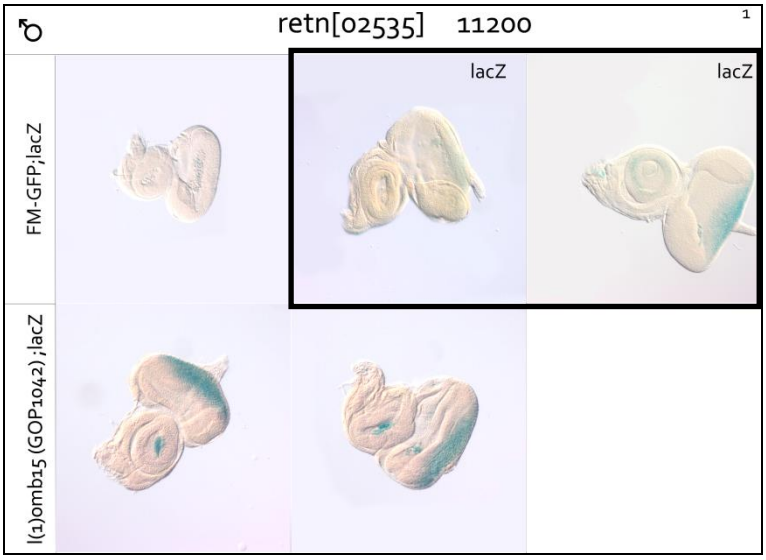


Figure 42. *retn-lacZ* imaginal disc expression.
X-Gal staining of L3 imaginal discs of the fly strain retn^{02535}, after cross with *l(1)omb^{15}* mutant line (cross 1, appendix). Picture within frame: retn^{02535} -lacZ (homozygous). 1st row: *FMGFP/Y*; *retn^{02535}-lacZ/II.* 2nd row: *l(1)omb^{15} /Y*; *retn^{02535} -lacZ/II*.

In both genetic backgrounds, no *retn-lacZ* expression was detected in the wing and leg discs. In the *FMGFP/Y* eye discs *retn^{02535}-lacZ* was detected posteriorly to the MF, being more robust on the homozygous discs. In the *l(1)omb^{15}* eye discs the expression appeared unaffected.

3.1.14 *Syndecan (Sdc)*

Syndecans are transmembrane heparan sulfate proteoglycans (HSPGs) known to interact with a diversity of extracellular ligands, often in combination with other cell surface receptors [260]. They are supposed to play a dual role in adhesion and as regulators of signaling from the extracellular matrix [261]. Vertebrates have four *Syndecans* that differ in their tissue distribution, co-receptors, and intracellular signals. All four have a similar carboxyl terminal PDZ binding domain and a Src/Fyn binding domain [262]. The *Drosophila* homolog, Sdc, shares conserved intracellular sequences. This structural conservation is sufficient for human Syndecan-2 to rescue *Drosophila sdc* function [263].

Sdc, contributes to axon guidance by acting as a co-receptor with Robo for the repellent signal Slit [264],[265], enhancing Robo repulsive signaling [266]. Sdc, mantains as well the secreted Slit, on the axons of the developing nervous system [265], assuring ordered directional migration and branch fusion.

Sdc acts also as a ligand of LAR, a tyrosine phosphatase receptor [267]. Furthermore, Syndecan acts as a co-receptor for Slit in the *Drosophila* heart [268].

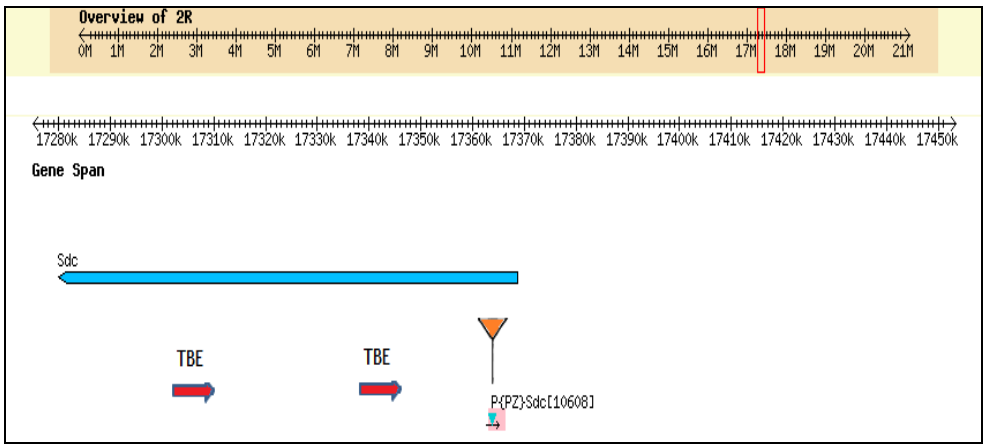


Figure 43. The potential TBE sites (red horizontal arrows) and the P-element insertion 10608 (orange) in the *sdc* locus.

Figure 44. *sdc-lacZ* imaginal disc expression.
X-Gal staining of L3 imaginal discs of the fly strain Sdc^{10608}, after cross with $l(1)omb^{15}$ mutant line (cross 108, appendix). 1st row: Sdc^{10608}*-lacZ* (homozygous). 2nd row: $l(1)omb^{15}$ */Y*; Sdc^{10608} *-lacZ/II*.

Homozygous Sdc^{10608}*-lacZ* wing and leg disc expression occurred quite ubiquitous. This wing pattern appeared to be lost in *l(1)omb* discs presumably due to heterozygosity of the lacZ construct.

3.1.15 *sloppy paired 2 (slp2)*

The *sloppy paired* gene cluster embraces two transcription units, *slp1* and *slp2*, both of which encode transcription factors with a forkhead domain [269]. As secondary pair-rule genes [270], they act, during segmentation of the germ band, downstream of primary pair-rule genes [271]. Expressed in 14 stripes in the embryo, *slp* expression supports the maintenance of the the parasegment boundary, preventing the expansion of the *en* stripes anteriorly into the *slp* domain, and retaining *wingless* expression in the *slp*-positive cells [272].

slp1 and *slp2* are expressed in the same striped pattern, with *slp2* expression starting a little later. Slp1 can rescue most of the segmentation defects caused by a deficiency of the entire *slp* locus [270], suggestive of *slp2* as not necessary for segmentation. About 2 kb 5′ of *slp2* several neuronal cis-regulatory modules have been identified [273].

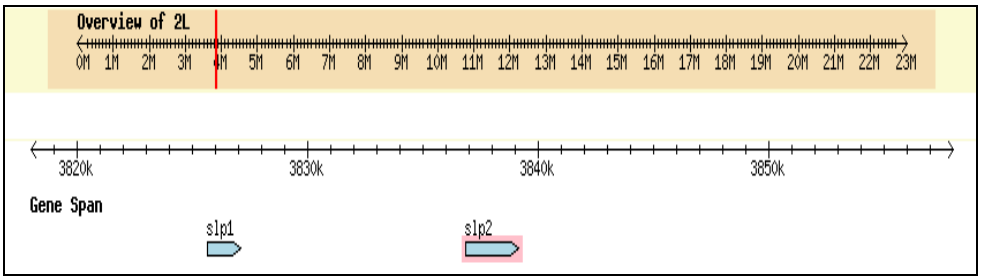


Figure 45. The *slp* locus.

In the *Drosophila* eye disc the *slp* complex is ventrally expressed, essential for the growth and survival of cells in the ventral region of the disc. In the third larval instar, the morphogenetic furrow begins to progress across the presumptive retina and *slp* transcription is located in post-furrow regions. However, *lacZ* reporters show persistent expression posterior to the MF, a likely consequence from perdurance of the long-living *lacZ* gene product [274].

The *slp2-lacZ* fly strain characterized below contains an enhancer trap insertion (MP8) just upstream of the *slp2* transcription unit. The line was obtained in a jump-starter induced mobilization from an X chromosome containing the PlacW insertions G0071 and G0099. The new insertion was noted because of its unusual eye pigmentation pattern, restricted to the anterior ventral quadrant, and was molecularly characterized {M. Porsch, 2002 doctoral thesis}.

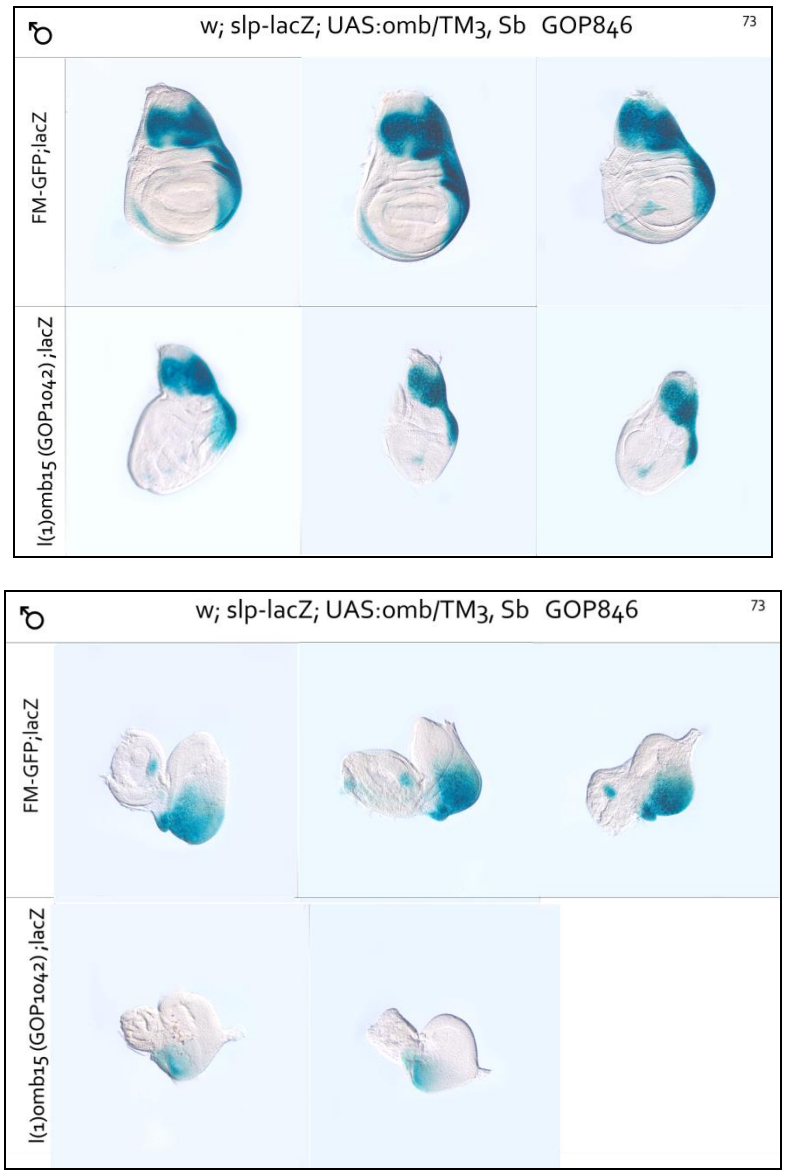


Figure 46. *slp2-lacZ* imaginal disc expression.
X-Gal staining of L3 imaginal discs of the fly strain *slp2,* after cross with *l(1)omb*^{15} mutant line (cross 73, appendix). 1st row: *FMGFP/Y*; *w; slp2-lacZ /II.* 2nd row: *l(1)omb*^{15} */Y*; *slp2-lacZ /II*.

In the *FMGFP/Y* genetic background, *slp2-lacZ* expression was observed in the wing notum and a posterior disc margin territory along with an anterior ventral pleura stripe. The expression was also detectable in the eye discs in the ventral half, in the antennal discs in a small patch of cells (Fig. 46), whereas the leg discs were unstained (data not shown).

In *l(1)omb*^{15} wing discs, expression along the ventral posterior disc margin and the anterior ventral pleura appeared reduced. The eye pattern looked reduced in extent and intensity while the antennal dot appeared to be missing. In all cases, the differences could be a consequence of the younger age of the mutant discs.

3.1.16 Signal-transducer/activator of transcription protein at 92E (Stat92E)

The evolutionarily conserved janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway controls responses to hematopoietic cytokines that coordinate inflammatory and immune responses in mammals, being of great importance in human diseases such as several types of human cancer, leukemia and myeloproliferative disorders [275],[276]. Vertebrates have seven STAT proteins, STAT1-4, 5a, 5b, and 6. They all have a conserved domain structure, including N-terminus, coiled-coil, DNA-binding, linker, SH2, and C-terminus [277],[278].

Drosophila JAK/STAT signaling pathway is conserved in evolution, with only one JAK, the gene *hopscotch* [279] and one STAT transcription factor, *Stat92E* [280]. It is involved in several processes, such as embryonic segmentation, larval hematopoiesis, regulation of stem cell maintenance and cellular proliferation. In addition, *Drosophila* JAK/STAT signaling is essential for immune and stress responses [281].

JAK/STAT pathway activation is mediated by secreted cytokine-like molecules *unpaired* or *outstretched (upd/ os), unpaired2* (*upd2), unpaired*3 (*upd3)* [282],[283], which bind to the transmembrane receptor *Domeless (Dome)* [284]. In the canonical model of JAK/STAT signaling cascade, ligand binding induces a change in the receptor leading to activation of the linked JAKs, which then phosphorylate the receptors tyrosine residues, thus creating docking sites for STATs. STATs subsequently dimerize and translocate in the nucleus, where they bind their target sites in DNA and act as transcriptional activators [285].

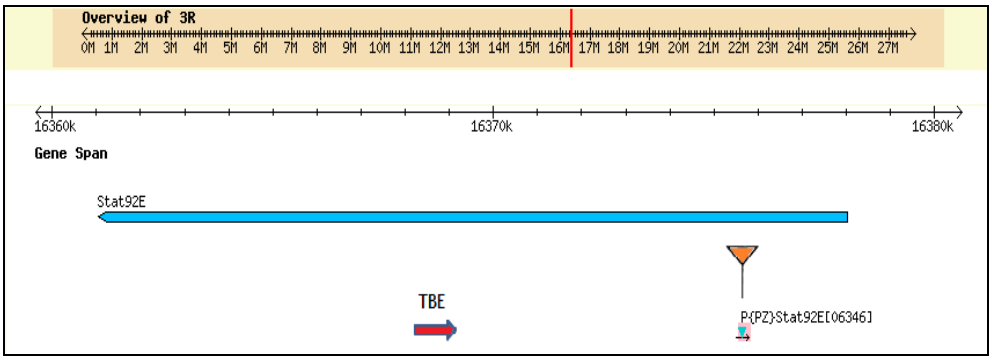


Figure 47. The potential TBE site (red horizontal arrow) and the P-element insertion 06346 (orange) in the Stat92E locus.

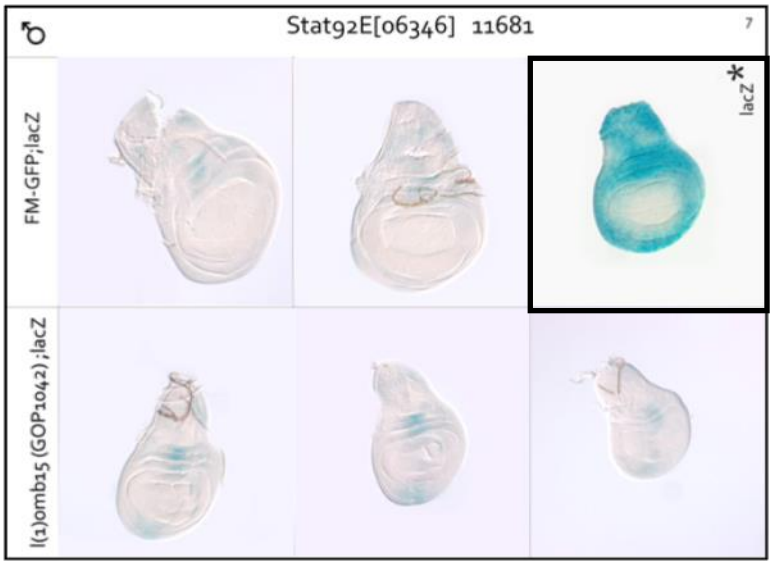
The JAK/STAT pathway is known to regulate vein formation in the wing blade. Abnormalities have been previously observed, including ectopic wing veins flanking distal L5 in JAK/STAT pathway mutants [280].

In the eye disc, the JAK/STAT pathway, during the first to early second instar larval stages, is activated ventrally, slightly broader than the region of Upd expression [286] and during the second to early third instar larval stages, in the entire eye disc [287]. Overexpression of the JAK/STAT signaling causes a large-eye phenotype [288], whereas, MF initiation is suppressed when the JAK/STAT pathway is down-regulated [289].

In the wing disc, the homozygous strain *Stat92E*^{06346}*-lacZ* exhibited staining which covered the entire disc with exception of pouch. LacZ expression in the wing disc of heterozygous larvae was faint and it was not expanded in the *l(1)omb*^{15} mutant background (Fig. 48).

Leg disc staining, consisting of a small set of cells in the center of the disc, did not differ between both genetic backgrounds (Fig. 48).

In the eye disc, *Stat92E*^{06346}*-lacZ* activity showed a gradient that was highest at the poles and decreased to an undetectable level at the DV midline. LacZ was also expressed in the ocellar region. In *l(1)omb* discs, only the dorsal margin expression remained. The wild-type expression pattern confirms previous observatons. However, in situ hybridization experiments undertaken with probes specific for the *Stat92E* transcript showed ubiquitous expression of *Stat92E* mRNA in third instar eye discs, suggesting that this enhancer detector might only report a subset of *Stat92E* transcript expression [290] (Fig. 48).



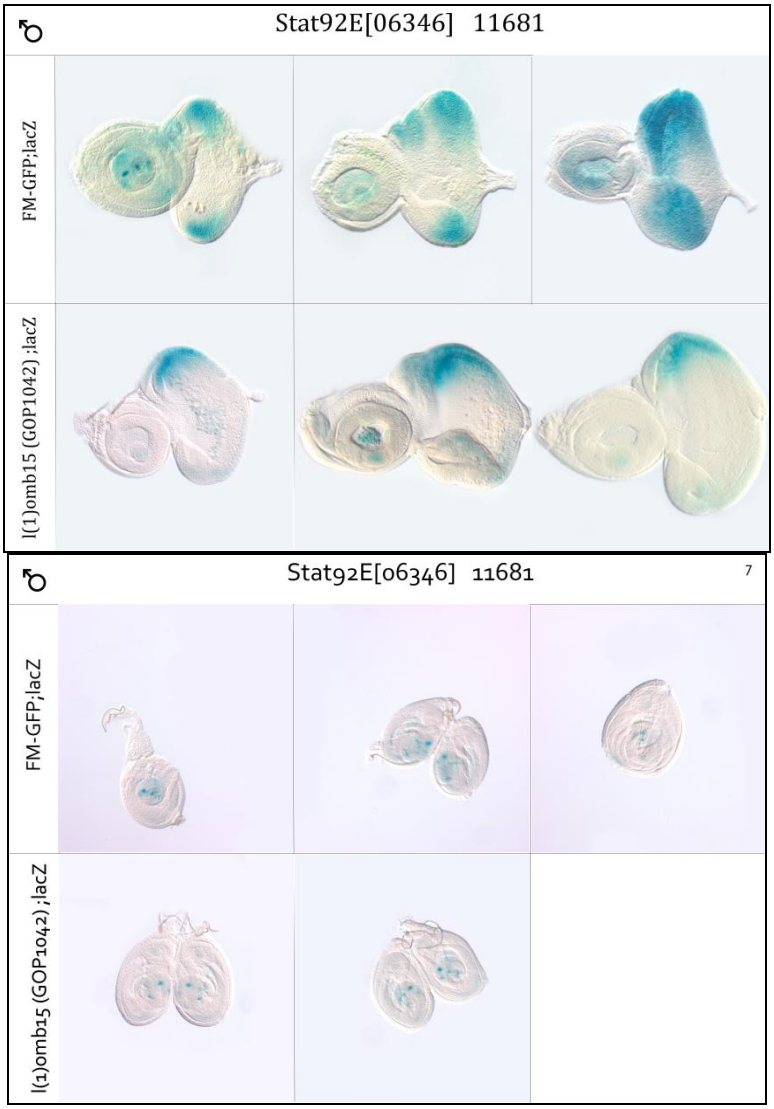


Figure 48. Stat92E-lacZ imaginal discs expression.
X-Gal staining of L3 imaginal discs of the fly strain *Stat92E*^{06346}, after cross with *l(1)omb*^{15} mutant line (cross 7, appendix). Picture within frame: *Stat92E*^{06346} -lacZ (homozygous). 1st row: *FMGFP/Y*; *w; Stat92E*^{06346}*-lacZ /III.* 2nd row: *l(1)omb*^{15} */Y*; *Stat92E*^{06346}*-lacZ /III*.

3.1.17 *wingless (wg)*

The Wnt pathway is a highly conserved signalling cascade, involved in several transcriptional and cellular responses [291],[106]. Wg acts as a morphogen activating a set of pathway-specific target genes in a gradient-dependent manner [292].

In the absence of Wg, cytoplasmic Armadillo (Arm), the *Drosophila* orthologue of β-catenin, is phosphorylated and targeted for degradation. Wg signaling stabilizes Arm, causing its cytosolic accumulation and translocation to the nucleus. There Arm relocates co-repressors such as Groucho and binds to lymphoid enhancer/T-cell transcription factor

(TCF, *Drosophila*: Pangolin, Pan), switching it from a repressor to a transcriptional activator and activates Wg target gene expression [293].

In addition to the main components of the pathway, there are further regulatory influences on Wnt pathway activity by members of other signalling pathways, such as the Hedgehog and Notch pathways [294]. Furthermore, *nkd* is a feedback inhibitor of Wg signaling in flies [295]. It is induced by Wg and interacts with Dishevelled to antagonize Wnt signal transduction [296].

In the leg disc, Wg is necessary, along with Dpp, for cell fate specification along the PD axis through direct activation of *Dll*, which is essential for leg development [214]. In the wing disc, *wg* is expressed in a narrow stripe along the DV boundary and a pair of stripes aroung the pouch. Wg overlaps with Dpp in the center of the wing pouch. Moreover, Wg is essential for wing pouch size regulation via *vestigial* [297], partly by inhibiting the apoptotic process [298],[299] and the epithelial cell-cell adhesion through the activation of *shotgun* [300], the *Drosophila* homolog of E-cadherin.

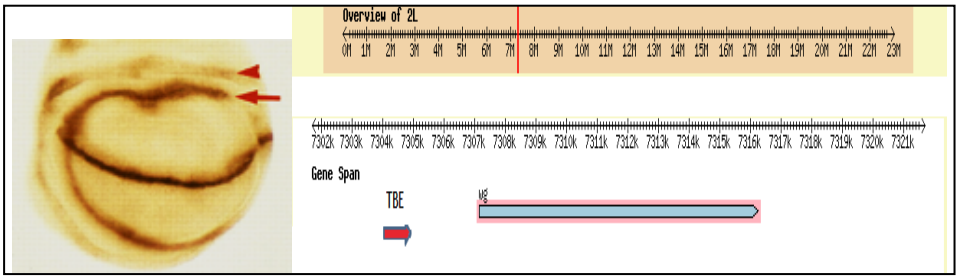
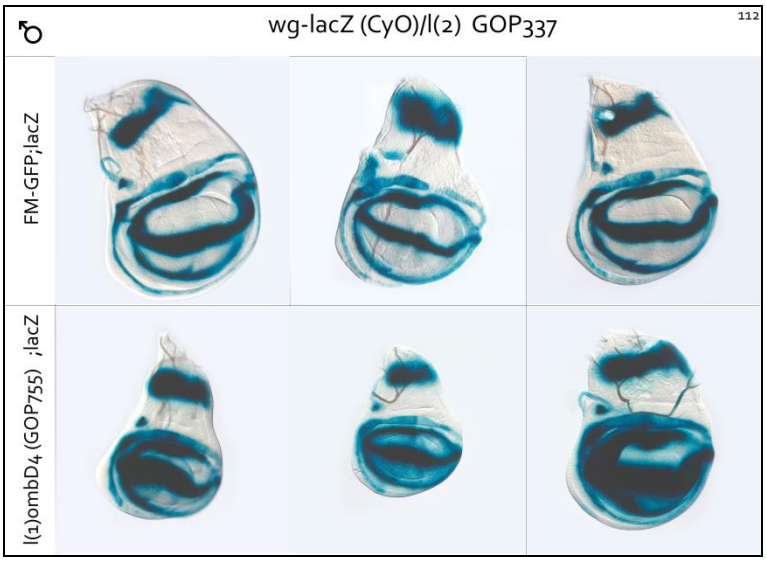


Figure 49. *wingless* wing disc expression and genetic locus.
Left: *wg* expression detected anti-Wg antibodyin the wing pouch [301]. Red arrows indicate the inner ring (IR), the arrowhead indicates the outer ring (OR). Right: The potential TBE site (red horizontal arrow) upstream of the *wg* locus.



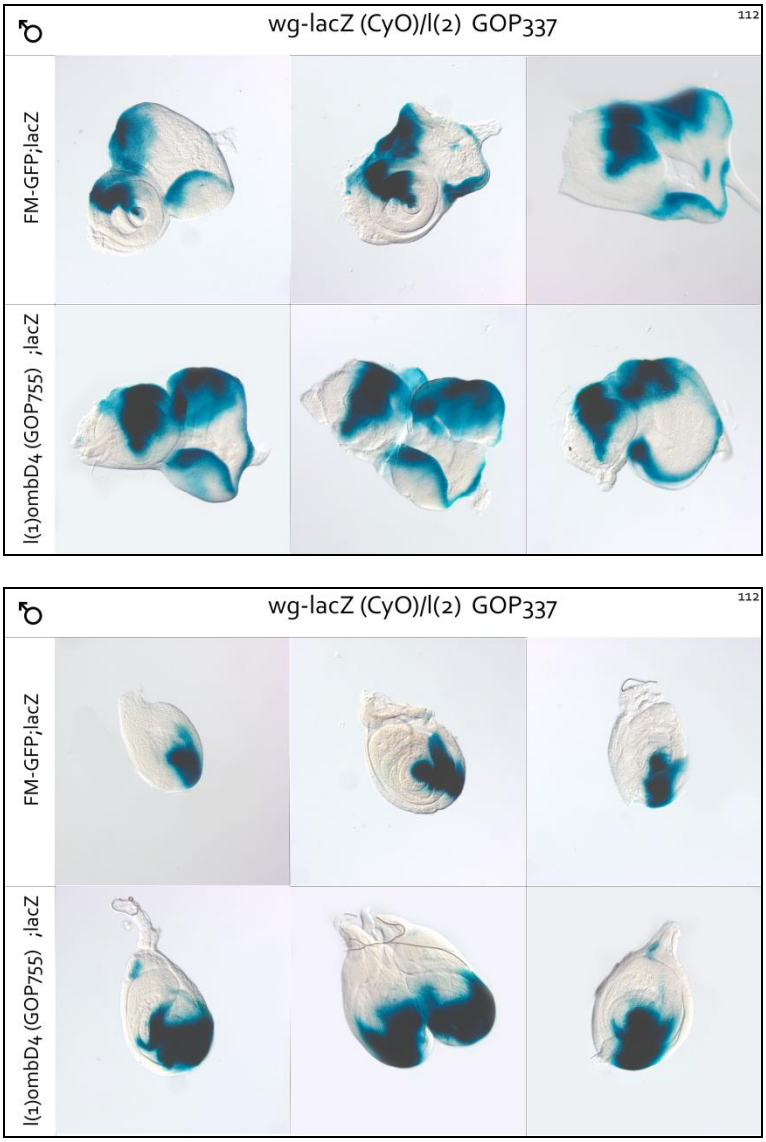
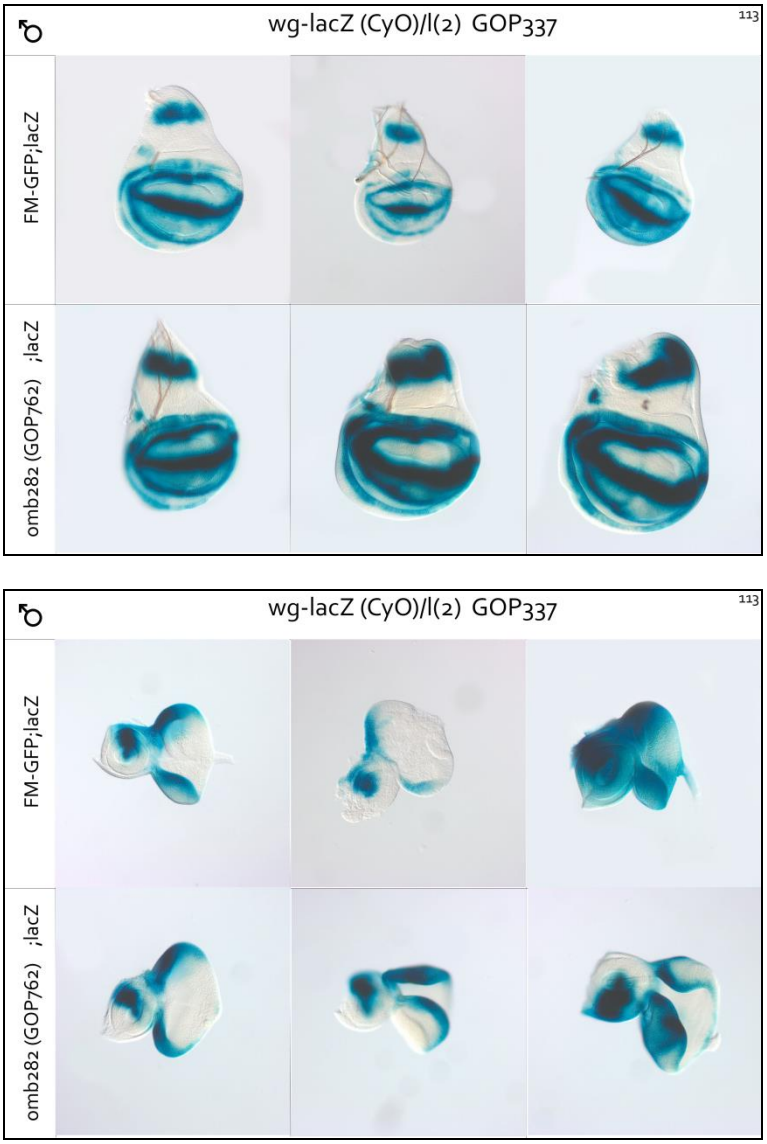


Figure 50. *wg-lacZ* imaginal disc expression.
X-Gal staining of L3 imaginal discs of the fly strain *wg-lacZ (CyO)/l(2),* after cross with *l(1)omb*^{D4} mutant line (cross 112, appendix). 1st row: *FMGFP/Y*; *wg-lacZ /II.* 2nd row: *l(1)omb*^{D4} */Y*; *wg-lacZ II*.

wg-lacZ refers to a lacZ-enhancer trap insertion in the wg gene located on the CyO balancer chromosome {obtained from Koni Basler, Zürich}. In the *FMGFP/Y* genetic background, *wg-lacZ* expression was detected in the expected territories. In a distinct D/V border cells stripe in the wing disc pouch and in an inner and outer ring in the surrounding hinge primordium (Fig. 50,51). *wg-lacZ* was also observed along the ventral and dorsal margins of the eye imaginal disc, where it is known to antagonize the Dpp-mediated morphogenetic furrow formation [302], (Fig. 50,51). In the leg disc, a subset of *wg-lacZ* cells appeared ventrally.

In neither *l(1)omb*^{D4} and *l(1)omb*^{282} discs obvious changes in *wg-lacZ* expression were observed (Fig. 50,51).

It is already known that when *wg* (or the *dpp*) signaling pathway is compromised, *omb* expression is lost [24]. In a previous study *wg* was detected misexpressed in the ventral compartment of *omb*^{3198} wing discs [146]. However, by blocking JNK-mediated apoptosis, though puc expression, *wg* expression reverted to wild-type.



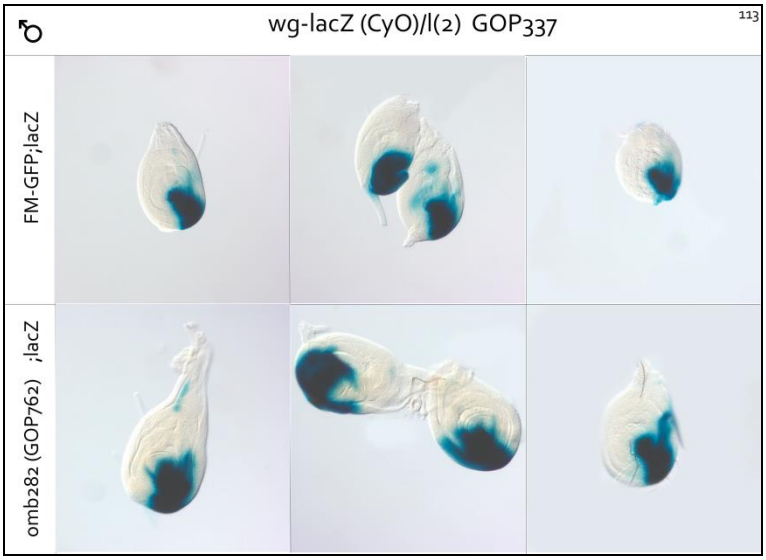


Figure 51. *wg-lacZ* imaginal disc expression.
X-Gal staining of L3 imaginal discs of the fly strain *wg-lacZ (CyO)/l(2),* after cross with *l(1)omb*^{282} mutant line (cross 113, appendix). 1st row: *FMGFP/Y*; *wg-lacZ /II.* 2nd row: *l(1)omb*^{282} */Y*; *wg-lacZ/II*.

3.2 Potential Omb target genes- Part II

Some of the investigated potential target genes were analysed in more detail.

3.2.1 *vestigial*: *vg* Boundary Enhancer (vgBE)

Following segregation of the *Drosophila* wing imaginal disc into dorsal and ventral compartments, *vestigial* activity promotes the formation of wings [303]. Along with the transcription factor Scalloped [304],[305], an active complex is formed that binds to specific DNA sequences and regulates gene expression in cooperation with several signaling pathways in a tissue-specific manner [306].

vg expression is driven by three known distinct inputs: short-range DSL (Delta/Serrate/LAG-2)-Notch signaling across the DV compartment boundary, long-range Wg signaling from the DV boundary cells and a short-range signal sent by *vg*-expressing cells arriving at the neighboring cells to upregulate *vg* in response to Wg. These inputs define a feed-forward mechanism of *vg* autoregulation that initiates in D-V border cells and propagates from cell to cell by reiterative cycles of *vg* upregulation [297]. This mechanism is required for normal wing growth and is mediated by two distinct enhancers in the *vg* gene. The boundary enhancer (BE), that provides low levels of Vg in most or all cells of the wing disc and the quadrant enhancer (QE), which is activated by the combined action of

Wg and the short-range *vg*-dependent entraining signal, but only if the responding cells are already primed by low-level Vg activity. Wg controls the expansion of the wing primordium following D-V segregation by fueling this autoregulatory mechanism [297].

The vgBE is first expressed during the second instar. Its expression pattern is similar to that of the Vestigial protein at this stage. vgBE encloses regions necessary for its activity directly upstream of the enhancer [303],[307]. At the beginning of the third instar, vgBE is expressed along the distal region of the wing disc, overlapping with the expression of *wingless*, while it also shows some activity in the neighboring notal regions of the wing disc [308].

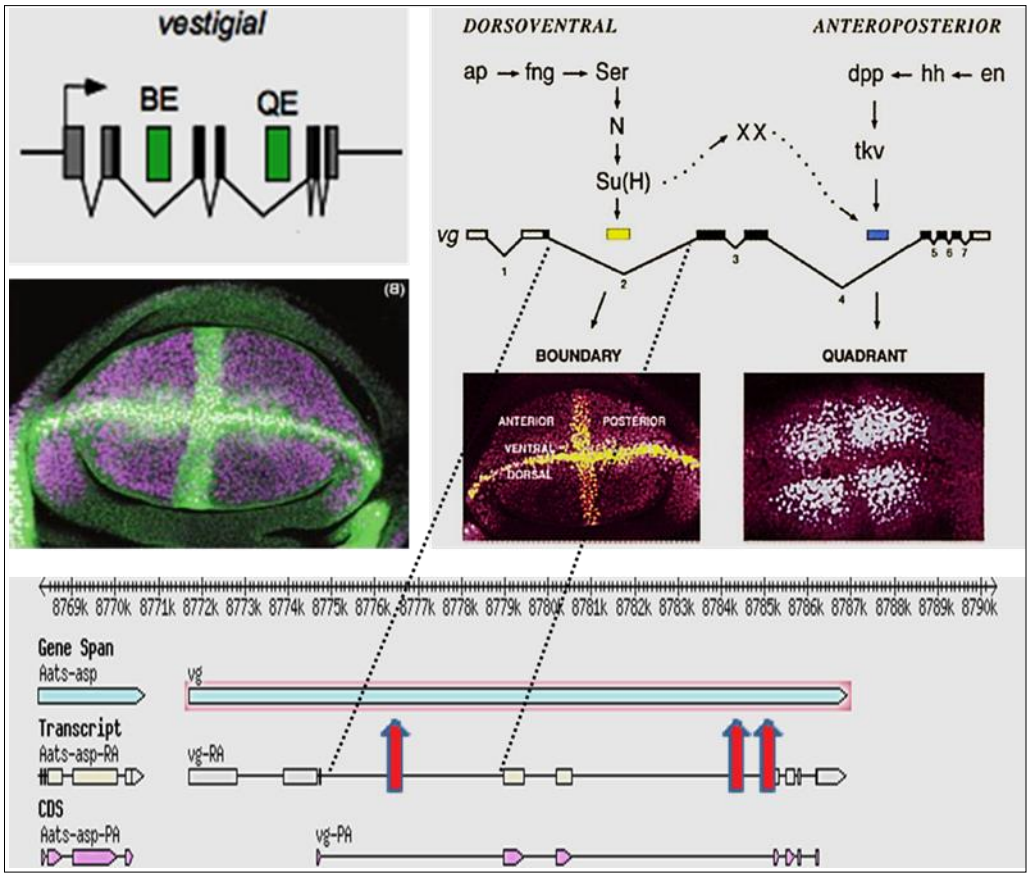


Figure 52. The vgBE in the second intron of the *vestigial* locus.
The position of the two *vestigial* enhancers {IMBA, Leonie Ringrose, online data} in the gene locus and their expression in the 3rd instar {Genetics, Daniel L. Hartl, 2011} wing disc are indicated. Potential TBE sites in the *vg* locus (red arrows).

A vgBE enhancer reporter line was characterized in In l(1)*omb*^{15} mutant background. No vgBE expression was detected in the eye antennal and the leg discs (data not shown). In FMGFP discs, the expected vgBE pattern was observed, along the DV and the wing A/P margins as well as more weakly along the AP boundary. In l(1)*omb*^{15} mutants, the medial

vgBE expression pattern was strongly reduced (Fig.53). The role of *omb* on this effect was further investigated by mutating a potential vgBE TBE site.

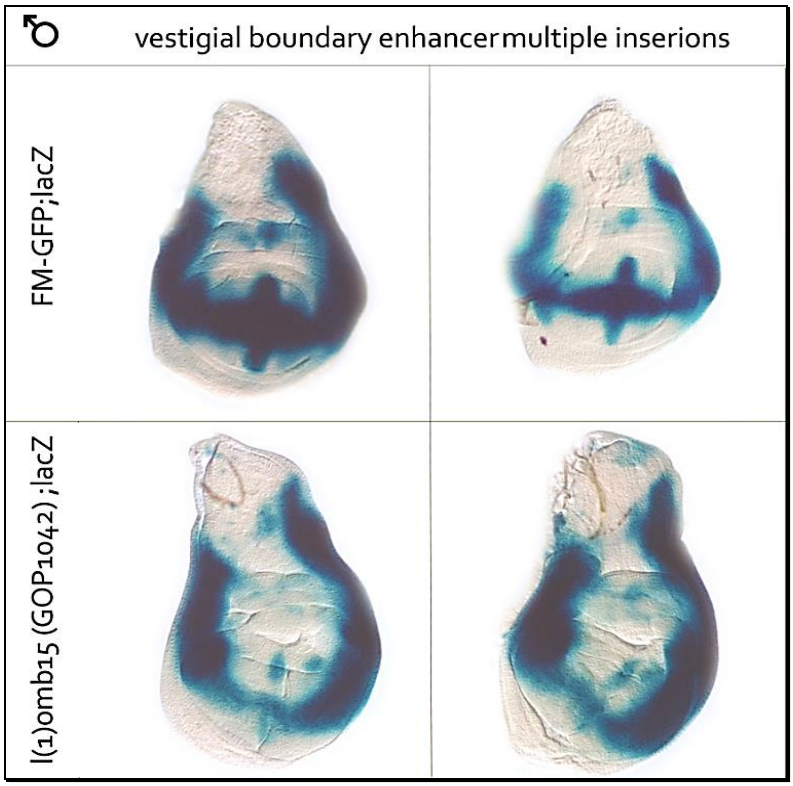


Figure 53. vgBE *-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain vgBE *-lacZ,* after cross with *l(1)omb*^{15} mutant line (cross 29, appendix). 1st row: *FMGFP/Y;* vgBE *-lacZ/II* . 2nd row: *l(1)omb*^{15} */Y*; vgBE *-lacZ/II.* In *omb* mutants the medial vgBE pattern was strongly reduced, at least the A/P part of that.

A 1055bp DNA fragment, encompassing the canonical 754 bp vgBE [303] was subcloned (for more details, see Appendix) into the pGWattBlacZ vector. This fragment (vgBE [1.1Kb], vgBE^{wt}) contains a weak predicted TBE which is only partly conserved among the 12 sequenced *Drosophila* species, unlike the Su(H) binding site which shows total conservation. The activity pattern of this fragment clearly deviated from the canonical vgBE indicating that the additionally included sequences exert a repressive effect predominantly on the D/V boundary of the pouch. Since the 1055bp fragment drove little expression along the pouch D/V boundary the expression in the *l(1)omb* background was not informative. The new expression in the eye-antennal imaginal disc presumably is an artifact often observed with the pGWattBlacZ vector integrated at the landing site 58A.

The potential TBE sequence was mutated and the new enhancer reporter construct was also introduced into the pGWattBlacZ vector. Transgenic flies carrying the corresponding

construct at the same insertion position were created (vgBE [1.1Kb], with TBE mutagenesis, vgBE$^{TBE\text{-}MUT}$) and characterized by X-gal staining.

For the below crosses *l(1)omb*^{DP6} was used, a presumptive null allele of *omb* obtained by imprecise P-element jump-out of the enhancer trap insertion omb^{P6} located upstream of *omb* exons Ib and II [309].

In *FMGFP* wing, vgBE^{TBEmut}*-lacZ* expression was detectable along the DV but not along the AP boundary, a pattern that was little affected in a *l(1)omb* mutant background. The *FMGFP* eye antennal discs looked as in vgBE^{wt}, showing expression along the ventral disc, anteriorly to MF and to the antenna (Fig. 54, 55).

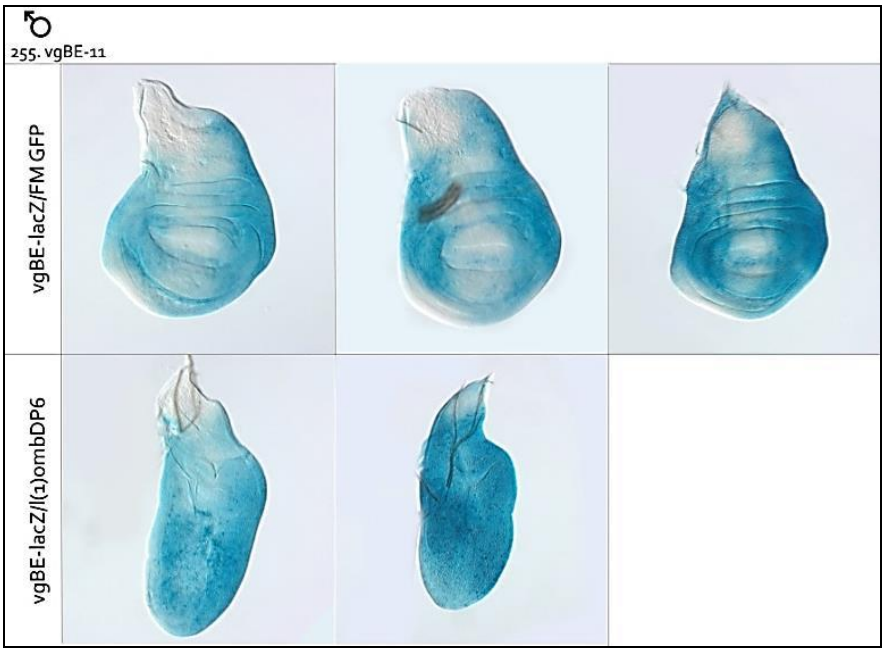
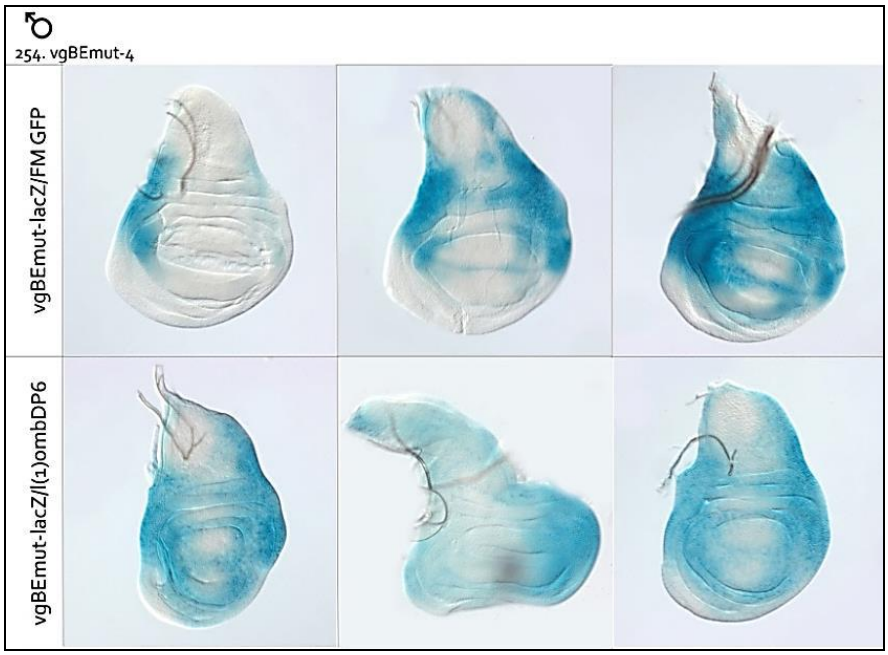


Figure 54. Imaginal disc expression pattern of the vgBE^{wt} in wt and *omb* mutant backround.
X-Gal staining of L3 imaginal discs of the fly strain vgBE^{wt} *-lacZ,* after cross with *l(1)omb*^{DP6} mutant line (cross 255, appendix). 1st row: *FMGFP/Y;* vgBE^{wt} *-lacZ/II* . 2nd row: *l(1)omb* ^{DP6} */Y*; vgBE^{wt} *-lacZ/II.*



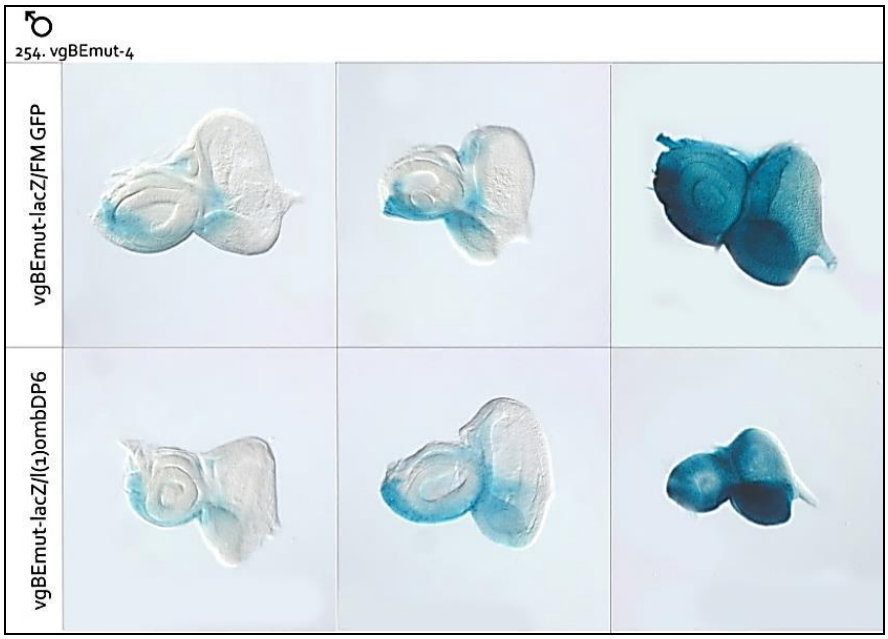


Figure 55. Imaginal disc expression pattern of the vgBE$^{\mathbf{TBEmut}}$ in wt and *omb* mutant backround.
X-Gal staining of L3 imaginal discs of the fly strain vgBE$^{\mathbf{TBEmut}}$ *–lacZ,* after cross with *l(1)omb*^{DP6} mutant line (cross 254, appendix). 1st row: *FMGFP/Y;* vgBE$^{\mathbf{TBEmut}}$ *-lacZ/II* . 2nd row: *l(1)omb* ^{DP6} */Y*; vgBE$^{\mathbf{TBEmut}}$ *-lacZ/II*. Wing disc: the *wt* vgBE^{TBEmut} pattern, which is detectable along the DV but not along the AP boundary, appears unaffected in the *omb* mutants. Eye antennal disc: the *wt* vgBE^{TBEmut} pattern appears faint but it is still possible to see the ventral eye expression, also detectable in the *omb* mutants.

Although the initial study of the original vgBE construct had shown that its activity depended on *omb* in the pouch region (Fig. 53), the results with the larger construct were non-conclusive.

vgBE was previously observed not to be affected in omb^{3198} clones [146]. Since I observed omb dependence in *l(1)omb* mutant discs, it is likely that *omb* is required for full activation of the original vgBE but not maintenance of its activity.

3.2.2 *spalt major (salm/ sal)*

SALL proteins are zinc-finger transcription factors conserved in metazoans. *Drosophila spalt major* and *spalt-related*, the first *SALL* described genes [310], participate in several developmental processes. *SALL* proteins are involved in various biological processes such as organogenesis, carcinogenesis and the maintenance of pluripotency in embryonic stem cells [311].

In vertebrates, there are four homologous genes, *SALL1–4,* defined as transcriptional repressors [312]. The importance of this family of proteins for human development and health is apparent from mutations that are linked to different inherited diseases characterized by abnormalities [313],[314] reminiscent of the limb and nervous system defects in *Drosophila sall* mutants. Sal, along with Salr, is necessary for the determination of neuronal fate in the peripheral nervous system [315] and regulate the expression of cell adhesion and cytoskeletal proteins in the central nervous system [316].

In the *Drosophila* wing imaginal discs, *sal* contributes to the wing growth and vein formation under the control of the TGFβ/Dpp pathway [317],[318]. The expression of *sal* in the wing blade, occurs in a broad central domain that ranges from the L2 provein to the L5 provein [319] (Fig. 57). Sal acts as a transcriptional repressor in *Drosophila* cells where it repress transcription through an AT-rich sequence [320]. *sal* genes show a complex pattern of expression regulated by independent enhancer regions [321],[319].

The *sal* wing blade-specific enhancer includes all the information needed to generate the *sal* expression domain in the wing. This enhancer contains Brk binding sequences, responsible for the repression of *sal* in the lateral regions of the wing, and activator sequences that drive reporter gene expression in the *sal* domain. The activation and repression regions do not overlap. Med and Sd bind to the activation region, although mutations in their binding sites do not abolish reporter expression. Moreover, endogenous *sal* is still expressed in wing discs mutant for *vg* [319].

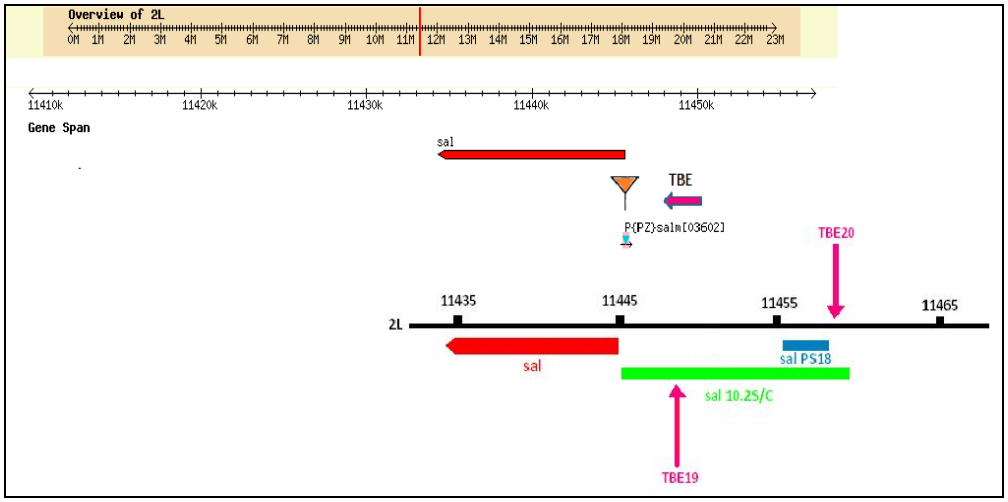


Figure 56. *sal* locus.
The potential TBE sites (pink arrows), the P-element insertion 03602 (orange) and the *sal* enhancers [322],[319], (green rectangles) near the *sal* locus.

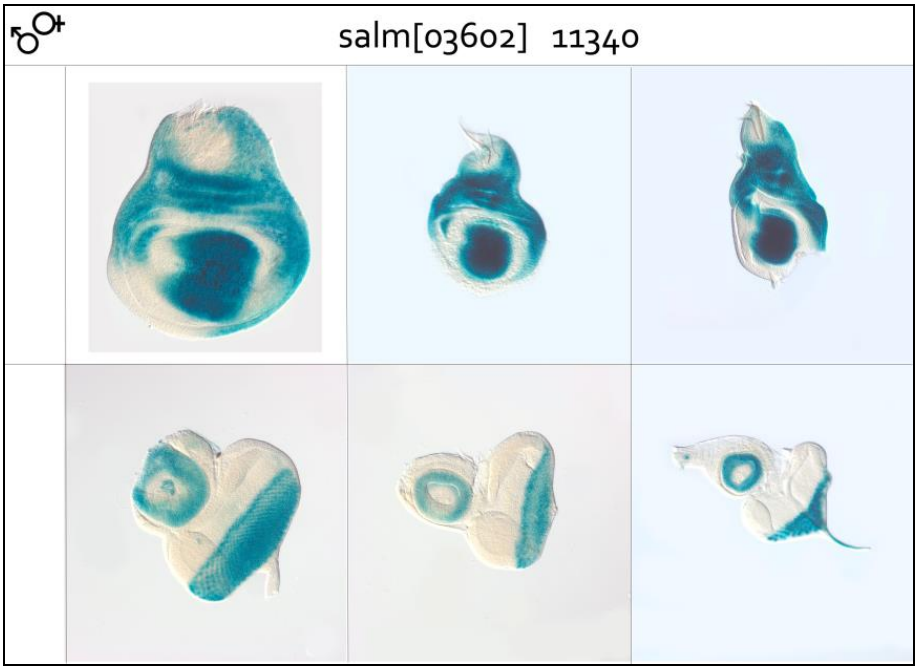


Figure 57. *sal-lacZ* expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain sal^{03602} (homozygous). 1st row: wing disc. 2nd row: eye antennal disc.

The enhancer trap line partially reproduced the expected *sal* wing, eye and leg pattern [323]. In the homozygous sal^{03602} eye antennal disc, strong expression was detected in a ring like domain in the antenna disc and posterior to the MF. In the wing disc, strong staining was apparent in the wing pouch, the lateral notum, and the proximal wing hinge (Fig. 57).

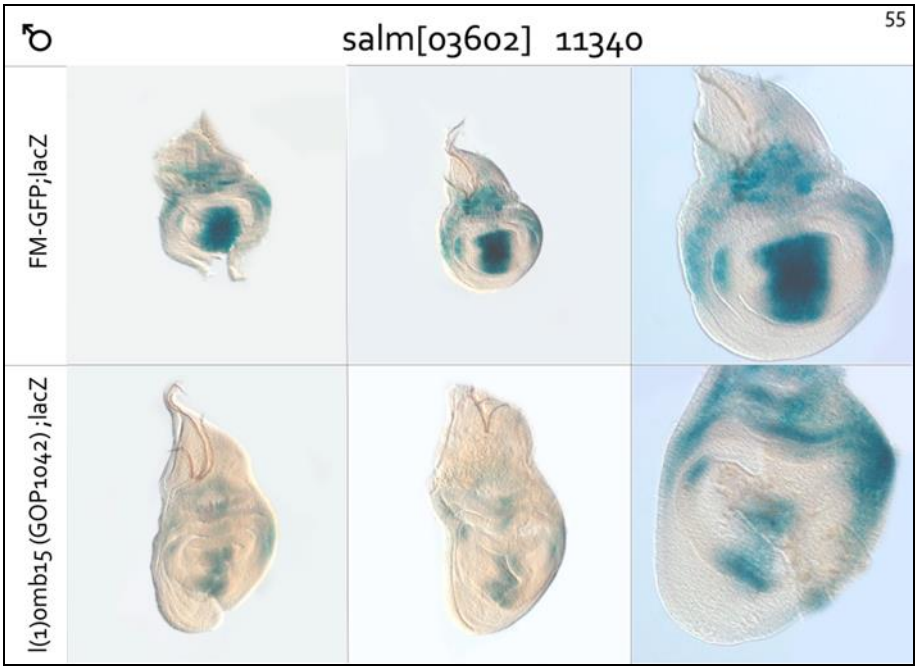


Figure 58. *sal-lacZ* expression in wt and *omb* mutant backround.
X-Gal staining of L3 imaginal discs of the fly strain *sal*^{03602}, after cross with *l(1)omb*^{15} mutant line (cross 55, appendix). 1st row: *FMGFP/Y*; *sal*^{03602}*-lacZ/II.* 2nd row: *l(1)omb*^{15} */Y*; *sal*^{03602} *-lacZ/II*.

In *l(1)omb*^{15} wing disc, *sal*^{03602}*-lacZ* pouch expression was reduced (Fig. 58). This was particularly obvious in late L3 wing discs when comparing the pouch expression to the other expression domains.

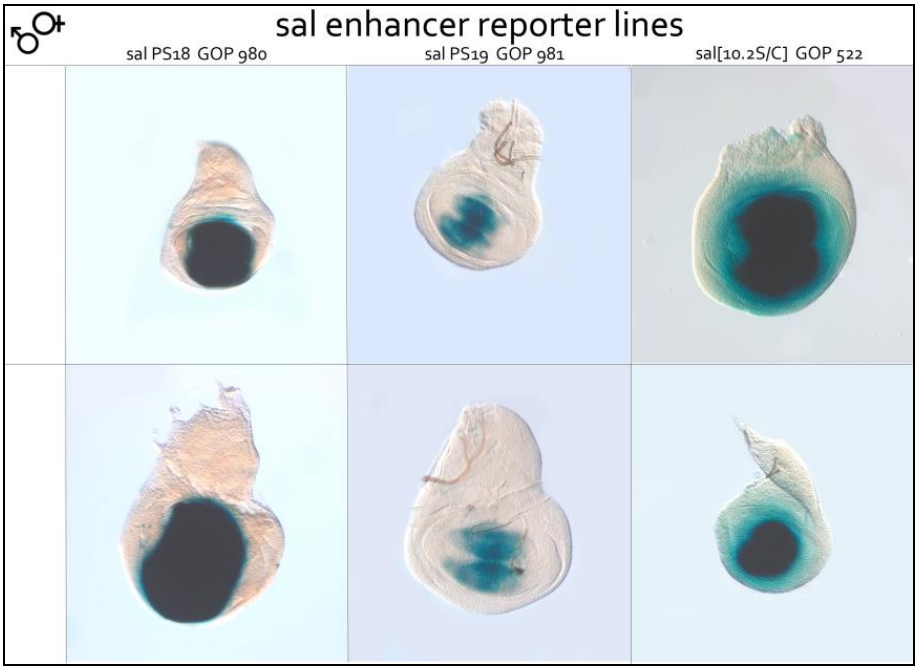


Figure 59. *sal-lacZ* enhancer reporter expression in imaginal discs.
X-Gal staining of L3 imaginal discs of the fly strain *salm*^{PS18}*-lacZ, salm*^{PS19}*-lacZ and salm*$^{10.2S/C}$*-lacZ.* 1st column: *salm*^{PS18}*-lacZ*, wing disc. 2nd column: *salm*^{PS19}*-lacZ*, wing disc. 3rd column: *salm*$^{10.2S/C}$*-lacZ*, wing disc. The strong wing blade staining is apparent.

In order to investigate the mechanism of Omb-dependence, I studied fly strains containing known *sal* wing pouch enhancer fragments. *salm^{PS18}-lacZ, salm^{PS19}-lacZ* [319] *and salm$^{10.2S/C}$-lacZ,* [322], were characterized by X-gal staining (see Fig. 59, 60, 61,62). The reporter lines reproduced the endogenous *sal* wing blade pattern [322],[319].

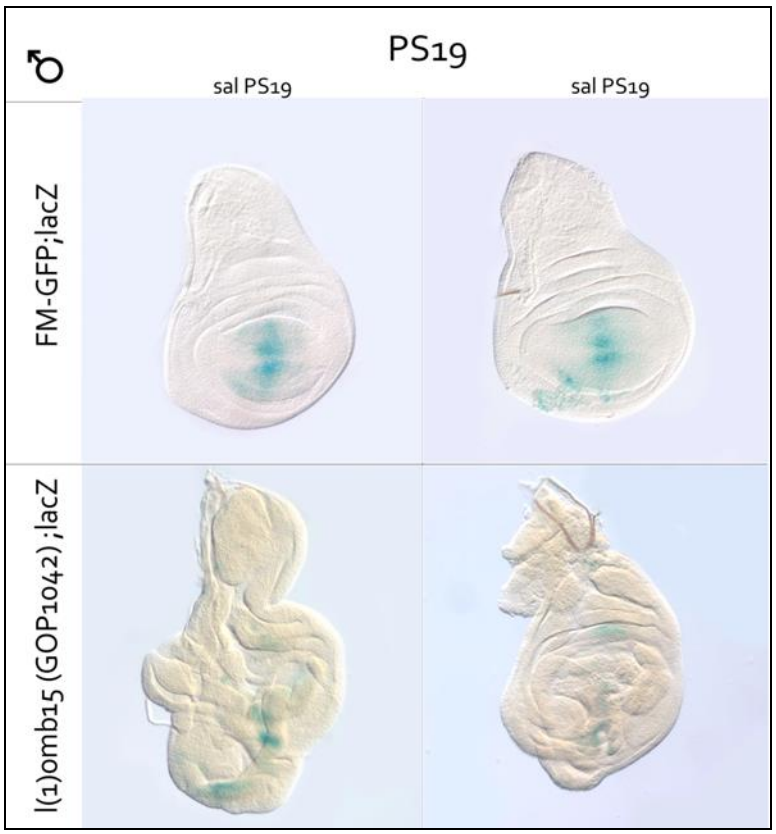


Figure 60. Imaginal disc expression pattern of the *salm^{PS19}-lacZ* in wt and *omb* mutant background.
X-Gal staining of L3 imaginal discs of the fly strain *salm^{PS19}-lacZ* after cross with *l(1)omb^{15}* mutant line (crosses 43, 44, appendix). 1st row: *FMGFP/Y*; *salm^{PS19}-lacZ /II.* 2nd row: *l(1)omb^{15} /Y*; *salm^{PS19}-lacZ /II*.

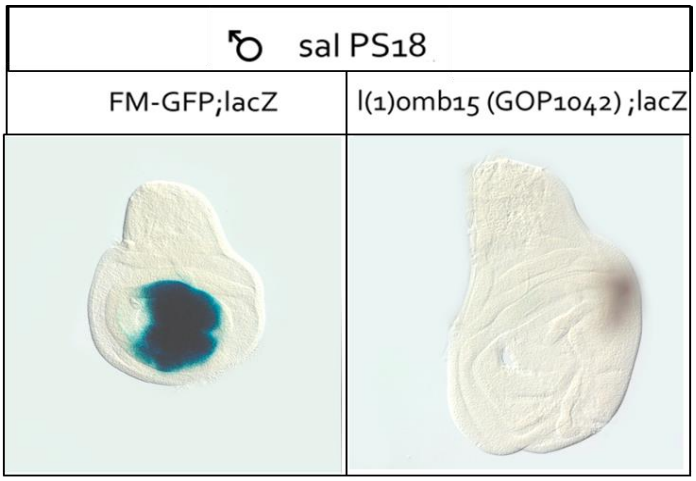


Figure 61. Imaginal disc expression pattern of the *salm^{PS18}-lacZ* in wt and *omb* mutant background.

X-Gal staining of L3 imaginal discs. of the fly strain *salm*^{PS18}*-lacZ* after cross with the *l(1)omb*^{15} mutant line (crosses 41, 42, appendix). 1st column: *FMGFP/Y*; *salm*^{PS18}*-lacZ /II.* 2nd column: *l(1)omb*^{15} */Y*; *salm*^{PS18}*-lacZ /II*.

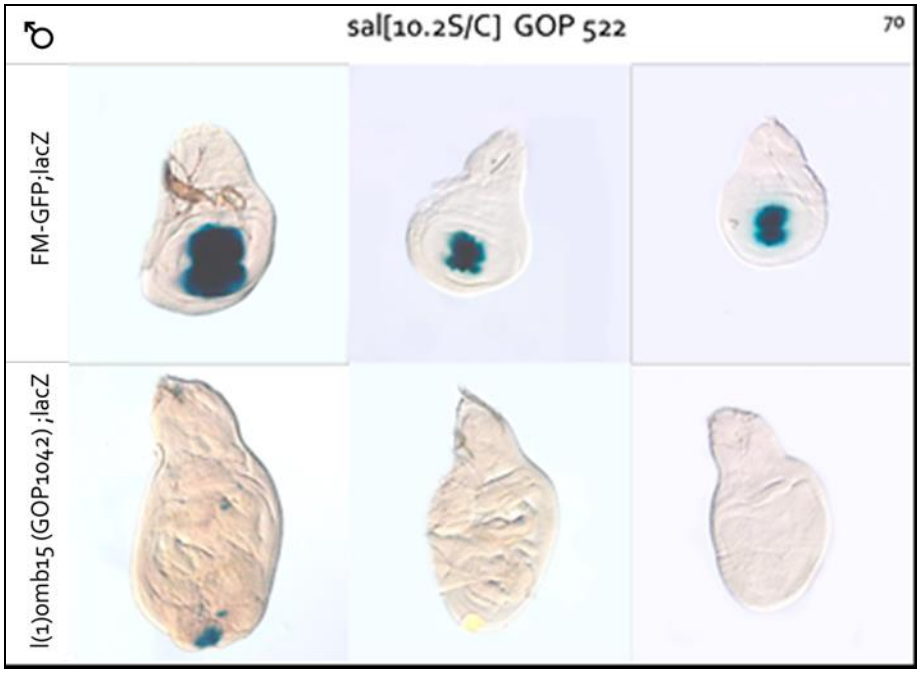


Figure 62. Imaginal disc expression pattern of the *salm*$^{10.2S/C}$*-lacZ* in wt and *omb* mutant backround.
X-Gal staining of L3 imaginal discs of the fly strain *salm*$^{10.2S/C}$*-lacZ* after cross with *l(1)omb*^{15} mutant line (cross 70 appendix). 1st row: *FMGFP/Y*; *salm*$^{10.2S/C}$*-lacZ /II.* 2nd row: *l(1)omb*^{15} */Y*; *salm*$^{10.2S/C}$*-lacZ /II*.

In all the above enhancer reporter lines, *salm*^{PS18}*-lacZ, salm*^{PS19}*-lacZ* and *salm*$^{10.2S/C}$*-lacZ,* the intense wing pouch expression was strongly reduced in *l(1)omb* discs.

It has been reported perviously that in l(1)*omb*^{3198} discs, *sal-lacZ* expression is lost in the wing pouch, although its expression in the notum is not affected [146]. The same results were obtained using anti-Sal antibody or the *sal*$^{10.2S/C}$*-lacZ* transgene construct [322].. *sal* expression in *omb*^{3198} clones generated in first instar larvae has been also tested, with the outcome of it being lost in most of the clone cells apart from some cells retaining low levels of expression [146]. In *omb* mutant discs also the expression *sal* target genes, *caps* and *trn* [324] appears affected.

The effect of Omb on *sal* expression seems to be indirect, as there is no potential TBE site contained in the small *salm*^{PS18}*-lacZ, salm*^{PS19}*-lacZ* (independent insertion of the same clone) enhancer fragment. A possible intermediating factor may be *brk*, the two lateral expression domains of which expand towards the AP compartment boundary in *omb*^{3198} mutant discs [146], a fact also observed in the current thesis for another *omb* mutant allele).

3.2.3 *ventral veins lacking/drifter (vvl/dfr) : the* vvl1+2 *enhancer*

ventral veins lacking, previously referred to as *Cf1a* or *drifter*, encodes a POU-homeodomain DNA-binding protein [325]. The POU domain proteins form a homeodomain proteins subfamily characterized by an additional DNA-binding domain, known as the POU domain [326]. In *Drosophila*, five POU protein- encoding genes are identified [327]. Dfr/Vvl, class III POU transcription factor is essential during development.

During *Drosophila* embryogenesis, *vvl* expression, under the control of *wg, hh* and *dpp,* is detectable in the developing tracheal system, the oenocytes, the CNS and the brain [325],[328]. As a neuron-specific transcription factor, Vvl regulates *dopa decarboxylase* (*ddc*) expression [329].

In addition to its expression in the embryo, *vvl* is also expressed in the wing imaginal discs [330]. During imaginal development, it is expressed in a dynamic pattern. Vvl synergizes with Mad in the regulation of *vestigial* [331]. Furthermore, through interaction with *torpedo, thick veins* and *Notch*, *vvl* regulates the wing vein differentiation between dorsal and ventral cells [330]. In a previous study, the *vvl* expression was found weaker in *omb* mutant discs and particularly in wing discs, limited to dorsal and ventral parts of wing pouch {Suche nach Zielgenen des T-Box-Transkriptionsfaktors Optomotor-blind aus Drosophila melanogaster, Gadomsky, Christian, Mainz, Univ., Diss., 2011}.

An 1055bp DNA fragment, incorporating the vvl1+2 trachea primordia enhancer [332] was subcloned (for more details, see appendix) into the pGWattBlacZ vector. The expression pattern of this fragment was previously observed in the tracheal primordia of st10 embryos [332], which I also confirmed (Fig. 64). This fragment adjacent to a STAT92E binding site contains a strong and fully conserved potential TBE (see appendix). Transgenic flies carrying the corresponding construct were created (line named vvl1+2 [1Kb]) and further characterized by abGal staining in the embryonic CNS and X-gal staining in the imaginal discs.

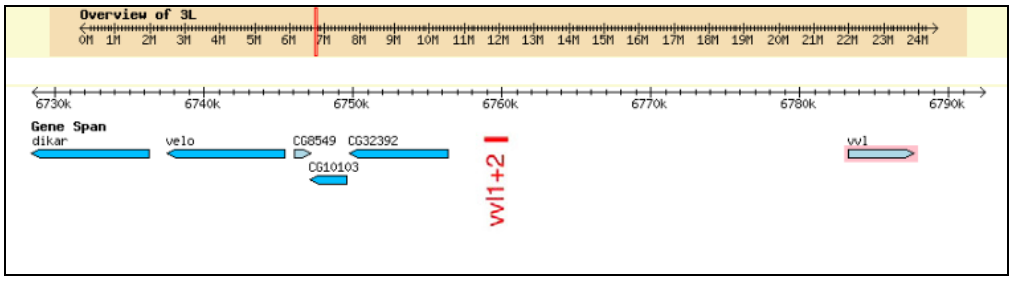
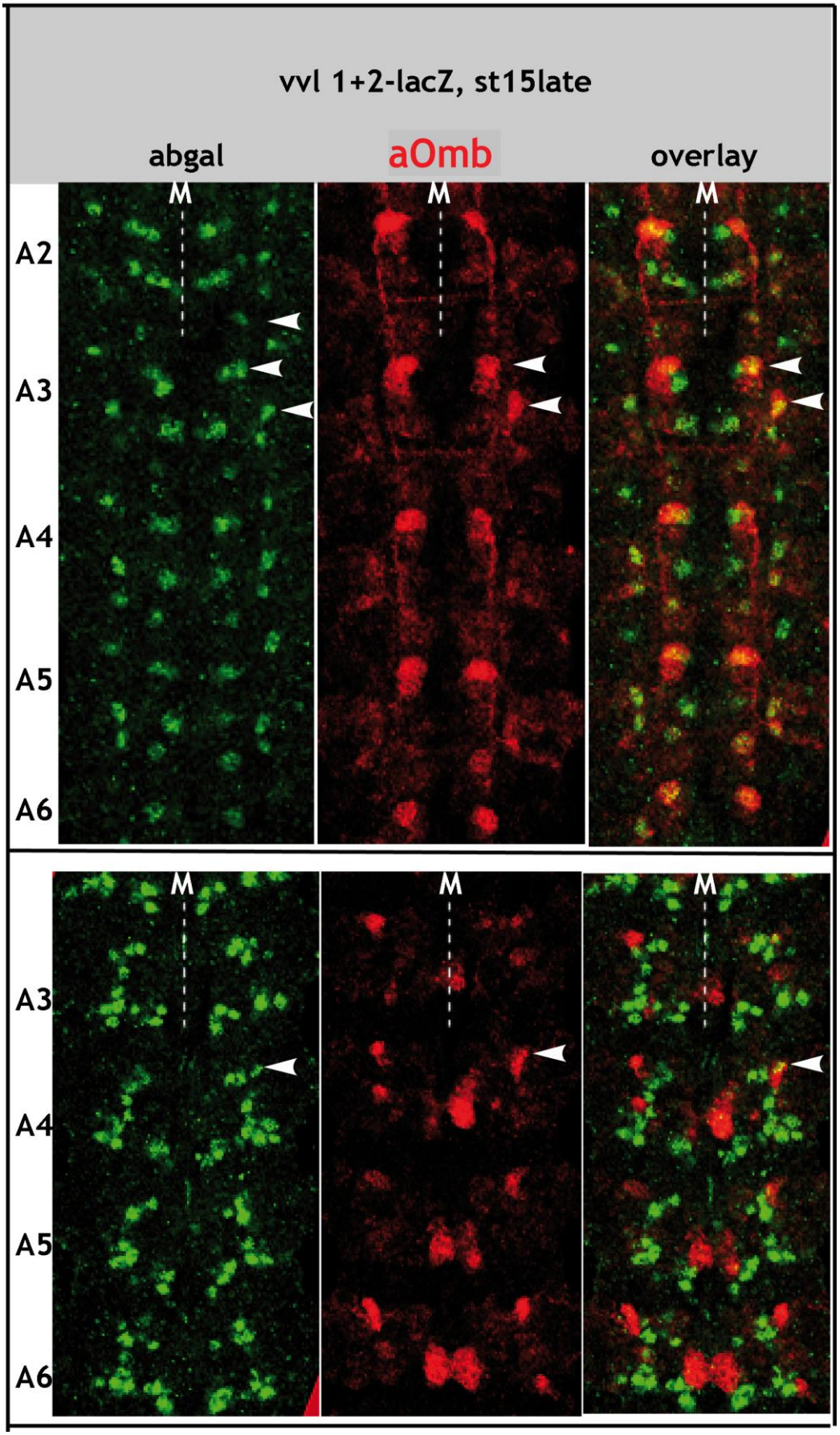


Figure 63. The potential TBE sites (red arrows), the vvl1+2 [1Kb] enhancer [332] (red bar) upstream of the *vvl* locus.


vvl 1+2-lacZ, st15late
abgal
aOmb
overlay
A2
A3
A4
A5
A6
M
A3
A4
A5
A6
M

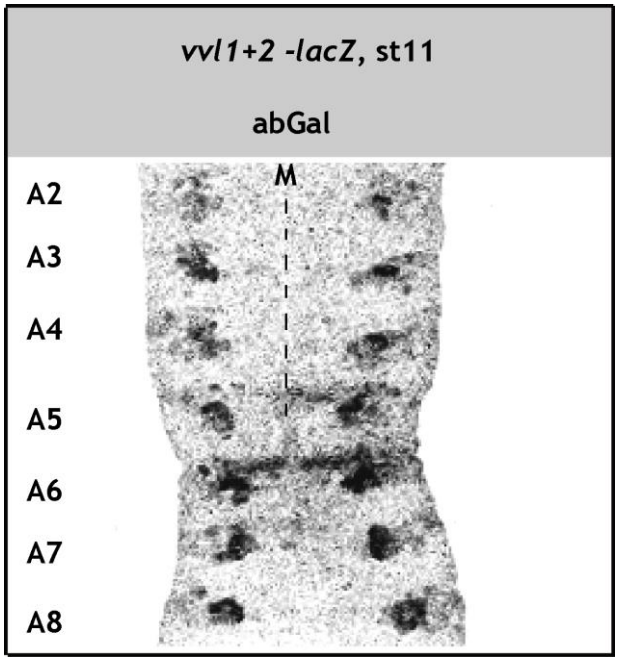
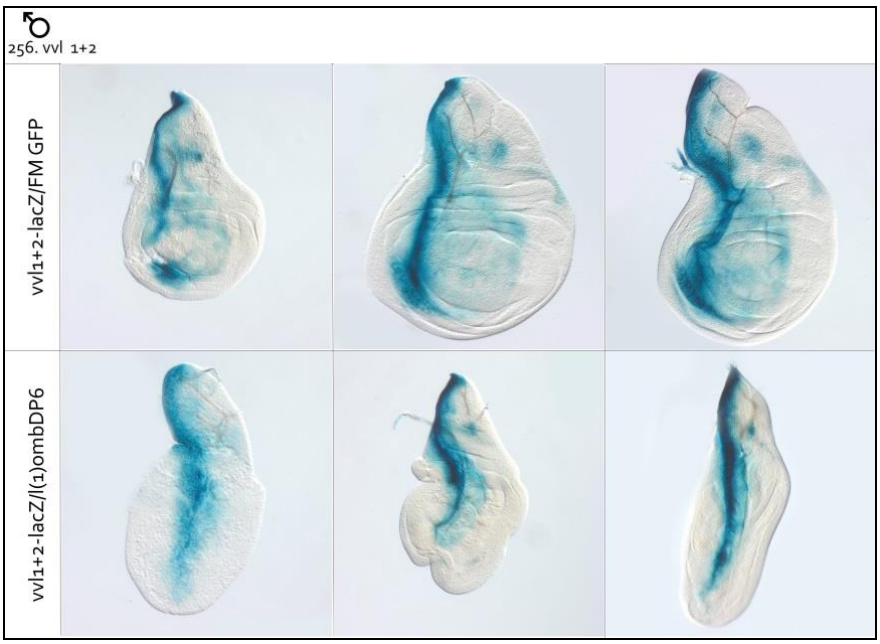


Figure group 64. Embryonic expression pattern of the vvl1+2 [1Kb] *-lacZ*. First figure: aOmb staining (red) along with abGal staining of stage 15 embryonic CNS of the fly strain vvl1+2 [1Kb] *-lacZ*. Dorsal (above), ventral (below) view; Abdominal segments A2-A6 are displayed; M vertical line: the midline; Anterior is up. Partial co-expression of Omb and the bGal.Second figure: abGal staining of stage 11 embryonic CNS of the fly strain *vvl1+2-lacZ*. Abdominal segments A2-A8 are displayed; M vertical line: the midline; Anterior is up. Expression observed in the tracheal primordia as it has previously been shown [332].



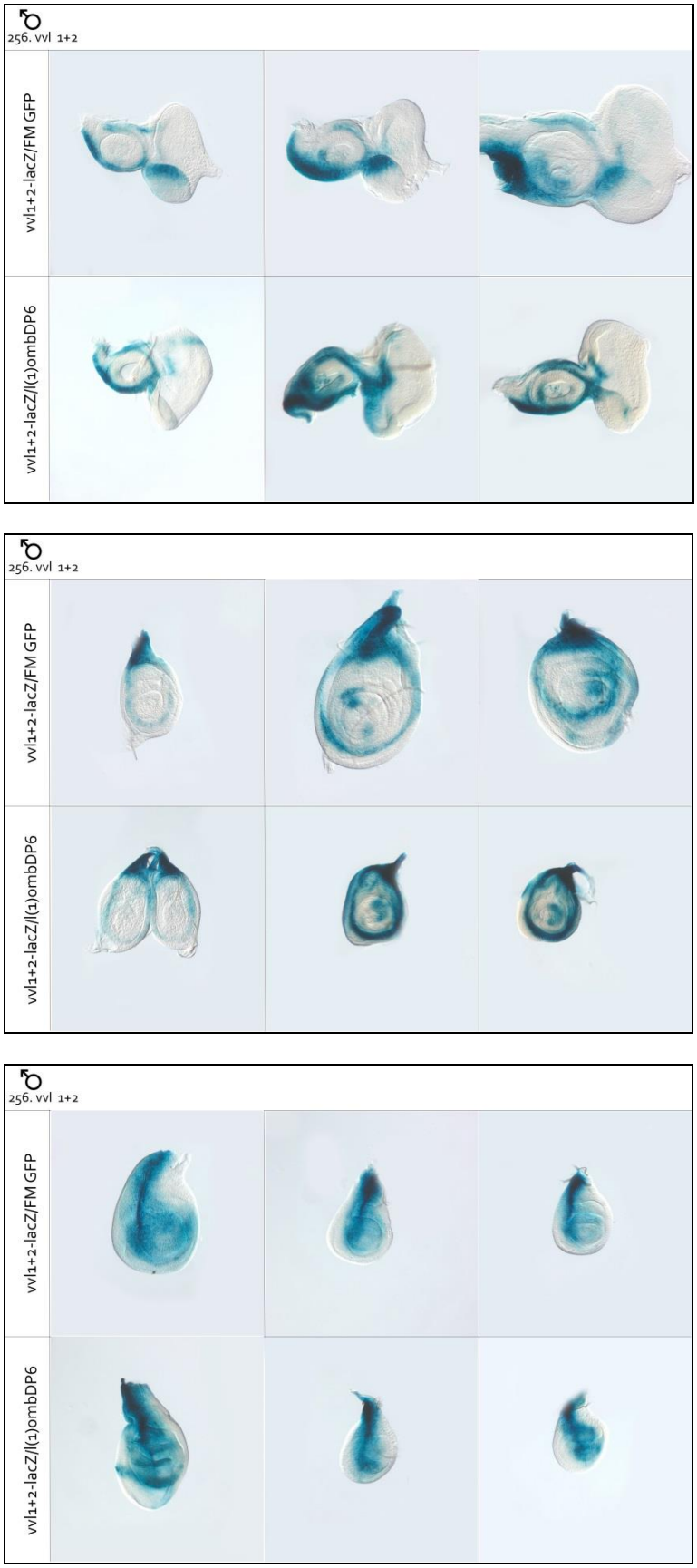


Figure 65. Imaginal disc expression pattern of the vvl1+2 [1Kb] *-lacZ* in wt and *omb* mutant backround.

X-Gal staining of L3 imaginal discs of the fly strain vvl1+2 [1Kb] *-lacZ* after cross with *l(1)omb*^{DP6} mutant line (cross 256 appendix). 1st row: *FMGFP/Y*; vvl1+2 [1Kb] *-lacZ /II.* 2nd row: *l(1)omb*^{DP6} */Y*; vvl1+2 [1Kb] *-lacZ /II*. Figures from above to below: wing, eye-antennal, leg, haltere disc.

vvl1+2 [1Kb]-lacZ during stage 11 is expressed at sites which possibly are the origins of the tracheal placodes [332]. Interesting expression of the enhancer is also found along the embryonic CNS in a complex pattern of expression, in individual Omb positive cells in the CNS of an advanced embryonic stage (Fig. 64).

The expression which I observed in the imaginal discs was also seen with other derivatives of the pGWattBlacZ vector inserted at the attP position 58A and is, therefore, considered an artifact. It has not yet been determined whether this construct is active in imaginal tracheal cells (Fig. 65).

3.2.4 *thickveins* (*tkv*): the *tkvA* enhancer

Dpp belongs to the BMP group of the Transforming Growth Factor family, the signaling of which is stimulated by the formation of complexes induced by their ligands. These involve four, two of type I and two of type II, receptors with serine-threonine kinase activity. The type II receptor kinase activates, through phosphorylation, the type I receptor, which then phosphorylates the Smad proteins. Phosphorylated Smad proteins translocate to the nucleus, where they regulate the transcription of target genes along with other transcription factors [333].

Three type I receptors (Thickveins, Saxophone and Baboon) have been identified in *Drosophila* [334]. Tkv is related to the vertebrate ALK-3/6. In the embryo, Tkv is required for the dorsoventral polarity establishment [335], mediating the signaling of Dpp and the dorsal patterning ligand Screw [336]. In the wing imaginal discs, Tkv is essential for anterior-posterior patterning [337], activating Dpp target genes like *omb, sal, vg* and repressing *brk* [338].

tkv is regulated through various mechanisms. It is negatively regulated through Dpp signaling, though it affects the activity range of the Dpp gradient [339]. Master of thickveins (Mtv, FlyBase: Scribbler) further downregulates the expression of *tkv* in response to Hh and En [340]. It has also been shown that the level of the Tkv protein is downregulated by a Dynamin-mediated endocytosis mechanism, independently of Dpp signaling [341].

In the wing imaginal disc, the *tkv* pattern extends laterally, complementary to that of *omb,* with maximal expression in the lateral wing while medially it fades [339].

It is already shown that *omb* regulates *tkv* expression, also in lateral wing regions, outside of the Dpp signaling range. In omb^{3198} discs, *tkv* expression expands medially, towards the central wing, which suggests that the control of *tkv* expression requires Omb. Additionally, within omb^{3198} clone cells, cell autonomous *tkv* overexpression is observed, whereas in cells that misexpress *omb,* while the consequent apoptosis is prevented*, tkv* expression is cell-autonomously lost within the clone cells [146].

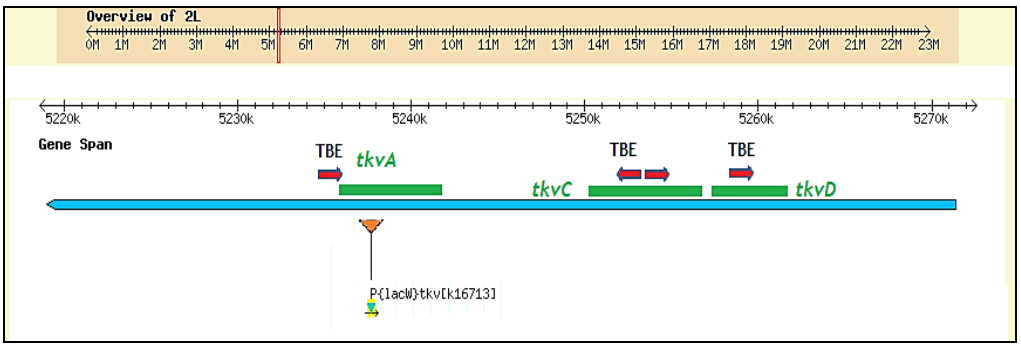


Figure 66. The potential TBE sites (red horizontal arrows), the subcloned fragments and the P-element insertion 01738 (orange) in the *tkv* locus.

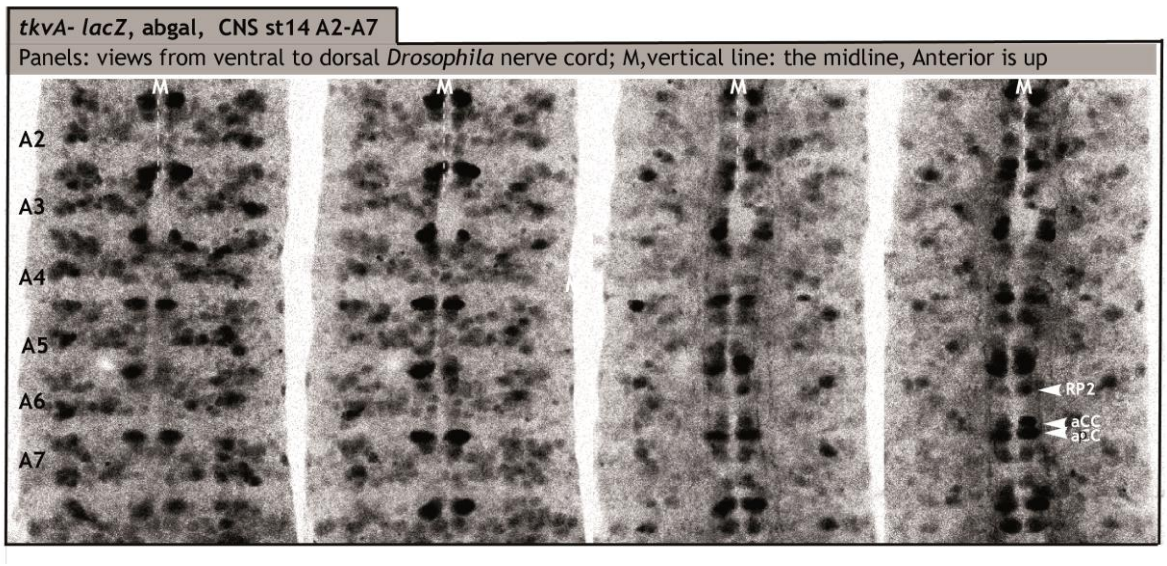


Figure 67. Embryonic expression pattern of the *tkv* A [5.2Kb]- lacZ.
Abgal staining of stage 14 embryonic CNS of the fly strain *tkv* A [5.2Kb]- lacZ. Abdominal segments A2-A7 are displayed; M vertical line: the midline; Anterior is up. Expression observed in the RP2 and aCC, pCC neurons and a lateral subset of neurons.

In addition to an trap line (*tkv^{k16713}-lacZ)*, three regulatory regions of the *tkv* locus were selected to be studied as potential targets of Omb due to their content in highly conserved TBEs.

The *FMGFP; tkv^{k16713}-lacZ* eye antennal control discs showed expression in the dorsal antennal periphery and in a central anterior eye region which in older discs is linked with a weak putative peripodial staining posteriorly to the MF. In *FMGFP* wing disc, *tkv^{k16713}-lacZ* expression was marginally detectable in a pattern rather complementary to that of Dpp. The consistent patterns were hardly affected in the *l(1)omb* mutant background (Fig. 68).

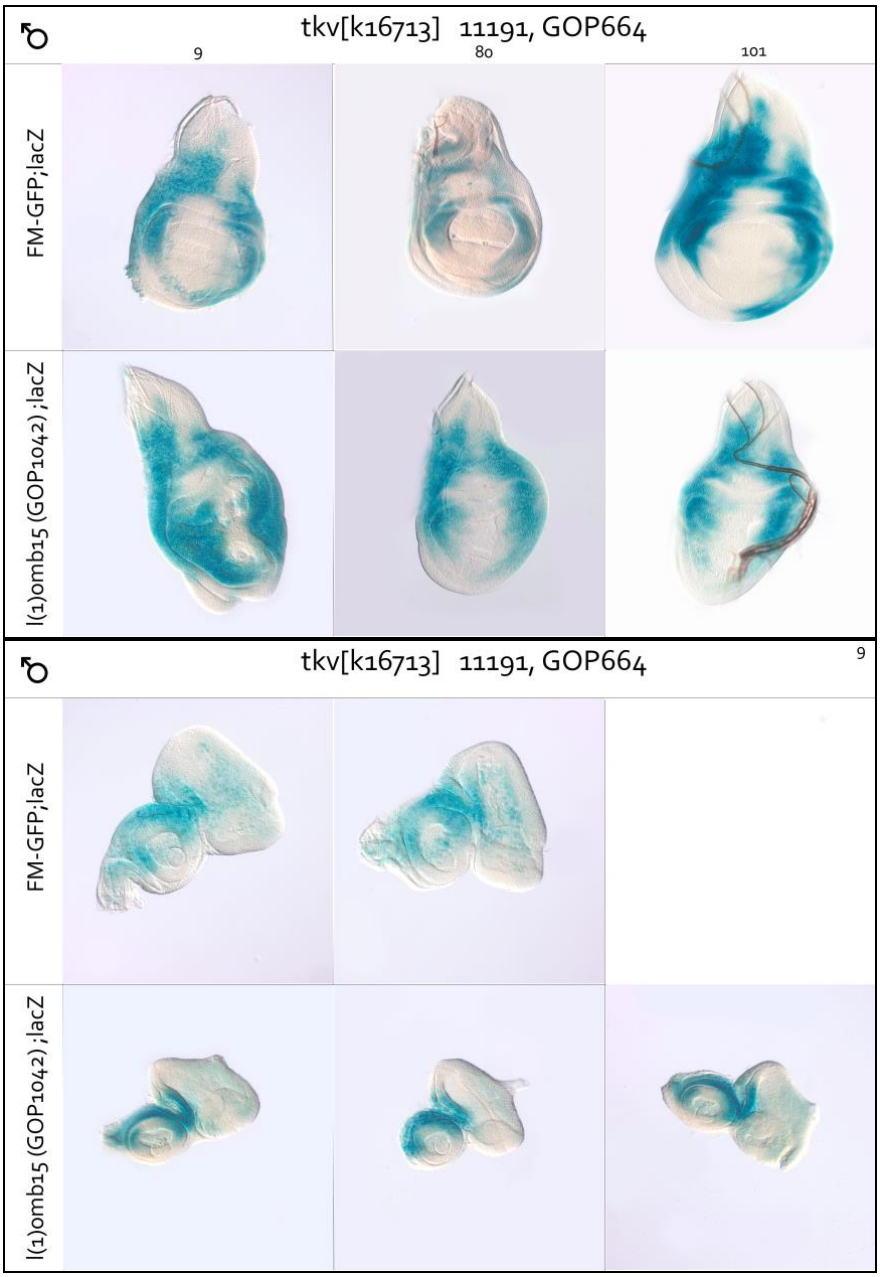


Figure 68. *tkv^{k16713}-lacZ* imaginal disc expression.
X-Gal staining of L3 imaginal discs of the fly strain *tkv^{k16713}-lacZ,* after cross with *l(1)omb^{15}* mutant line (cross 80, appendix). 1st row: *FMGFP/Y*; *tkv^{k16713}-lacZ/II.* 2nd row: *l(1)omb^{15} /Y*; *tkv^{k16713}-lacZ /II*.

In *FMGFP* wing discs, the enhancer trap line partially reproduced the expected *tkv* lateral wing pattern [342]. In *l(1)omb^{15}* wing disc, the lateral *tkv^{k16713}-lacZ* wing expression domain

appeared to invade the medial wing region. The effect was particularly strong in one of the discs (Fig. 68).

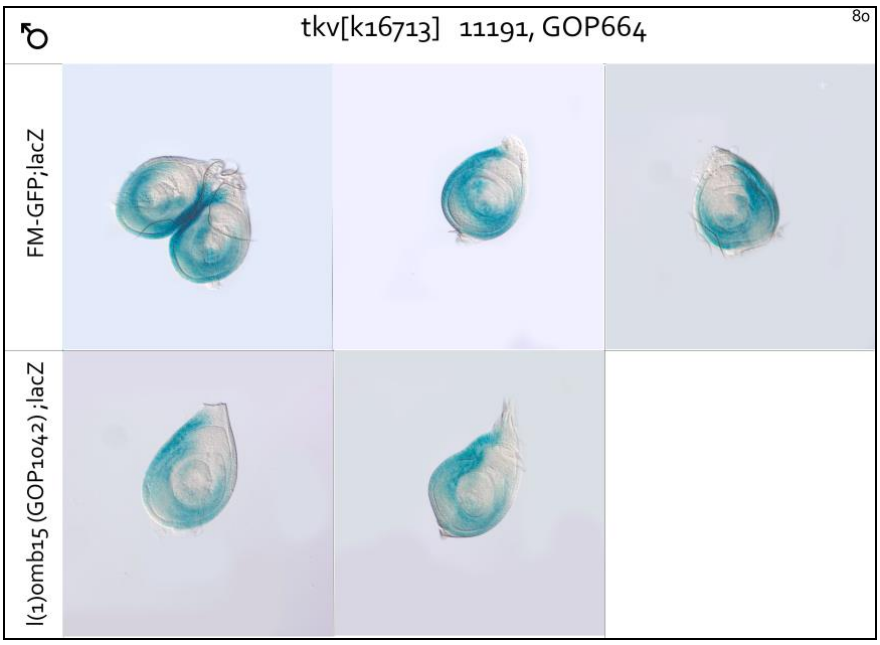


Figure 69. *tkv*^{k16713}*-lacZ* leg disc expression.
X-Gal staining of L3 imaginal discs of the fly strain *tkv*^{k16713}*-lacZ,* after cross with *l(1)omb*^{15} mutant line (cross 80, appendix). 1st row: *FMGFP/Y*; *tkv*^{k16713}*-lacZ/II.* 2nd row: *l(1)omb*^{15} */Y*; *tkv*^{k16713}*-lacZ /II*.

In order to investigate the mechanism of Omb-dependence, three regulatory regions of the *tkv* locus were selected to be further studied as putative *omb* targets. The DNA fragments *tkvA* (5177bp), *tkvC* (3925bp), *tkvD* (3363bp), were subcloned (for more details, see Appendix) into the pGWattBlacZ vector. These fragments contain several predicted TBEs, with most of them highly conserved among the 12 sequenced *Drosophila* species. Transgenic flies carrying the *tkvA* construct were created and characterized by X-gal staining.

tkv A [5.2Kb]- lacZ expression deviated from the known *tkv* expression. In FMGFP wing discs, a ring like pattern around the pouch was observed, which was not so strong in l(1)*omb*^{3198} mutants. In *FMGFP* eye antennal discs, *tkvA-lacZ* expression was widespread but reduced posteriorly to the MF. The pattern seemed not to be affected in the *l(1)omb* mutant background (Fig. 70).

Although *tkv* was previously observed to be affected in *omb*^{3198} clones [146] and the enhancer trap line *tkv*^{k16713}*-lacZ* had shown that *tkv* activity depended on *omb* in the pouch region (Fig. 68), the *tkvA* construct apparently contributes little to the endogenous pattern (Fig. 70). The *tkv*C [3.9Kb]- lacZ construct could, however, not be integrated into the

pGWattBlacZ vector, so it is still in the entry pCR8GWtopo vector. The *tkv*D [3.4Kb]- lacZ construct was already subcloned (for more details, see Appendix) into the pGWattBlacZ vector. The creation of transgenic flies was not possible in the time frame of this study.

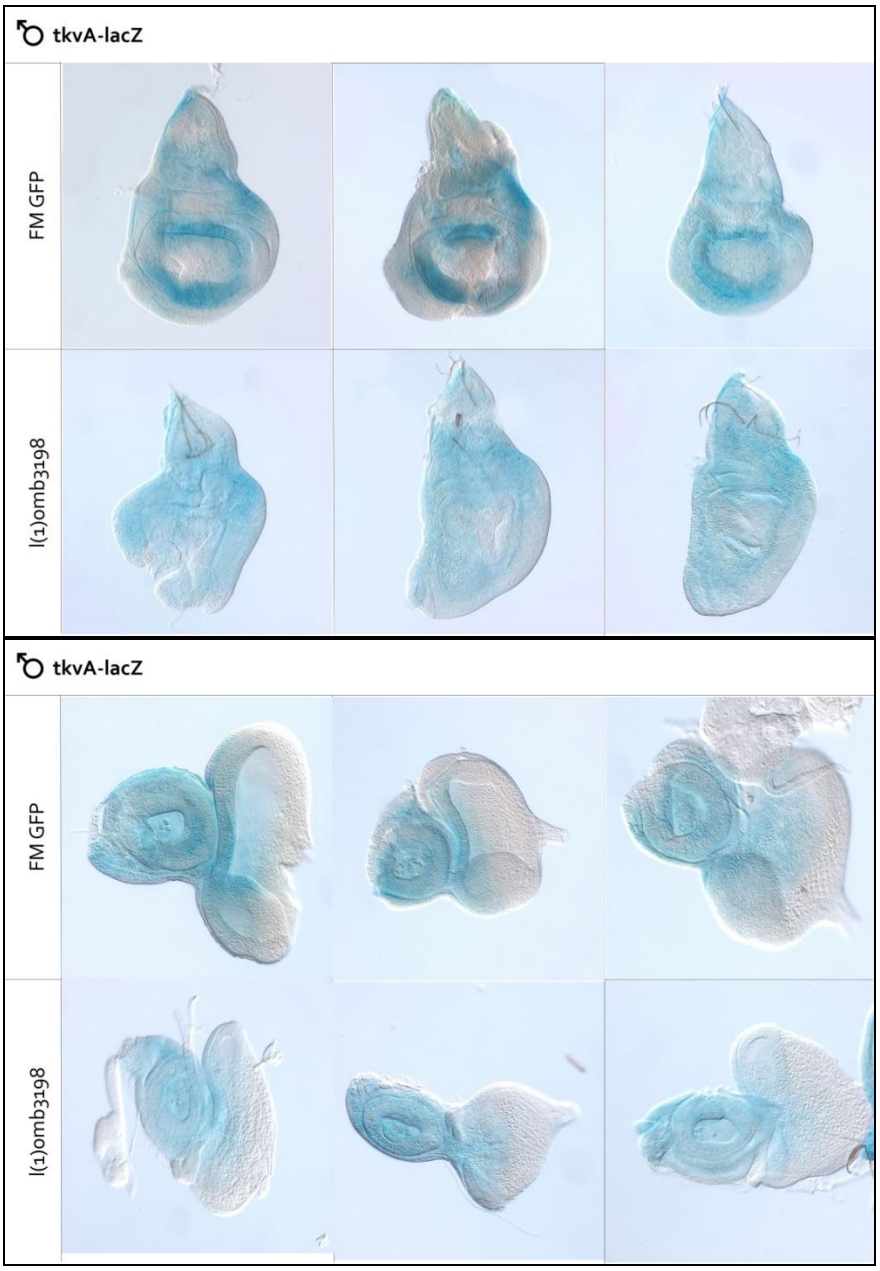


Figure 70. Imaginal disc expression pattern of the *tkv* A [5.2Kb]- lacZ in wt and *omb* mutant backround.
X-Gal staining of L3 imaginal discs of the fly strain *tkvA-lacZ* after cross with *l(1)omb*^{3198} mutant line (cross 289 appendix). 1st row: *FMGFP/Y*; *tkvA-lacZ /II.* 2nd row: *l(1)omb*^{3198} */Y*; *tkvA-lacZ /II*. Figures from above to below: wing, eye-antennal, leg, haltere disc.

3.2.5 hedgehog (hh): hh [3.1Kb]- TBE3*, hh* [2.4Kb]- TBE5 enhancers

Hedgehog signaling is important in embryonic development and adult tissue homeostasis, and its mis-regulation leads to several human disorders including cancer [343]. Vertebrate

hh family members are the Sonic Hedgehog (Shh), the Indian Hedgehog (Ihh) and the Desert Hedgehog (Dhh). Hh proteins are dual lipid modified and form soluble protein complexes that promote its long-range signaling [344].

Hh signaling is extensively studied. Hh is secreted from the posterior cells of the *Drosophila* wing imaginal disc and patterns the central wing region [345], such as the L3-L4 intervein region [346],[93]. Hh in the anterior compartment cells binds to Patched, allowing the activation of Smo. Further, through Smo interaction with intracellular signaling complexes, the transcription factor Cubitus interruptus (Ci) activates Hh target genes, in the anterior cells near the A/P boundary.

In the eye disc, *hh* is expressed at the posterior margin and is involved in the initiation of the morphogenetic furrow, inducing the differentiation of the anterior eye cells to photoreceptor cells [347]. Hh signaling pathway is subject to extensive feedback among elements of the pathway [348], which have effects in the dynamics of Hh gradient formation and the individual patterns of its target genes expression [349],[350],[351].

In the two introns of *hh* two TBEs were identified, TBE3 and TBE5 (Fig. 71), with high binding score (PWM) and almost total conservation in the 12 sequenced *Drosophila* genomes. TBE5 is particularly interesting, since a TBE with the same sequence along with an adjacent Homeodomain binding site are also conserved in the *hh* vertebrate homologue, *shh* [352]. *omb* interaction with Hh has been reported [144]. According to Umemori et al. 2007, *omb* participates in a negative feedback loop, between Dpp and Hh. Loss of *omb* expression upregulates Hh signaling causing an ectopic local overgrowth in the wing columnar epithelium.

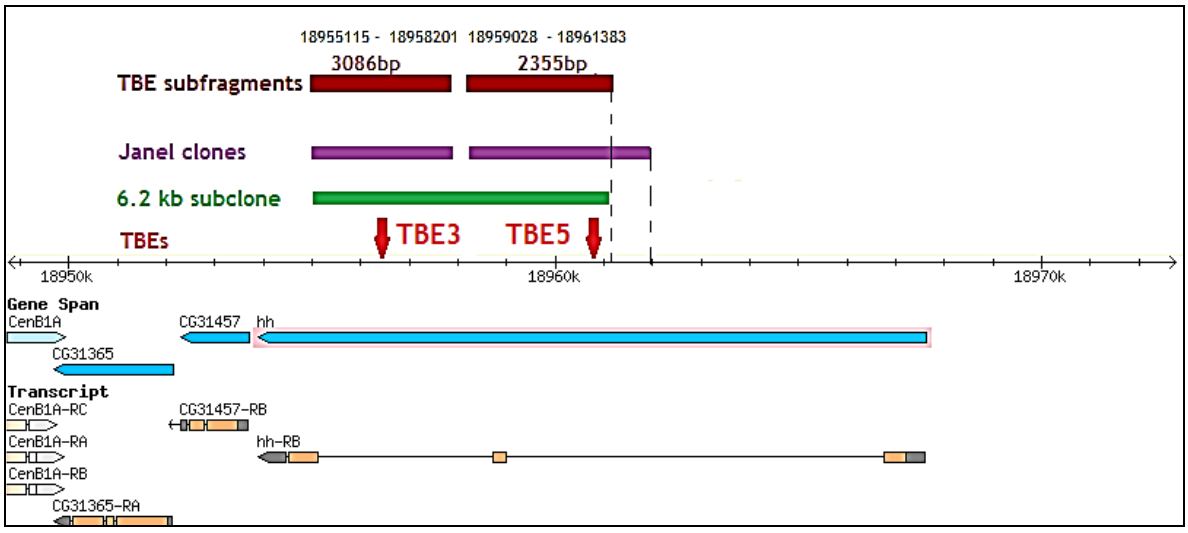


Figure 71. Genetic map of the *hh* locus (blue) with transcripts on chromosome 3R.

The green bar represents the already studied 6.2 kb*hh-lacZ* transgene {Suche nach Zielgenen des T-Box-Transkriptionsfaktors Optomotor-blind aus Drosophila melanogaster, Gadomsky, Christian, Mainz, Univ., Diss., 2011}, (green) containing the second intron, the second exon and parts of the first intron. The red vertical arrows point to the presence of two conserved potential TBEs. The lila bars represent the Janelia clones (Janel 45169 3R:18,955,107..18,958,749 and Janelia 48081 3R:18,958,960..18,962,674). The dark red bars represent the subcloned enhancer fragments. The first containing the TBE3 and the second intron (3086bp) and the second containing the TBE5 and part of the first intron (2355bp).

A *hh-lacZ* transgene (6.2 kb subclone) has been previously characterized {Suche nach Zielgenen des T-Box-Transkriptionsfaktors Optomotor-blind aus Drosophila melanogaster, Gadomsky, Christian, Mainz, Univ., Diss., 2011}. It is active in a subpattern of the endogenous *hh*. Its expression reveals that this enhancer region has the potential to induce the endogenous expression in the eye and a part of the wing disc, indicating that it could constitute a part of the *hh* wing enhancer.

Subsequently, the 6.2 kb region has been subcloned in 2 smaller fragments. The first containing the TBE3 in the second intron (*hh* [3.1Kb]- TBE3, 3086bp) and the second containing the TBE5 and part of the first intron (*hh* [2.4Kb]- TBE5, 2355bp). Each of them was cloned in the enhancer-reporter vector (pGWattBlacZ, for more details, see Appendix).

Figure 72. X-gal staining of L3 imaginal discs of the fly strain UAS-lacZ; $hh^{6.2\ kb}$-Gal4.
The different rows contain independent transformants from the same construct. In both the wing and the eye-antennal discs dorsal structures are stained. In the eye discs the staining extends along the D/V boundary. In the wing the $hh^{6.2\ kb}$-Gal4 enhncer drives the expression to a posterior stripe. Data from C.Gadomsky 2011.

Before transgenesis of our constructs, two Janelia lines became available that similarly covered our two TBE sites (Janelia 45169, Janelia 48081). The lines have been characterised by K. Oden. The line Janelia 45169, contains the TBE3. The enhancer expression in the wing imaginal disc, visualized with Xgal staining, is running along posterior margin of disc in notum, hinge and pleura. At the anterior margin, the expression is restricted to around the hinge level. The line Janelia 48081, containing the TBE5, shows expression only in the eye antennal disc.

3.2.6 *invected (inv):* inv [2Kb] **enhancer**

invected along with its homologue, *engrailed* belong to a gene complex [353]. Both are expressed in posterior developmental compartments [354], in several *Drosophila* tissues, including the embryo, imaginal discs, histoblasts nest, the hindgut and the CNS. *inv* encodes a protein of a similar size as *en*, and comprises a well conserved homeodomain near its carboxyl terminus. *en* and *inv* are found to be regulated by a common set of *cis* regulatory sequences [353].

en and *inv,* through overlapping and redundant expression [353], specify the posterior cell fate and create the AP compartment border [355],[356]. It is known that *en* acts cell autonomously, repressing anterior compartment genes, such as *ci* [80], *ptc* and *dpp* [357], inhibiting the posterior cells response to Hh, thus defining the posterior compartment [82]. In addition, *en* non-autonomously affects the anterior compartment, by regulating *hh* [358], which in turn activates *dpp,* consequently affecting growth and patterning of both wing compartments [359].

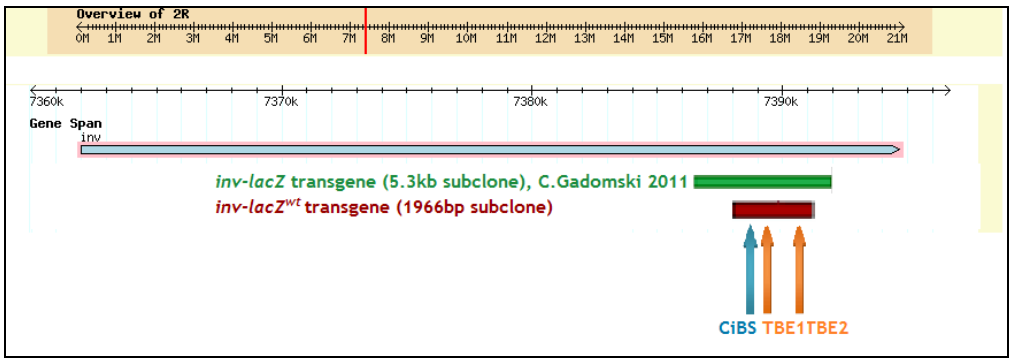


Figure 73. Molecular map of the *inv* locus (light blue) with transcripts on chromosome 2R.
The green bar represents the already studied 5.3kb *inv* [5.3Kb]-lacZ transgene {Suche nach Zielgenen des T-Box-Transkriptionsfaktors Optomotor-blind aus Drosophila melanogaster, Gadomsky, Christian, Mainz, Univ., Diss., 2011}, (green). The red bar represents the 1966bp *inv* [2Kb]- lacZ subcloned enhancer fragment, containing two well conserved potential TBEs (the first, TBE1, at 1073bp) and a conserved Ci binding site (at

862).. The orange vertical arrows indicate the presence of the two TBEs. The blue vertical arrow indicates the presence of the Ci binding site.

An *inv-lacZ* transgene (5.3 kb subclone) has been previously characterized (Gadomsky, 2011). I also tested the expression pattern of this fragment in embryonic CNS (Fig. 75).

In late L3 [360], the *inv* [5.3Kb]-lacZ AP- boundary expression present in the wild type wing pouch, disappeared in the *omb* mutant background. It has, therefore, been assumed that Omb activates *inv*. This effect of Omb loss failing to activate the enhancer expression, might be indirect, as it is known that a decline of the posterior *omb* wing disc expression leads to the formation of a distinctive AP boundary folding [25].

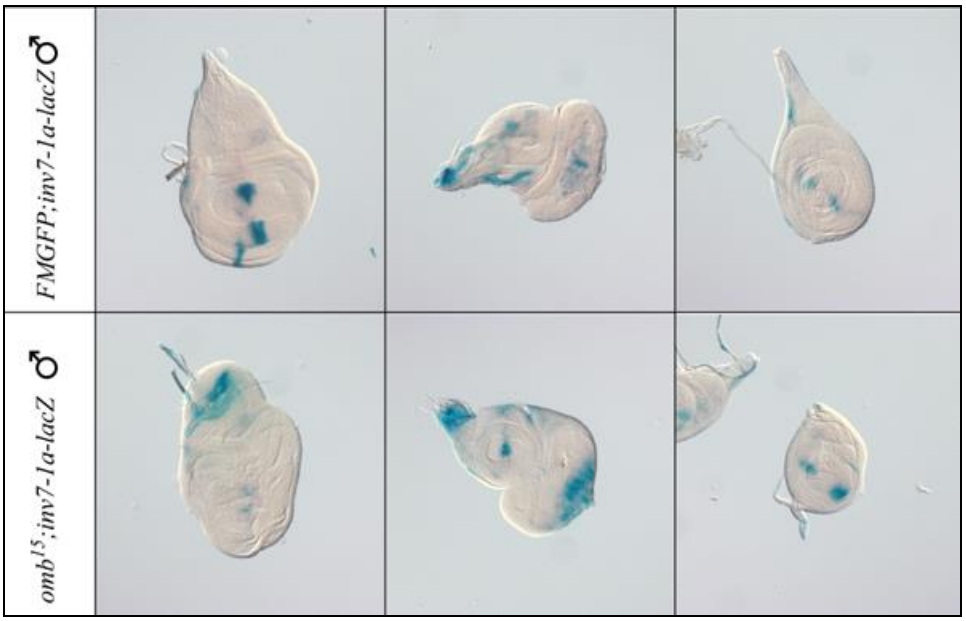


Figure 74. X-gal staining of L3 imaginal discs of the line *inv* [5.3Kb]-lacZ in late L3 stage.
1st row: *FMGFP/Y*; *inv* [5.3Kb]-lacZ */II.* 2nd row: *l(1)omb*^{3198} */Y*; *inv* [5.3Kb]-lacZ */II*. Data from C.Gadomsky 2011.

A 1966bp DNA fragment, *inv* [2Kb]- lacZ, smaller than the above mentioned one, was subcloned (for more details, see Appendix) into the pGWattBlacZ vector. This fragment contains two conserved TBEs (TBE1, TBE2) with high PWM values and around 200 bp upstream of TBE1 a well conserved Ci binding site [361]. The construct could, however, not be integrated into the fly genome.

However, in the *inv* [2Kb]- lacZ fragment, the potential TBE1 (at 1073) was mutated and the new enhancer reporter construct was introduced into the pGWattBlacZ vector.

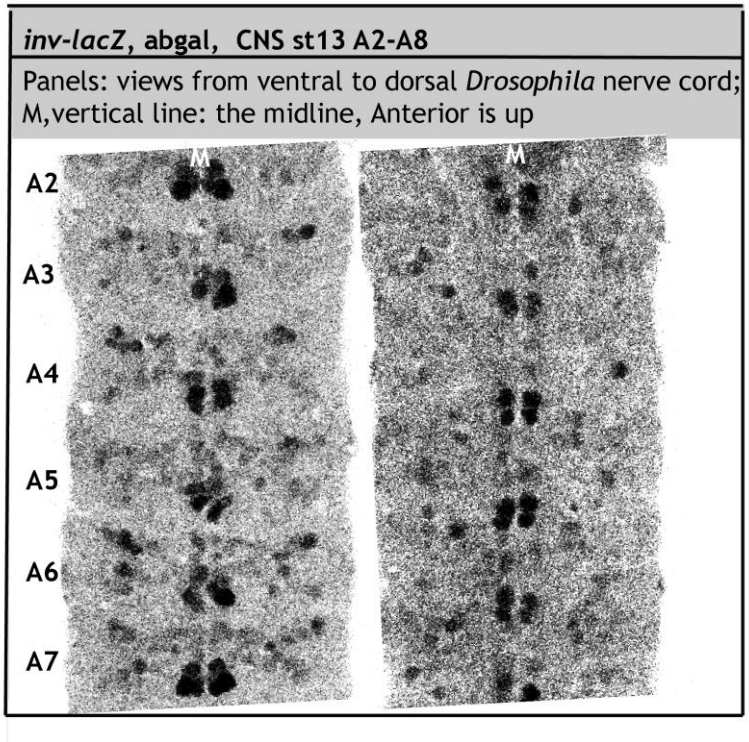


Figure 75. Embryonic expression pattern of the *inv* [5.3Kb]-lacZ.
Abgal staining of stage 13 embryonic CNS of the fly strain *inv* [5.3Kb]-lacZ. Abdominal segments A2-A7 are displayed; M vertical line: the midline; Anterior is up.

Transgenic flies carrying the corresponding construct at the same insertion position were created (*inv* [2Kb]- lacZ^{TBEMUT}) and characterized by X-gal staining. I also tested the expression pattern of this fragment in embryonic CNS (Fig. 76).

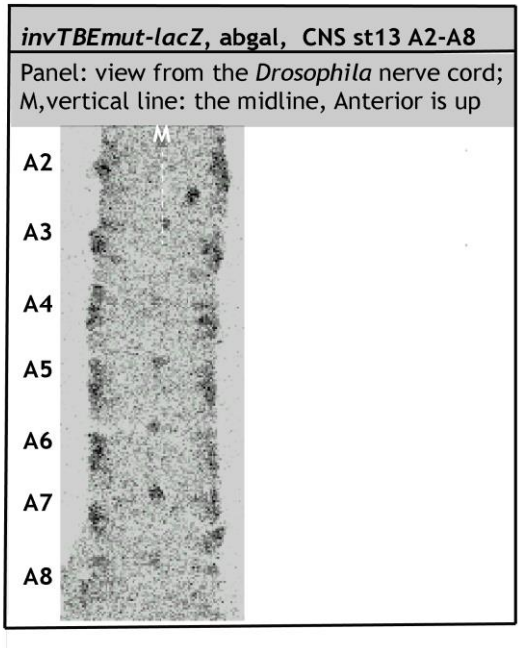
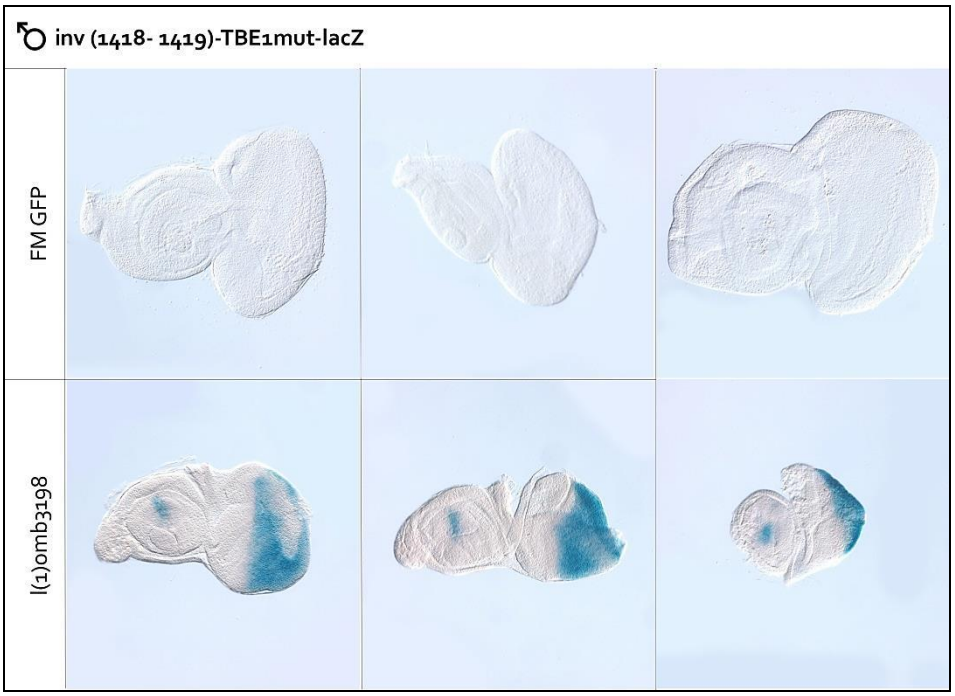


Figure 76. Embryonic expression pattern of the *inv* [2Kb]- lacZ^{TBEMUT}.
Abgal staining of stage 13 embryonic CNS of the fly strain *inv* [2Kb]- lacZ^{TBEMUT}. Abdominal segments A2-A8 are displayed; M vertical line: the midline; Anterior is up.



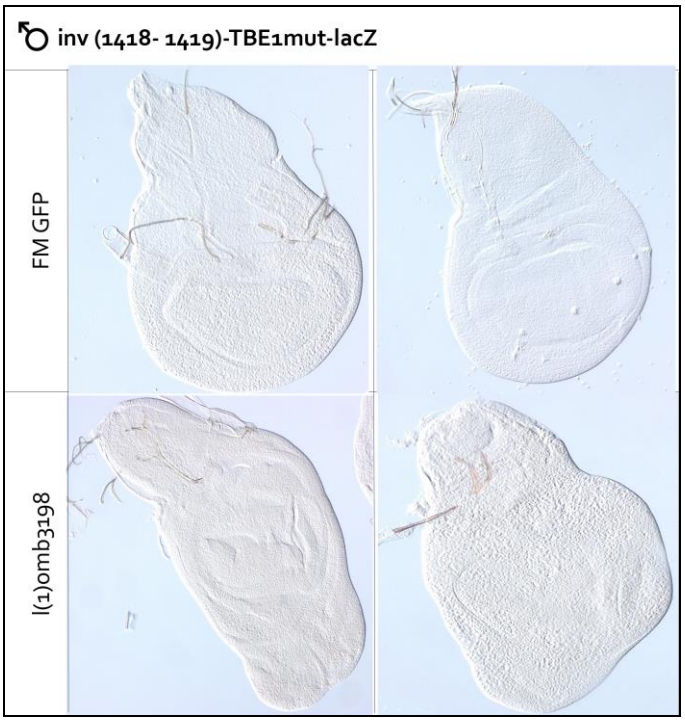


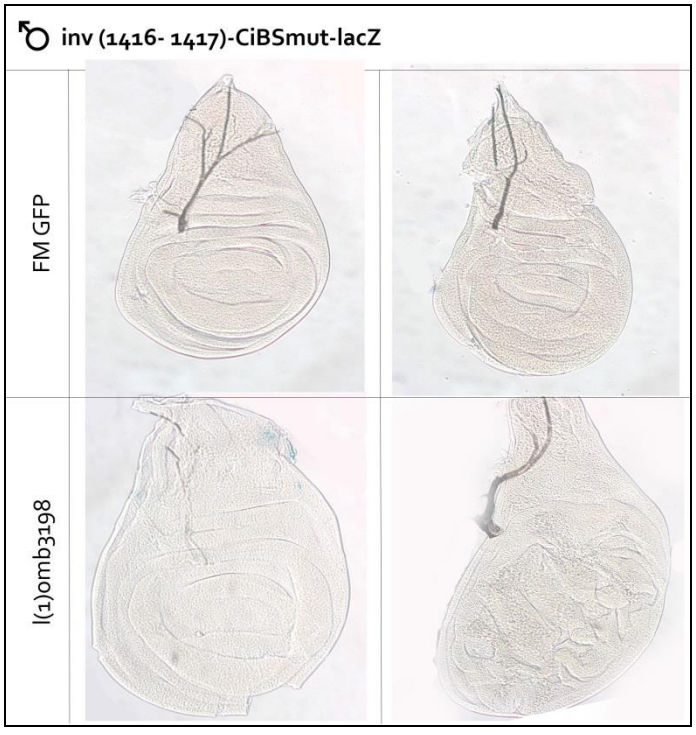
Figure 77. Imaginal disc expression pattern of the *inv* [2Kb]- lacZ^{TBEMUT} in wt and *omb* mutant backround.
X-Gal staining of L3 imaginal discs of the fly strain *inv* [2Kb]- lacZ^{TBEMUT} *–lacZ*, after cross with *l(1)omb*^{3198} mutant line. 1st row: *FMGFP/Y; inv* [2Kb]- lacZ^{TBEMUT} *-lacZ/II* . 2nd row: *l(1)omb* ^{3198} */Y*; *inv* [2Kb]- lacZ^{TBEMUT} */II*. Wing disc: no *inv* [2Kb]- lacZ^{TBEMUT} expression was detectable in FM-GFP or the *omb* mutants. Eye antennal disc: in FM-GFP no *inv* [2Kb]- lacZ^{TBEMUT} was seen, but in the *omb* mutants, it was possible to detect posterior eye expression.

The expression differed from the original *inv* [5.3Kb]- lacZconstruct (Fig.74). In *FMGFP* wing disc, no *inv* [2Kb]- lacZ^{TBEMUT} expression was detectable, a pattern that was not altered in the *l(1)omb* mutant background. In the *FMGFP* eye antennal discs, no inv [2Kb]- lacZ^{TBEMUT} was observed, but in the *omb* mutants, posterior eye expression arose (Fig. 77).

The initial study of the original *inv* [5.3Kb]- lacZ construct had shown that its activity depended on *omb* in the pouch region (Fig.74). This is confirmed by *inv* [2Kb]- lacZ^{TBEMUT} (Fig.77).

Furthermore, in the *inv* [2Kb]- lacZ, the potential CiBS (at 862) was mutated and the new enhancer reporter construct was also introduced into the pGWattBlacZ vector. Transgenic flies carrying the corresponding construct at the same insertion position were created (*inv* [2Kb]- lacZ^{CiBSMUT}) and characterized by X-gal staining.

In *FMGFP* wing, leg and haltere discs, no *inv-lacZ*^{CiBSMUT} expression was visible, a fact that remained unchanged in the *l(1)omb* mutant background. In the *FMGFP* eye antennal disc, no *inv* [2Kb]- lacZ^{CiBSMUT} was observed, nonetheless, in the *omb* mutants, particular posterior eye expression was detectable (Fig. 78).



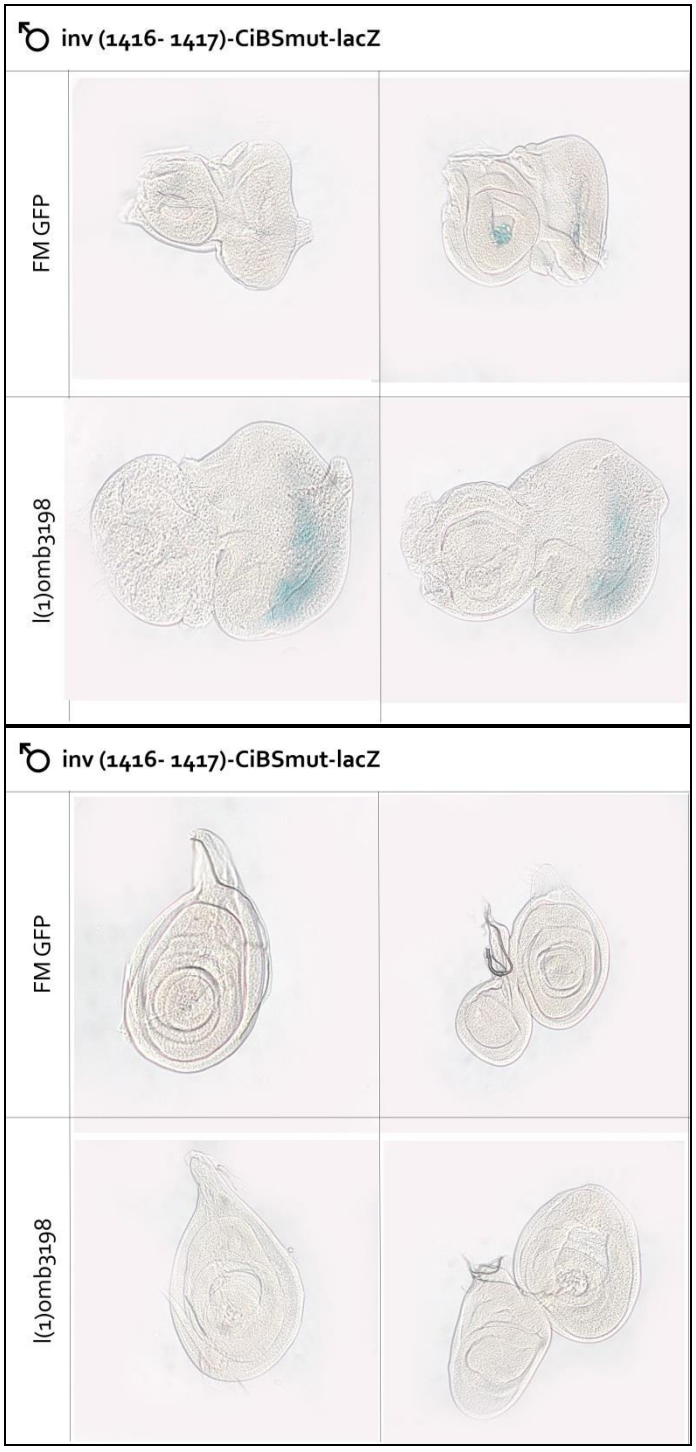


Figure 78. Wing, eye-antennal, leg imaginal disc expression pattern of the *inv* [2Kb]- lacZ^{CiBSMUT} in wt and *omb* mutant backround.
X-Gal staining of L3 imaginal discs of the fly strain *inv* [2Kb]- lacZ^{CiBSMUT}, after cross with *l(1)omb*^{3198} mutant line. 1st row: *FMGFP/Y; inv* [2Kb]- lacZ^{CiBSMUT}*/II*. 2nd row: *l(1)omb* ^{3198} */Y*; *inv* [2Kb]- lacZ^{CiBSMUT} */II*. Wing/leg/haltere disc: no *inv* [2Kb]- lacZ^{CiBSMUT} expression was detectable in FM-GFP or the *omb* mutants. Eye antennal disc: in FM-GFP no *inv-lacZ*^{CiBSMUT} was seen, but in the *omb* mutants, it was possible to detect posterior eye expression.

en distant enhancer elements are located in the first intron of *inv* locus as well as in the *inv* [5.3Kb]- lacZ (Fig. 74*)* fragment [362]. Therefore, also the above fragment may enclose cis-regulatory sequences. In this case, as the control construct *inv* [2Kb]- lacZ is missing, no further conclusions are permitted.

invected is known to inhibit the formation of innervated bristles between the third and fourth longitudinal vein [363], a phenotype also observed when *omb-RNAi* is expressed in this region {Suche nach Zielgenen des T-Box-Transkriptionsfaktors Optomotor-blind aus Drosophila melanogaster, Gadomsky, Christian, Mainz, Univ., Diss., 2011}, leading to the assumption that the lack of *inv* activation in these genotypes could be responsible for the phenotype. Moreover, anterior *omb* clones present a segregation behavior in the center of the pouch [26], similar to that of En-/Inv- double mutant clones [364].

Whatever the case may be, the production and characterization of a transgenic fly carrying the *inv* [2Kb]- lacZ DNA fragment, may possibly further elucidate the case.

3.3 Omb in the *Drosophila* embryonic central nervous system

CNS cells derive from precursors, called neuroblasts. Thirty neuroblasts develop per hemisegment, each of them dividing in a stem-cell-like way to produce a clone of neurons and/or glia cells [365]. A combination of several transcription factors, are expressed within the neurons of each lineage, regulating neuronal specification. Most of these transcription factors are expressed in a complex, to some extent overlapping pattern, with the specific differentiated identity of a neuron being determined by the precise group of transcription factors it expresses [366].

Segmentally repeated motoneurons, sets of cholinergic interneurons, a cuticular exoskeleton and arrays of body wall muscles compose the neuromuscular system of the *Drosophila* [367]. Motoneurons are determined early in the neuroblast lineage and their axons leave the CNS in one of the two main nerve tracks in each hemisegment, the segmental (SN) or the intersegmental nerve (ISN), [368]. From these nerves, secondary branches deviate at specific points in the periphery, developing in response to the presence of target muscles [369], each innervating a discrete muscle set [370]. In each abdominal hemisegment 30–40 motoneurons innervate 30 muscles [149].

3.3.1 Omb in embryonic CNS

It is already known that *omb* is required not only for patterning of the wing imaginal disc [371], but also for determining specific neuronal fates in the adult brain [30]. This study analyzed the role of Omb in the *Drosophila* embryonic central nervous system. In order to identify subsets of cells expressing Omb, embryos were immunohistochemically stained against Omb in combination with neuronal and glial markers. Subsequently, the embryos were dissected and their CNS was flattened. Subsets of neurons expressing Omb were identified.

As described previously, the first *omb* transcription is detectable in the cephalic region of embryos at stage 8 in two bilaterally symmetric domains. Later, during germ band elongation, a narrow strip of cells connecting the two initial domains expresses Omb. At stage 11, Omb is observed in a segmentally repeated pattern, the staining intensity of which decreases from anterior to posterior. Upon stage 13, sets of cells begin to express Omb and expression within the segments increases. In the second half of embryonic development, Omb expression in most of the peripheral and ventral nervous system gradually declines. Just before hatching, Omb pattern is limited to the antennomaxillary

complex and to a caudolateral region within the larval brain, posterior to the commissure connecting both hemispheres [372].

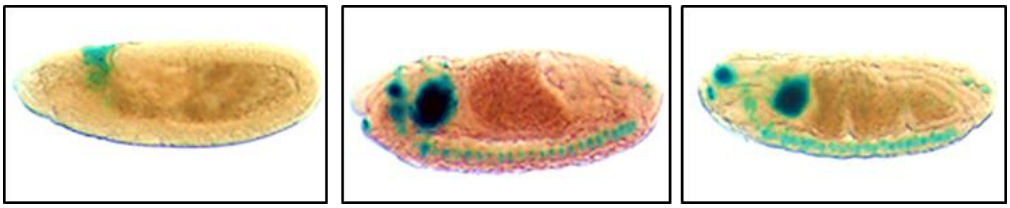


Figure 79. Omb embryonic expression.
X-Gal staining in whole mount embryos of the ombP1-lacZ fly strain. Panels from left to right: stage 10, stage 13, stage 16; anterior is left. Data from Nathalie Reinhard., practical course.

3.3.2 Omb and Even skipped (Eve)

even skipped encodes a homeodomain transcription factor required during *Drosophila* segmentation for activation of *engrailed* [373] and the organization of the odd-numbered parasegments [271]. *eve* is activated by *gap* genes in a striped pattern that develops subsequently into narrow stripes that correspond with the odd-numbered parasegment boundaries [374]. Eve has been considered as a transcriptional repressor due to its alanine/proline-rich repressor domain [375]. Eve, along with Islet and Lim3, regulate motoneuron axon targeting [376]. Eve expression is necessary and sufficient to direct motor axons into the dorsal muscle field [377], [378].

After segmentation, Eve is expressed in later *Drosophila* developmental stages, such as specific lineages within the dorsal mesoderm [379], the anal plate ring [380], and the nervous system [377],[381]. In the CNS, it is expressed in approximately 16 cells per abdominal hemisegment. Medially, in the pCC and fpCC interneurons and the aCC and RP2 motoneurons [382], mediolaterally, four CQ neurons and laterally, the eight to ten EL interneurons [73],[67].

The neuron aCC develops in stage 11 in one segment and then migrates anteriorly to the segment border [383]. From there, aCC innervates muscle DA1 intersegmentally. RP2 develops and remains in its segment of birth and innervates muscles DA1 and DA2 segmentally [384]. CQ-U motoneurons (U1 to U5) innervate the muscles DO1 (U1), DO2, DA1, DA2 and LL1 [149].

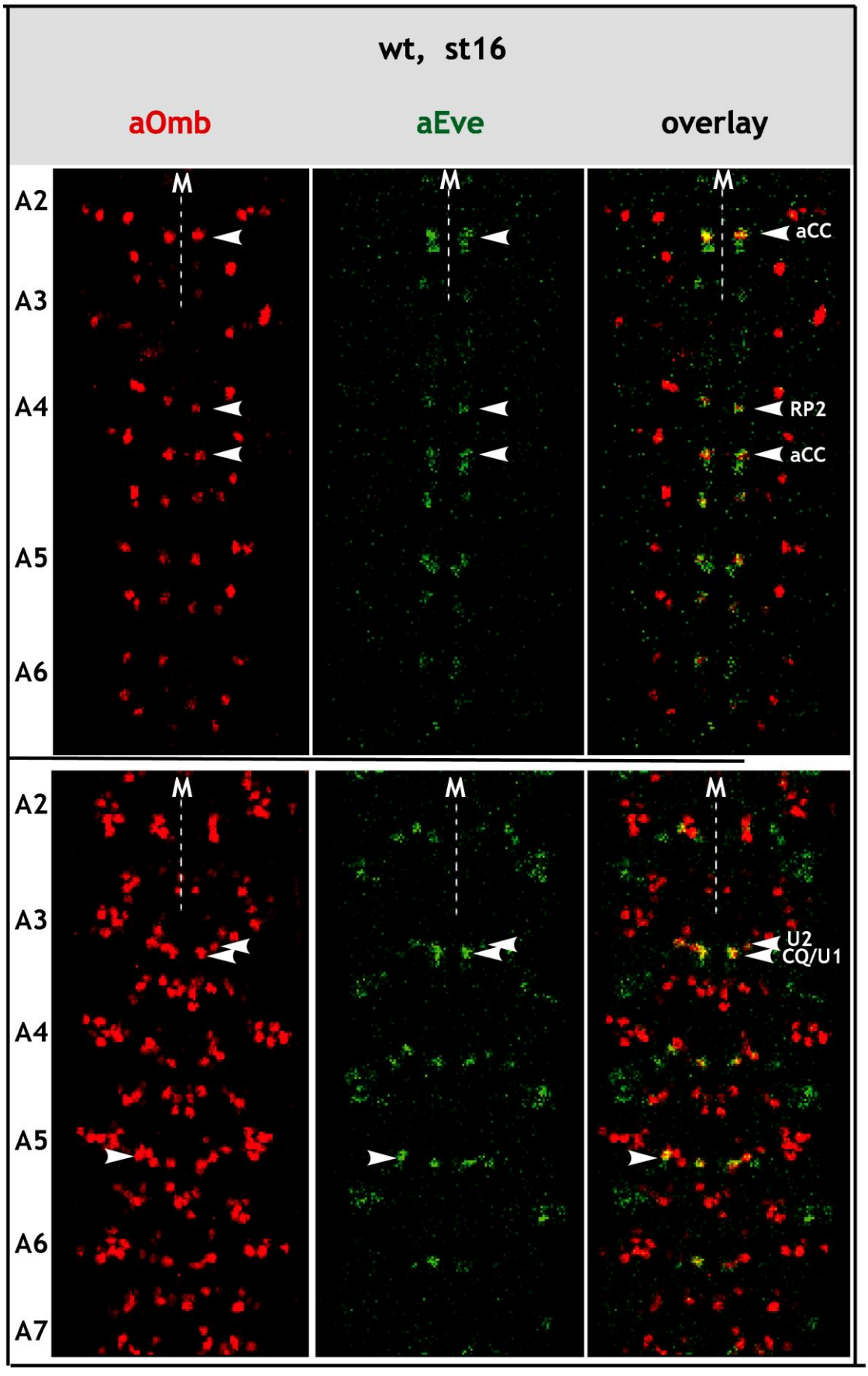


Figure 80. Omb and Eve co-expression in neuronal cells of wt fly strain.

CNS, dorsal (above) and ventral (below) view of stage 16 wild-type embryo; Panels from left to right: Omb channel, Eve channel, channel overlay; M,vertical line: the midline; anterior is up. Omb is expressed in a subset of the Eve neuronal cells: the medial aCC and RP2 motorneurons as well as the mediolateral CQ neurons.

Immunostaining of Omb on wt embryonic CNS along with Eve showed that, at late embryonic stages, they are co-expressed in a subset of neuronal cells: The medial aCC and RP2 motorneurons as well as the fpCC interneuron and mediolateral four CQ neurons (Fig.80). The aCC motoneuron is specified by a genetic cascade involving *even-skipped, grain* and *zfh1* [227]. The expression of Omb in this neuronal cell indicates possible interaction with *grain* and *zfh1*. Both *grn* and *zfh1* contain high-score, high-conservation TBEs.

The results presented above show that Omb is expressed in motoneurons, suggesting the possibility of an Omb role in the process of motor axon projection or identity specification.

3.3.3 Omb and Eagle (Eg)

Eg is a zinc finger transcription factor [385], member of the steroid receptor family [386]. It is expressed in only four neuronal lineages including NB 7-3 and temporarily in the embryonic gonad [387]. Eg expression is negatively controlled by Dpp, directly but also indirectly through repression of EGFR signaling during embryonic neurogenesis [388].

In the central nervous system, just after delamination, during stages 10 and 11, *eagle* expression is observed in four neuroblasts, NB2-4, NB3-3, NB6-4 and NB7-3. Eg expression reaches a maximum during the period of neuroblast delamination, apart from NB6-4. By the end of stage 12, Eg expression in the glial progenies of NB 6-4 is no longer detectable [389]. However, Eg is present in all NB7-3 serotonergic neurons [390] until late stage 17 [385]. The lineage of NB 7-3 progeny contains 4 mediodorsal neurons: the three 7-3I- EW interneurons, which project contralaterally across the posterior commissure and one motoneuron, the 7-3M- GW neuron, which projects ipsilaterally [385]. Mutant alleles of *eg* show the correct number of progeny early in development, but later the few remaining neurons have abnormal axon projections [389].

Immunostaining of Omb on the embryonic CNS of the Eg-GFP fly strain along with GFP showed that, at the late embryonic stages, Omb is expressed in a subset of Eg positive neuronal cells.

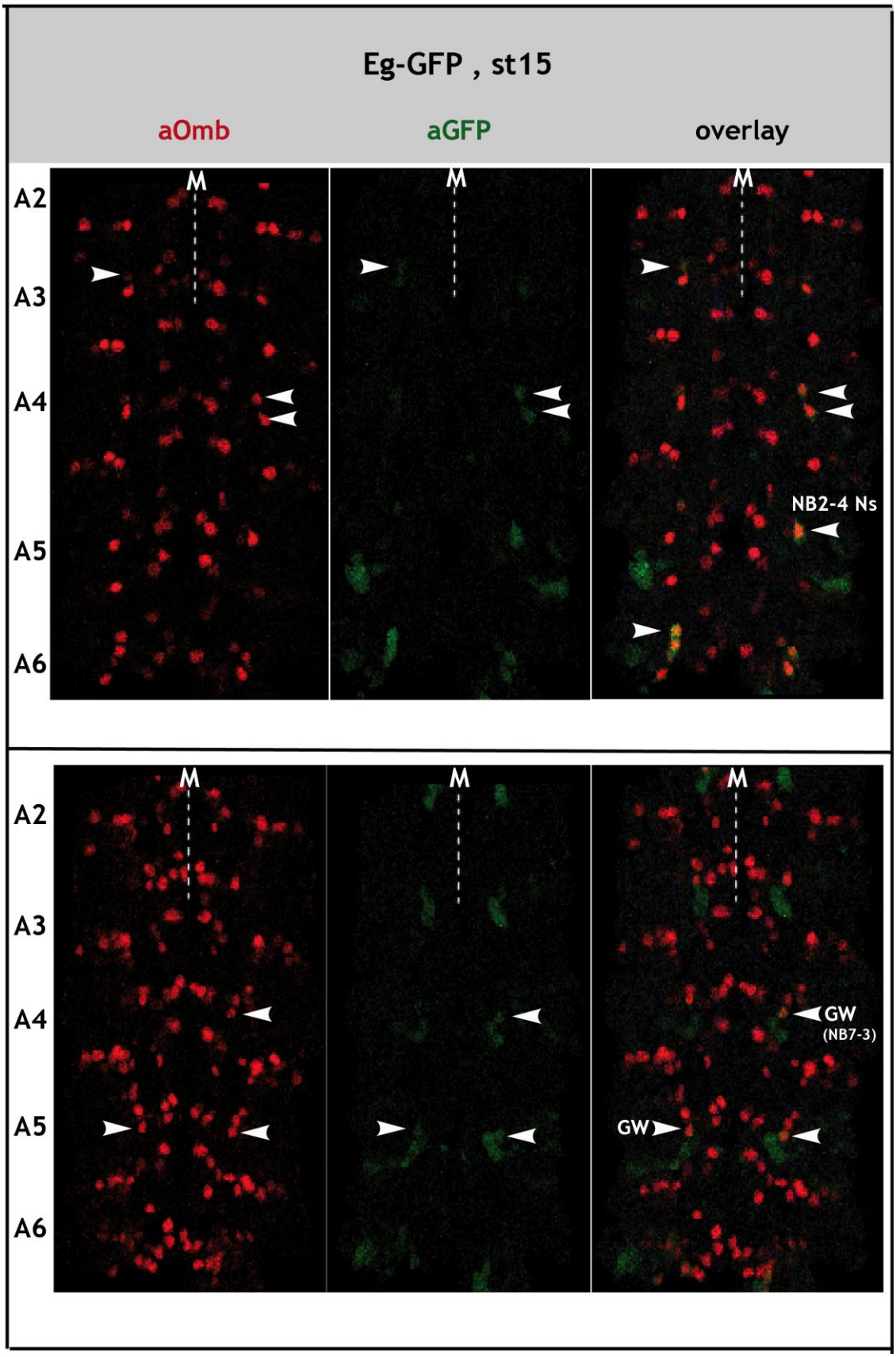


Figure 81. Omb and GFP co-expression in neuronal cells of the fly strain eg-GFP.

CNS, dorsal (above) and ventral (below) view of stage 15 wild-type embryo; Panels from left to right: Omb channel, GFP channel, channel overlay; M,vertical line: the midline; anterior is up. Omb is expressed in a subset of Eg neuronal cells: the NB7-3 GW serotonergic motoneuron and the NB2-4 neuronal progeny.

The following subsets of motoneurons expressing Omb were identified: the NB7-3 GW serotonergic motoneuron and the N2-4 neuronal progeny (Fig.81).

In the *Drosophila* NB 7-3 lineage, activation of Notch signaling induces Zfh-1, necessary for development of the GW motor neuron cell fate. Eagle acts antagonistically to Notch signaling, suppressing Zfh-1 [391]. Double antibody staining against Omb and Zfh1 shows that most Omb positive cells express also Zfh1 {Funktionelle Analyse der beiden Zinkfinger- Homöodomänentranskriptionsfaktoren Zfh-1 und Zfh-2 während der Entwicklung des Nervensystems von *Drosophila melanogaster*, Georg Vogler, Mainz, Univ., Diss., 2007}. *zfh1* contains high-score, high-conservation TBEs. Thus, along with Eg, Omb may affect Zfh1 expression and subsequently the development of the motoneuronal cell fate.

3.3.4 Omb and Reversed polarity (Repo)

repo encodes a homeobox protein, expressed in all lateral glial cells in the embryonic CNS and PNS [392]. *Repo,* being expressed exclusively in Gcm (Glial cells missing) positive glia, is a putative target gene of Gcm [393], the primary regulator of glial cell fate in *Drosophila* [394]. Repo also auto regulates itself; nonetheless, *repo* expression is controlled by additional glial-specific factors [395],[396]. A role has been suggested for *repo* in the migration and differentiation of embryonic glial cells as well as for maintenance of their function [397],[398].

Tested in different embryonic stages*,* similar to stage 14 (Fig. 82), ombP1-lacZ expression resembles the endogenous Omb expression.

Expression of Omb in glial cells is previously reported, in larval optic lobe [30]. There Omb is expressed in the inner optic chiasm glial cells, in subsets of Repo positive cells and in marginal glial cells, being required for glial cell migration and morphology as well as for axonal pathfinding. *omb* transcript is also detected in the eye imaginal disc, in a group of presumptive glia precursor cells posterior to the morphogenetic furrow and in the optic stalk [372].

However, immunostaining of Omb, along with Repo, on embryonic CNS of the ombP1-lacZ fly strain, at stage 14, showed that, Omb is not expressed in any Repo positive glial cells

(Fig. 82). There is furthermore no co-expression of Omb and Repo found, einther in earlier or later stages (data not shown).

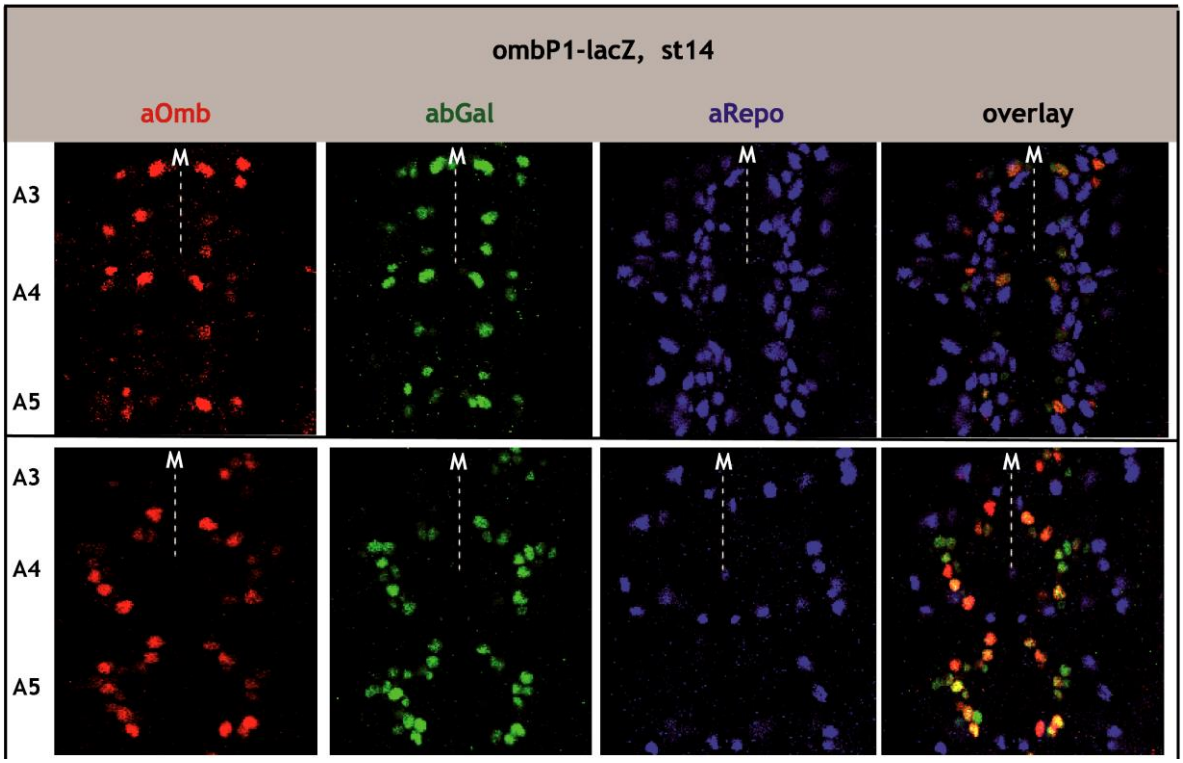


Figure 82. Omb, bGal and Repo expression in the CNS of the fly strain ombP1-lacZ.
Stage 14 embryos dorsal (above) and ventral (below) view; Panels from left to right: Omb channel, bGal channel, Repo channel, channel overlay; M,vertical line: the midline; anterior is up. bGal resembles the endogenous Omb expression. Omb and Repo show no co-expression.

3.3.5 Omb and Deadpan (Dpn), Prospero (Pros)

pros encodes a nuclear protein containing a homeodomain-like sequence. In neuronal lineages of the central nervous system, Pros is specifically detected in a subset of neuroblasts and GMCs, where it is necessary for neuroblast cell lineage and GMC fate specification [399].

Pros acts as a transcriptional regulator in GMCs and young neurons where it down-regulates cell cycle genes and switches on differentiation genes [400]. Neuroblasts lacking Prospero generate abnormal cell lineages that form tumors [63] and neurons lacking Pros differentiate into motoneurons and sensory neurons with axon pathfinding defects [401]. Prospero is conserved in vertebrates, where the *Pr*ospero-related homeob*ox* family affects the activation of precursor differentiation in several tissues [402].

dpn encodes a basic-helix–loop–helix protein [403] related to the vertebrate Hes family of transcription factors. Dpn is expressed in all neural precursors. Loss of Dpn results in weak motor activities, lethargic behavior, and death [404]. *dpn* and is recently reported to contribute to Notch activity in various *Drosophila* tissues [404].

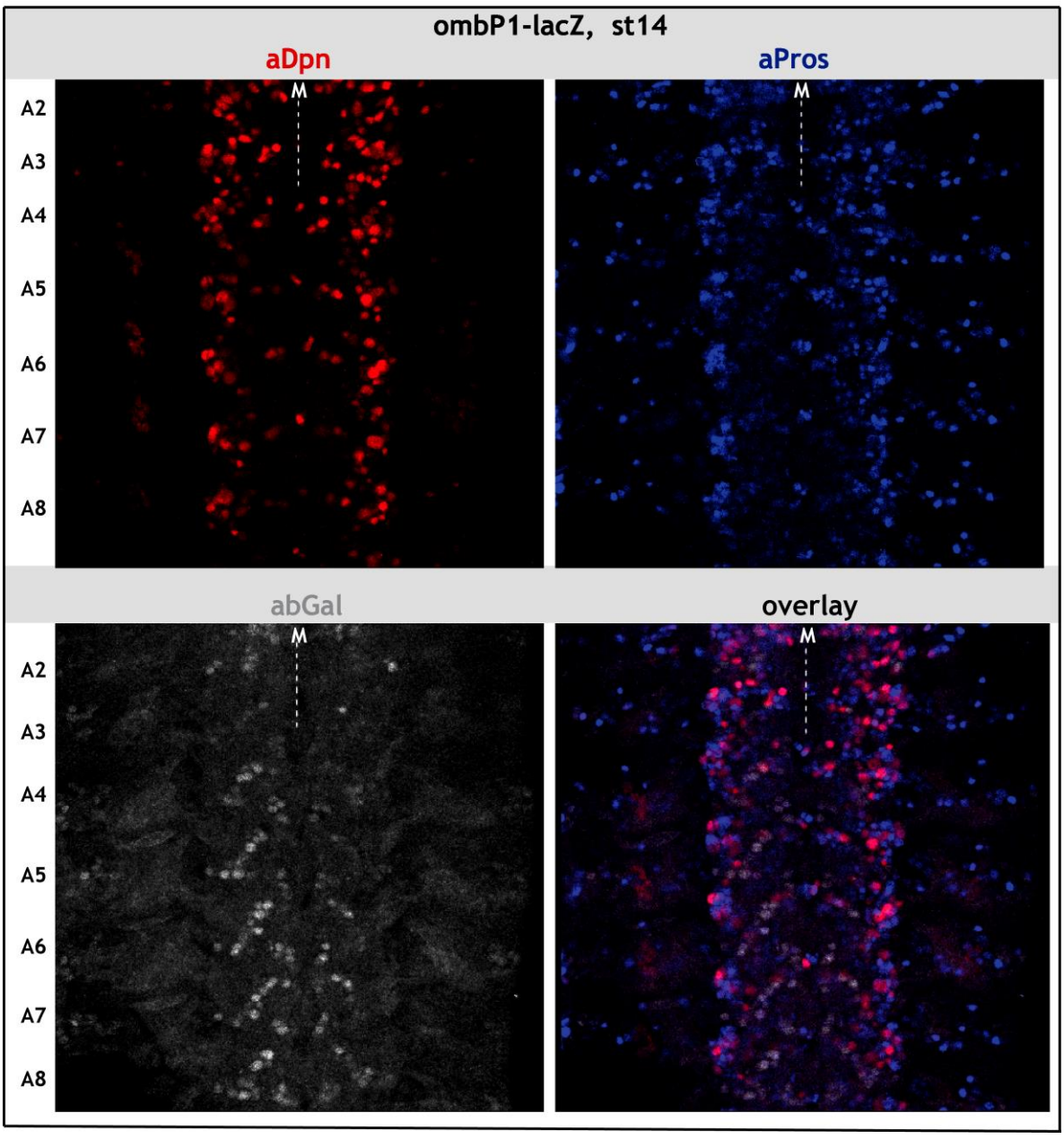


Figure 83. Pros, bGal and Dpn expression in the CNS of the fly strain ombP1-lacZ.
Stage 14; 1st row: Panels from left to right: Dpn channel, Pros channel; 2nd row: Panels from left to right: bGal channel, , channel overlay; M,vertical line: the midline; anterior is up. bGal shows barely co-expression with Dpn nor with Pros at this stage.

Immunostaining of bGal, along with Dpn and Pros, in the embryonic CNS of the fly strain ombP1-lacZ, at stage 14, showed at this stage, Omb is barely co-expressed with Pros or with Dpn (Fig. 83).

A previous study reported that Prospero is required for *omb* transcription, as Omb expression decreased in the *prospero* mutant cells [63]. In this study, DamID was used to identify genomic Pros binding sites. A binding site was identified within the *omb* transcription unit. In addition, a genome-wide expression profiling on wild-type and *prospero* mutant embryos was performed, in neural stem cells of late stage 12 embryonic ventral nerve cords of target genes that contain a Prospero consensus sequence within 1 kb of the transcription unit. *omb* expression shows significant reproducible reduction in *prospero* mutants, a fact consistent with Prospero being required for *omb* transcription.

The staining I performed was during the embryonic stage 14, considerably later than the above mentioned experiment. Although, aPros staining, along with Omb, at stage 14 (Fig.83), shows that, Omb is hardly expressed in any Pros positive cells, the different stage cannot be disregarded. To further study a possible co-expression of Omb and Pros and thus a dependency of Omb expression on Pros, more stainings in different embryonic stages are essential and may lead to a final conclusion.

3.3.6 Omb and Wingless (Wg)

In the *Drosophila* embryo, Wg is secreted in a narrow stripe of cells at their anterior margin of each parasegment. *wingless* is a member of the class of segment polarity genes, vital for several processes including segmentation [405].

Early expression of *wg* is regulated by gap and pair-rule proteins. Later, *wingless* signaling is controlled by Hh and Ci [406], while Ptc acts as a negative regulator of *wg* expression [407]. After gastrulation, *wg* transcription is also dependent on cell-cell communication. Posteriorly, *wingless* is required to maintain engrailed expression in adjacent cells [408]. Anteriorly, *wingless* is necessary for local distribution of the *armadillo* protein [409]. Later *wg* activity seems to be essential to suppress the differentiation of denticles by epidermal cells in the ventral part of each segment [405].

wg, apart from its role in the ectoderm patterning [410] and the neuroectoderm diversity [411], has also a role in heart formation [412]. It has been, furthermore, assumed that *wg* may take part in the skeletal muscle development [413]. The wingless gene is highly conserved in vertebrate and *Drosophila* [414].

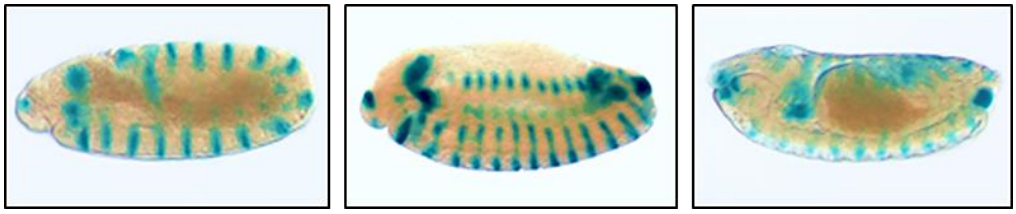


Figure 84. X-Gal staining in embryos of the wg lacZ- fly strain.
Panels from left to right: stage 11late, stage 12late, stage 16; anterior is left. Data from Nathalie Reinhard., practical course.

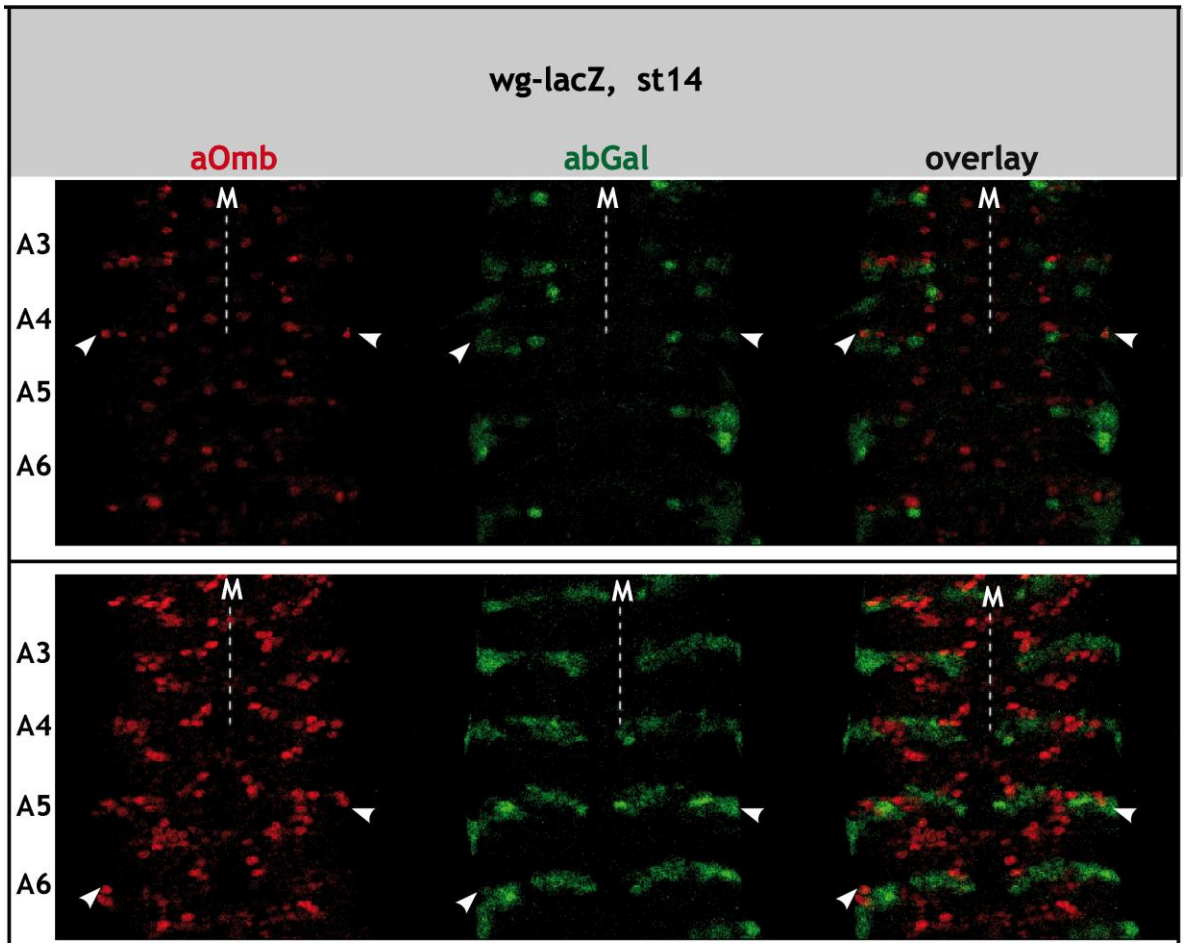


Figure 85. Omb and bGal expression in the CNS of the fly strain wg-lacZ.
Stage 14: dorsal (above) and ventral (below) view; Panels from left to right: Omb channel, bGal channel, channel overlay; M,vertical line: the midline; anterior is up. One bGal positive cell expresses Omb.

In the *Drosophila* wing disc, Omb expression is required for the distal wing development and is controlled by both Dpp and Wg signaling [24]. This kind of interaction between Omb and Wg homologues, Tbx and Wnt, respectively, is also seen in vertebrate appendages [415],[416]. Moreover, the *wg* locus contains a high-score, highly conserved potential TBE.

Immunostaining of bGal, along with Omb, in the embryonic CNS of the fly strain *wg*-lacZ at the embryonic stage 14, showed that Omb is expressed in one *wg*-lacZ positive lateral cell cluster per hemisegment (Fig.85).

Considering that Wg signaling is shown to be active at NB4-2 and its progeny, regulating its formation and identity specification of axon projection [417], and Wg affects, via the segmentation genes *naked cuticle (nkd)* and *gooseberry (gsb),* NB 6-4 and NB 7-3 cell fates [418], further analysis of possible association between the two factors appears appealing.

3.3.7 Omb and Midline/ Neuromancer 2 (Mid/ Nmr2)

mid (also known as *neuromancer 2*) encodes a T-box transcription factor homologue to the Tbx20 family [419]. Adjacent to *mid* lies its paralog *H15* and both genes are required in several developmental processes [420]. In the *Drosophila* leg, *mid* functions*,* along with *H15,* as a ventral fate selector gene [42]. *mid* has also an important role in cardiac patterning and cardioblast differentiation [421].

Even so, *mid* and *H15* are essential in the formation and specification of neural progenitor cells [123]. *mid* is expressed in the anterior part of each neuroectodermal segment. There *mid* is required for the formation and specification of NBs, where it acts redundantly with *H15*, in the regulation of neuroblast marker gene expression. Early *mid* mutations cause segmentation defects [422], while mutations in the mature ventral nerve cord cause loss of neurons and misspecification of individual neuronal and glial fates [123].

Explicitly, Mid is identified, in the *Drosophila* nerve cord, as a neuronal fate determinant, repressing *eve* expression. Mid is also detected in a few Zfh-1-expressing motor neurons. Along with H15, Mid is expressed in neurons deriving from the NB lineages 2–2, 6–1, and 6–2 [129].

It is known that the T-box *omb* and *H15* collaborate for the dorsoventral axis formation of the *Drosophila* leg [423] and arthropod appendages [133]. Interestingly, Omb is, at embryonic stage 16, expressed in a number of Mid positive cells (Fig. 86).

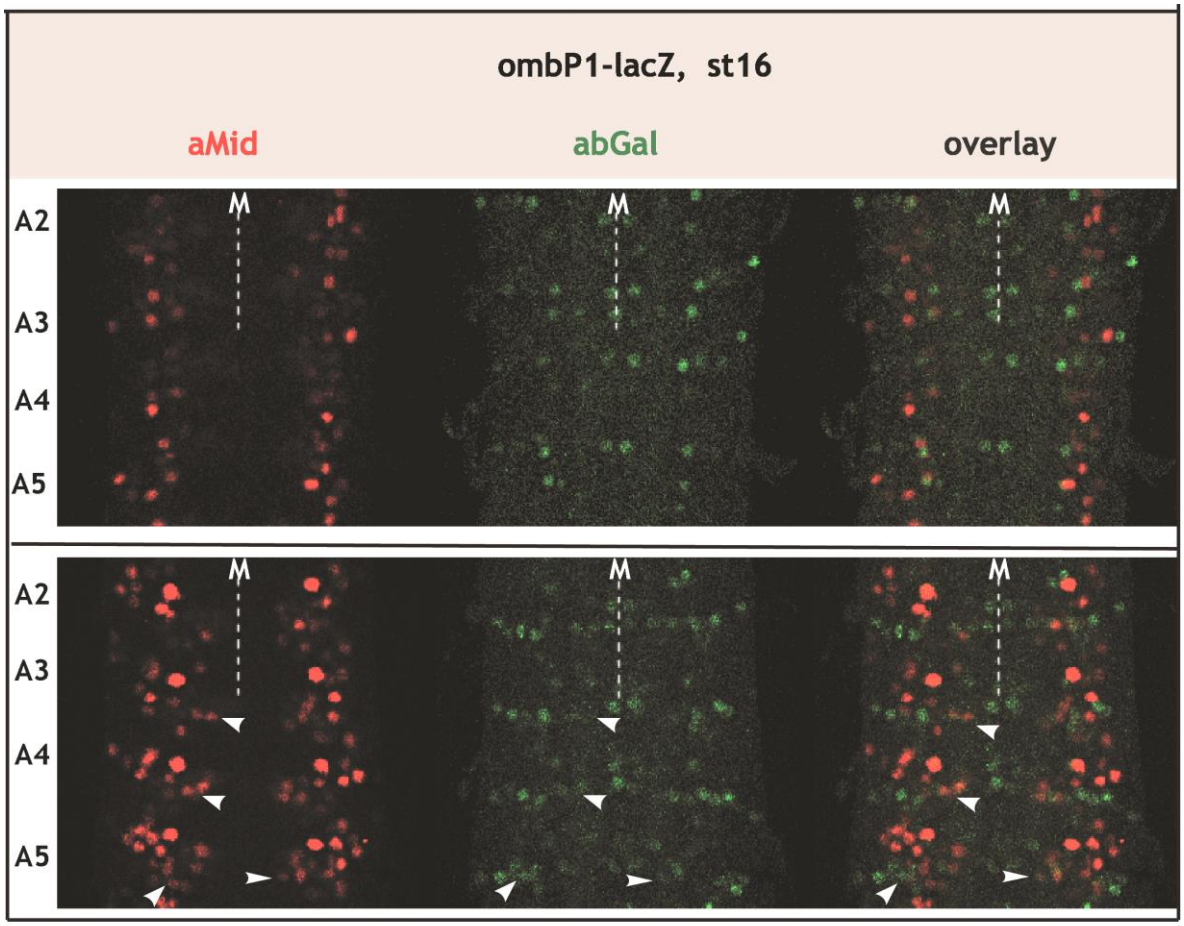


Figure 86. Omb and Mid expression in wt CNS.
Dorsal (above) and ventral (below) view of stage 16 wild-type embryo; Panels from left to right: Mid channel, Omb channel, channel overlay; M,vertical line: the midline; anterior is up.

3.3.8 Omb and OK371Gal4

The OK371 enhancer trap element is inserted in the proximity of the annotated gene *CG*9887, detecting an enhancer of the *Drosophila* vesicular glutamate transporter DVGLUT. DVGLUT is expressed in the glutamatergic motoneurons, initially in their cytoplasmic borders and later along motor axons and at neuromuscular junctions [424].

The enhancer trap line OK371-Gal4 is expressed in a defined subset of neurons from embryonic stage 15 to adulthood. In the ventral nerve chord, it is expressed almost exclusively in motoneurons and in the brain in a limited number of neuronal clusters [424].

Use of OK371Gal4 offers the possibility to test Omb expression in the OK371 positive neurons. Immunostaining of GFP and Omb, in the embryonic CNS of the fly strain OK371CD8GFP (OK371Gal4 x UASCD8GFP), at the embryonic stage 16, showed that at this stage, Omb is co-expressed with GFP in a subset of neuronal cells.

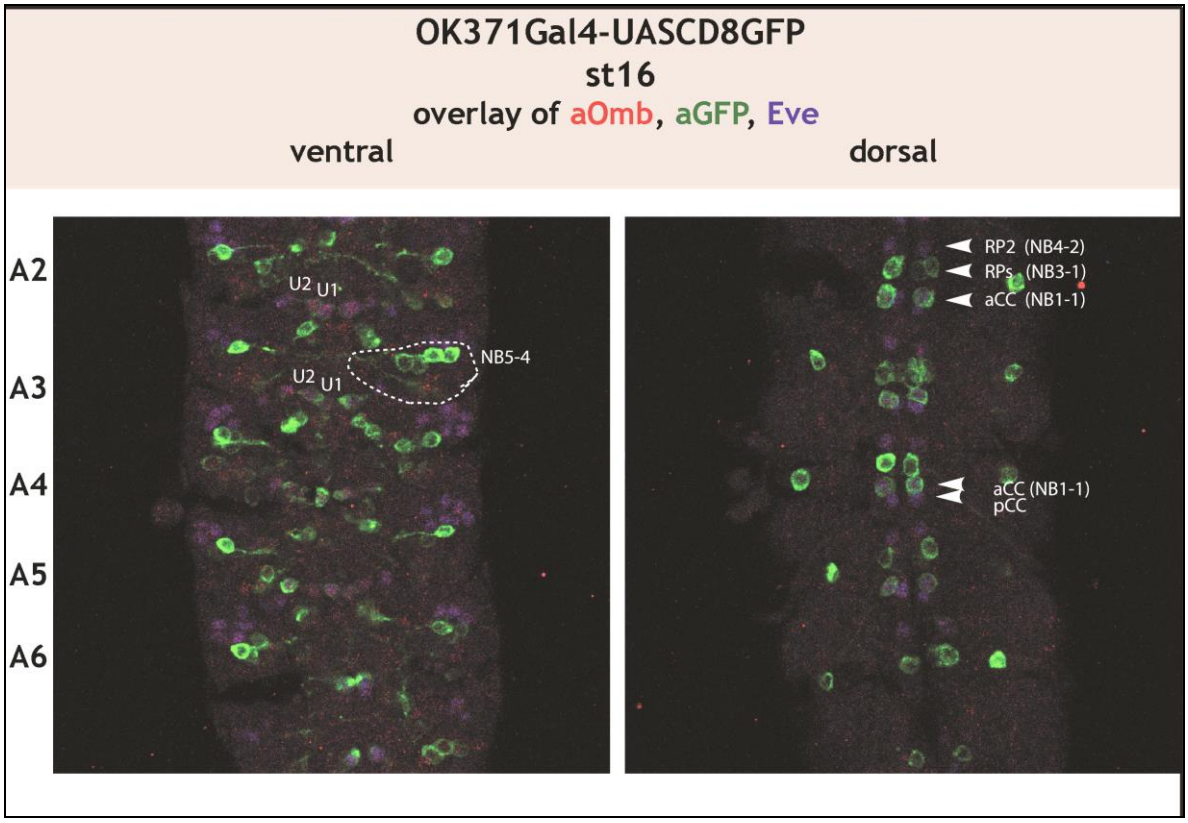


Figure 87. Omb, Eve and GFP co-expression in neuronal cells of the fly strain OK371Gal4>UASCD8GFP. CNS, stage 16 embryos, ventral (left) and dorsal (right) view; channel overlay; M,vertical line: the midline; anterior is up. Omb, Eve and GFP are co-expressed in a subset of neuronal cells.

The results presented above show that during embryonic stage 16, Omb is expressed in OK371 positive cells, like the aCC, and CQ/U. These are also Eve positive, dorsally projecting motoneurons. The CQ set consists of five U motoneurons, U1 to U5, per hemisegment. U1 and U2 are the earliest to express Gal4[1] and can be easier identified (Fig. 87).

The DVGluT widely is expressed all over the Drosophila nervous system consistent with the idea that glutamate is the main neurotransmitter in neuromuscular junctions [424]. Subsequent of Omb co-expression with Eve, the conception of Omb having a role in the motor axon projection arose. Its detection in glutamatergic motoneurons supports this idea.

4 Conclusions

In a prior bioinformatic analysis by Hüyseyin Binbas, potential Tbx targets sequences in wing-related genes have been identified. Guided by this information, enhancer trap/reporter lacZ insertions were characterized by X-gal staining first in wildtype and then in *l(1)omb* imaginal discs.

In several lines I observed an increase in reporter expression in a *l(1)omb* mutant background. Since Omb is assumed to function predominantly as a transcriptional repressor, this may indicate direct regulation. Repression by Omb was observed e.g. for *brk* and *tkv*. These genes are negatively regulated by Dpp, while *omb* is induced by Dpp. Omb which mediates the effects of Dpp on proliferation could, thus, also mediate the Dpp effect on patterning of the wing disc. However, *brk* and *tkv* were not completely derepressed in *l(1)omb* indicating that Dpp represses these genes also by an Omb-independent mechanism.

More frequently I observed loss of reporter expression in an *l(1)omb* mutant background. In these cases, regulation by Omb presumably is indirect. For example, STAT92E-lacZ expression in the wildtype eye was symmetrically expressed at the dorsal and ventral margins. In *l(1)omb*, ventral expression was selectively lost. Loss of *omb* is known to cause ventral overproliferation of the eye by activation of the Jak/STAT pathway. *STAT92E* expression is negatively regulated by Jak/STAT signaling suggesting that loss of *omb* activates Jak/STAT further upstream in the pathway.

Regional overproliferation of eye and wing in the *l(1)omb* mutant background proved a complicating issue in the search for Omb targets. This effect made it difficult to decide whether an expanded reporter expression pattern was due to tissue expansion or reporter gene derepression. For instance hth-lacZ appeared to expand along the ventral eye disc margin in *l(1)omb*. Without addtional experiments it cannot be concluded whether this is due to de-repression or to activation in association with the proliferative state. Parallel to my experiments, evidence accumulated in our laboratory that loss of *omb* may attenuate Wg and Hegehog signaling. Since these diffusible proteins are the main patterning molecules in the wing imaginal disc, with *dpp* being downstream of Hh, many of the observed effects could be secondary to reduced Wg and Hh activity. Examples are ab-lacZ, Dll-lacZ and vgBE-lacZ (reduced expression on the dorso-ventral boundary) and inv-lacZ (late larval expression in the anterior wing disc compartment is lost) or sal-lacZ. Epistasis experiment will be required to clarifiy these issues.

Furthermore, loss of *omb* appeared to induce cell fate changes. It was reported previously that in an *omb* null mutant, the dorsal determinant *apterous* (*ap*) is ectopically expressed in the ventral compartment (an effect I did not observe with the strongly hypomorphic *l(1)omb*^{15}, indicating strong dose dependence). Ventral repression of *ap* is maintained by epigenetic mechanisms. The patchy and variable nature of ectopic expression of *ap* or grn-1.1-lacZ points to an effect of *omb* on epigenetic stability.

In the second part of my thesis, an analysis of Omb expression in the *Drosophila* embryonic ventral nervous system was performed. Omb was found co-expressed with Eve in the medial aCC and RP2 motorneurons as well as the fpCC interneuron and the mediolateral CQ neurons. Additionally, Omb was detected in the Eg positive NB7-3 GW serotonergic motoneuron and the N2-4 neurons. Omb was not found in Repo positive glial cells. During embryonic stage 14, Omb showed some coepression with Dpn or Pros. At the embryonic stage 16, Omb was expressed in minor subset of Mid and Wg positive cells.

5 Appendix

5.1 List of Abbreviations

A	anterior
AP	anterior-posterior
BM	branchiomotor
BMP	bone morphogenetic protein
CNS	central nervous system
D	dorsal
DV	dorsal-ventral
DVGLUT	vesicular glutamate transporter
EGF	epidermal growth factor
EGFR/ DER	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
GMC	ganglion mother cell
IN	interneurons
ind	intermediate neuroblasts defective
ISN	Intersegmental nerve
JAK/STAT	janus tyrosine kinase/signal transducer
LB	Luria broth
MF	morphogenetic furrow
MN	motoneurons
msh	muscle segment homeobox
NB	neuroblast
nkd	naked cuticle
nmr	neuromancer
P	posterior
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PD	proximodistal
PWM	conventional binding score
SN	segmental nerve
slou	slouch

Tbx	T-box
V	ventral
vg	vestigial
VM	visceromotor
VNC	ventral nerve cord
X-Gal	for 5-bromo-4-chloro-3-indolyl-β-D-

Table 8. General abbreviations used during this study.

ac	achaete
ap	apterous
byn	brachyenteron
bi	bifid
Brk	Brinker
Ci	Cubitus interruptus
col	collier
DER	Drosophila EGF receptor
Dhh	Desert Hedgehog
Dll	Distalless
Doc	Dorsocross
Dome	Domeless
Dpp	Decapentaplegic
en	engrailed
Fj	Four-jointed
Ft	Fat
Fz	Frizzled
gcm	glial cells missing
Hh	Hedgehog
Ihh	Indian Hedgehog
iro	iroquois
lb	ladybird
Mad	Mothers against dpp
Med	Medea
mid	midline
Mtv	Master of thichveins
omb	optomotor blind

org-1	optomotor-blind-related
Ptc	Patched
Punt	Put
Sax	Saxophone
Shh	Sonic Hedgehog
Tkv	Thickveins
upd	unpaired
vg	vestigial
vnd	ventral nervous system defective
Wg	Wingless

Table 9. Gene name abbreviations used during this study.

5.2 TBE List

Gene	Ch	BSd	BSseq	ScoreBs	BSposWoFlaLen	D. mel Score	AvAllSpecScore	AvUp %	AvBS %	AvDown %
ap	2R	rev	ATTCACACTT	AAGTGTGAAT	1623432	8,14	8,14	69,27	100	63,82
argos	3L	for	AGGTGTCAAA	AGGTGTCAAA	16455125	8,72	8,18	46	94,55	71,64
	3L	rev	TTTCACACCT	AGGTGTGAAA	16473099	10,34	9,19	65,11	88,89	72,45
brk	X	rev	TTTCACACCT	AGGTGTGAAA	7214632	10,34	10,34	68,4	100	57,2
bun	2L	for	AGGTGCGAAA	AGGTGCGAAA	12525389	8,02	7,56	76	95,71	64
dac	2L	for	GGGTGTGAAA	GGGTGTGAAA	16481745	9,51	9,51	68,55	100	62,54
Dll	2R	for	AAGTGTGAAA	AAGTGTGAAA	20698330	9,06	8,86	73,64	94,55	60,55
ds	2L	rev	GTTCACACTT	AAGTGTGAAC	632709	8,38	8,38	66	100	50,55
	2L	rev	TTTCACACCT	AGGTGTGAAA	660558	10,34	8,47	63,11	91,11	75,56
dve	2R	for	AGGTGTGAGA	AGGTGTGAGA	18165193	10,93	8,55	64,55	94,55	70,91
grn	3R	rev	GTTCACACTT	AAGTGTGAAC	3995394	8,38	8,63	77,27	93,64	78,73
neur	3R	rev	GCTGACACCT	AGGTGTCAGC	4838976	8,63	8,48	92,36	97,27	54,36
salm	2L	rev	TTTCACACTT	AAGTGTGAAA	11449223	9,06	8,9	72	98,18	64,91
Sdc	2R	for	AGGTGTCAAG	AGGTGTCAAG	17303324	8,44	8,34	67,64	94,55	81,09
	2R	for	GAGTGTGAAA	GAGTGTGAAA	17341013	8,23	8,23	68,22	100	89,11
Stat92E	3R	for	AGGTGTGAAA	AGGTGTGAAA	16368590	10,34	8,11	69,64	90,91	65,09
wg	2L	rev	TTTCACACCC	GGGTGTGAAA	7304515	9,51	9,51	90,18	100	68,73

Gene	Ch	BSd	BSseq	ScoreBs	BSposWoFlaLen	D. mel Score	AvAll Sp	AvUp %	AvBS %	AvDown %
vvl	3L	for	GGGTGTGAAC	GGGTGTGAAC	6778400	8,83	8,83	73,64	100	73,64
		for	AGGTGTGAGA	AGGTGTGAGA	6780774	10,93	8,51	89,27	82,73	57,45
hh	3R Intron 2 (TBE3)	for	TGGTGTGAAA	TGGTGTGAAA	18956417	8,46	8,46	72,55	100	73,46
	3R Intron 1 (TBE5)	for	AGGTGTGAAC	AGGTGTGAAC	18960798	9,66	9,64	80,36	99,09	58,18
tkv	2L	for	TGGTGTGAGC	TGGTGTGAGC	5236952	8,37	8,37	63,56	100	99,33
	2L	rev	TTTCACACCA	TGGTGTGAAA	5252533	8,46	8,46	97,78	100	71,33
tkv	2L	for	AGGTGTGAAA	AGGTGTGAAA	5253613	10,34	9,68	79,78	97,78	85,11
	2L	for	AGGTGTGAAG	AGGTGTGAAG	5259079	10,06	10,06	87,56	100	86,45
vg	3L	for	GGGTGTGAAC	GGGTGTGAAC	6778400	8,83	8,83	73,64	100	73,64
inv	2R	rev	GTTCACACCT	AGGTGTGAAC	7389071	9,66	9,66	95,64	100	78,91
		rev	AGGTGTGAGA	AGGTGTGAGA	7389449	10,93	8,39	74,89	81,11	60,89

Gene	Chr	BSdir	BSseq	ScoreBs	BSposWoFlaLen	D. mel Score	AvAll Spec Score	AvUp %	AvBS %	AvDown %
hth	3R	rev	TTTCACACTT	AAGTGTGAAA	6332256	9,06	9,06	89,09	100	80,55
		for	AAGTGTGAGT	AAGTGTGAGT	6355510	8,73	8,51	88,91	87,27	86,91
		for	GGGTGTGAAT	GGGTGTGAAT	6384361	8,59	8,09	76,8	87	54,2
		for	AGGTGTGAAG	AGGTGTGAAG	6387276	10,06	8,09	85,6	95	64
		rev	TTTCACACTT	AAGTGTGAAA	6389415	9,06	9,06	78	100	71,45
		rev	CCTCACACTT	AAGTGTGAGG	6398368	9,37	8,04	93,45	90,91	75,64
		rev	TCTCACACCT	AGGTGTGAGA	6423319	10,93	8,88	64,55	90	70,73
		rev	ACTCACACCC	GGGTGTGAGT	6443649	9,18	8,76	87,5	97,5	43
		for	AAGTGTGAAT	AAGTGTGAAT	6457314	8,14	8,09	62,18	98,18	91,45
		for	AAGTGTGAGG	AAGTGTGAGG	6459996	9,37	8,32	65,09	89,09	57,46
		rev	TTTCACACCC	GGGTGTGAAA	6471194	9,51	9,51	83,46	100	98,73

Table Group 10. TBE lists.

5.3 Sequence Data for the cloning projects

5.3.1 *vg* boundary enhancer fragment (vgBE**[1.1Kb]**)

Fragment	lenght	Oligos used	Oligo Sequence	TBE sequence
vgBE[1.1Kb] ^{wt}	1055 bp	1159 vgBE-1534for	AATTTGTTTGAACGGGCTTGG	TTCGACACTT
		1160 vgBE-2568rev	TATTAGCTGCAGGCGATATTT	

Fragment	lenght	Oligos used	Oligo Sequence	TBE sequence
vgBE [1.1Kb] ^{TBEMUT}	1055 bp	1161 vgBEmut-for	CGTCCGCCCTTTCGAAACTTTGGCCAGCCAGC	TTCGAAACTT
		1162 vgBEmut-rev	GCTGGCTGGCCAAAGTTTCGAAAGGGCGGACG	

Table 11. Information about the vg boundary subcloned enhancer fragment (vgBE[1.1Kb] wt) and its mutagenesis (vgBE [1.1Kb]TBEMUT) .
Sequence length, oligonucleotides used for its amplification, and in red the nucleotide mutated in the encompassed TBE sequence.

vgBE**[1.1Kb]**^{wt} **(oligos , TBE site)**

```
   1 AATTTGTTTG AACGGGCTTG GAGCCTGAAT AATTAGACGC TTCTAACAAA
  51 TCATAAGAAT TCACGCAGAA CGCAAATCGA TTTGGGAAAC CCGAAACAAT
 101 AAAGAACCGA AAAAAAGAGA AGTTGCCAAA CTAAAGGGGC AACATTTCAA
 151 GGAATTCCGC AACTCAATGT TGGCTTTGTT TCGCCTCTCC CGCTTTTTGC
 201 TAACATTGAT TTCGAAGATT TCGCTGTGAT TTCTGTGACA AGTACAGAAA
 251 AGTTCTCACG ATCGCTGGTT TCCAGTGTCC AGTTTCGAGG GCCCAGAACT
 301 CAGGCCGACC ATGGTCAAAA GGCGGCTGGC AGTGCGGCCA ATATCCATGA
 351 GGCAGTTGCC GTTGCTGCTG CCGCTGGGGC ACAAAGAAAT TCTTGTGTCG
 401 CAAGTTCCAA TTATACAAGG CCGCGGCACC AAATCGAAAT TGGCCTTTTA
 451 TGTAAGTAAC CGATACGACA CGATTCTGAT TCTATGCTTT TGCATGCCCA
 501 TAAAGAGGGA CCGCGATAAA CCCGCCTGGA TATTGCGGCT TCGGTTGCTC
 551 CTTTCTGCTC CTTTCTGCAC CTCCGCCATG GAAAGCCAAT CGCAGATTGA
 601 GTCAGCAGCT TTGGCTACTC AGCTGGATGA CCGGTTTATT TTGTCCCACA
 651 AACGCACAAT CCTCGTCCGC CCTTTCGACA CTTTGGCCAG CCAGCCAGCC
 701 AGCCAGTCCC GAAACCTAAA GTTAAACTAA AACAAATAAA CAAACCAAAA
 751 ACCTTGGTCC GCTCGGTTAA TTTATTGTGT GTATCGCCAT TAGTTCTTGC
 801 CGATCTTGCA GCTCTCCCCG CCTACAACGC CATAACATAA CCATACCGAT
 851 TCGATCCTTG GGACCTTTTG TCAACCGATC GCAGATAAAC CAGGGAGCTG
 901 AATTCAAAAA ACAAAGAACG AATGAAAAAA AAAAGCGAAA AGTTTTGGGG
 951 CTCCGAAATT TTGTCGCTGG TTCGTCTGGC GATTCCCGGT GATTTATGCC
1001 GTGTGATCGA AGGTCTGACT GCTCGGCATA ACCGAAATAT CGCCTGCAGC
1051 TAATA
```

vgBE fragment encloses a reverse TBE (grey underlining) with conventional binding score (PWM) higher than 4.46 and almost total preservation of its sequence (ttcgacactt) in all *Drosophilidae* except *D. ananassae.*

TBE conservation_alignment (8776857 – 8776928), (**TBE**)

```
D. melanogaster  cacaaacgcacaatcctcgtccgccct----------ttcgacacttt----------------g----g
   D. simulans   cacaaacgcacaatcctcgtccgccct----------ttcgacacttt----------------ggccag
  D. sechellia   cacaaacgcacaatcctcgtccgccct----------ttcgacacttt----------------g-----
     D. yakuba   cacaaacgcacaa-----------------------tcgacacttt----------------c-----
     D. erecta   cacaaacgcacaatctttggccgccct----------ttcgacacttt----------------g-----
  D. ananassae   ttccaatacccgattcccgattcccttagaaaccaaaacgcacaattt----------------t-----
D. pseudoobscura ggacaacgcacaat-tttgtcctcccg----------tcgacacttt----------------g-----
  D. persimilis  ggacaacgcacaat-tttgtcctcccg----------tcgacacttt----------------g-----
  D. willistoni  agcagacgcacaat-tttgtccaaccg----------tcgacacttt----------------g-----
     D. virilis  aagttgcgcacagt-tttgtcgtcccg----------tcgacactttgctttttggcgggcc------
  D. mojavensis  gag-------cagt-tttgtcgtcccg----------tcgacacttt----------------------
   D. grimshawi  ==========================================================
     A. gambiae  ==========================================================
```

5.3.2 vvl1+2 **enhancer fragment (**vvl1+2 ***[1Kb])***

Fragment	lenght	Oligos used	Oligo Sequence	TBE sequence
vvl1+2 [1Kb]	1000 bp	1333 vvl-1334for	TAATTGCGTCCGA AGCGAACC	AGGTGTGAGC
		1334 vvl-2313rev	GGAGAGGGCCAG CATGTAGAT	

Table 12. Information about the vvl1+2 [1Kb] subcloned enhancer fragment.
Sequence length, oligonucleotides used for its amplification and the encompassed TBE sequence.

vvl1+2 ***[1Kb],*** **(oligos , TBE site, Sotillos vvl1+2enhancer core)**

```
  1 TAATTGCGTC CGAAGCGAAC CCCTTGAAAT TCCCGAAAAT GCCTAATAAG
 51 ATCACAGGAA CAACAGTCCG CACTCGATAA GTGGCCATGA CGTTTGACAT
101 TCGCTGTGGT CCTTTGGCCA TTTAGTGAGT TCAAAGGTTA ATGATGGCCA
151 CACAGGCGAC AACTGGCGAC TGAGATCTTG TCCCGCAATT TCCCGATCAT
201 TTGCTCAGAT ACGATACGGA TTCTGCGAAG TCACCGGGCA AAGGCATCCC
251 ACTGAGTTTG TTGGCTTCTT GTCAAACAAA TCATTTACTG CGGATACTCC
301 TCGATATTCC TCAAGATACT ATCCATTTCA CTGTGTAGGT GTGAGCTGCA
351 ATTTTCCCTG GAAAAATTAC GTCCAGCACG GGATTTGATT AATTTATTAC
401 CGCTTGTCGA AGGGAAAGTG ATCTTCGGGT TCCTAACGGT TGCGGATCGT
451 AAAAAACATT CGGCAGACAC AATTGTTGAA TTATCTGCGG GCTGCTGTTG
501 TGGGCACTTT TTTATGAGTT ATTTATGTGC GACTGTGGCG CCAACAGGAT
551 CATAAAATAT GTAGTTATGG GTAAATCTGT GAAAATAAAT GTAAGCGTAA
601 TCTGGAAAAT ATTGAGTGGC CTAGATTCAC CACATTAGTC ATTTCAAAGA
651 AATTAAAGAA AACTAAAGTA GAAGTATAAA AATTTTAAAT TGTAATGATT
701 TAAATATATC AAATAATGCA AATTTTAAAA AATTAATTTG ATTACTGCTT
751 TATTAAAATT TTTATTTCAT ATTAATATGA TTTATCGTGC ATACGGAAAT
801 TAAACTGGAT TATGGTAATG AAATTTATAA ATAAATCAAA TACAGAGTAC
851 TTGTAGTAAT GTTAAAACAA AACTGAAAAC TGATTGTTGA TTAATTGCAT
901 GCCATATTTG AAGAAGTTAT AGAAAGTAAT TCATGGGTAT AGAACTGTCT
951 TAAATTGTCA GATAATATTT CTGTTGATAA TCTACATGCT GGCCCTCTCC
```

TBE conservation_alignment (6757786 - 6758358), (**TBE**)

5.3.3 *tkv* **enhancer fragments (*tkvA, C, D*)**

Fragment	lenght	Oligos	Oligo Sequence	Chr. location	TBE sequence
tkv A [5.2Kb]	5177 bp	970 tkvfor235	CTAATATGC CCCCAATA GAATG	2L: 5236001-5241500	TGGTGTGAGC
		971	TATGAGGA		AGGTGTGATT

		tkvrev5390	TGCCGACA ACGAGA		AGGTGTTAAA

Table 13. Information about the *tkv* A [5.2Kb] subcloned enhancer fragment.
Sequence length, oligonucleotides used for its amplification and the encompassed TBE sequences.

tkv A [5.2Kb] *vvl1+2,* (oligos , TBE site)

```
   1 CTAATATGCC CCCAATAGAA TGCCGGACAA TTGTTTTTCC CCCTGGTTTC
  51 AAGGTGCACG AGAGATAAAA AAAAGCAAAG AAATGTTATA TTAATATTTA
 101 CCATGAGCCT TCTTCTTTCT GGATTTCGGC GCCATGTCTG ATGGCTACAA
 151 CAAGAGCAAC AACAACAACA GCAACGACGT GGGCCAAAGG CTGATGATTT
 201 TTATACGGCG GCAACAACTG CAATGCAACT AAGTTGTTGT TGCCCCAACC
 251 GATCGTTCTT GGTTGTTTTT GTTGGCCTTC CTCTCTATAT AGAGAGGGTA
 301 TCGGGATCCC GGGTGTTTTG GGGCCAGTTT CCCCTTCGAT ATCCCCTCGT
 351 TGCCACGTTT ATTTTTTCCG GGCTATGCAG CAAAACAATA AACGCGAGGC
 401 AGCCAGCAGC AACGAAAGCA AAACAAAAGC ACCAGGAGAA CTGGATTCGT
 451 TGTATCTCGT CGAGTCTTCG CGTTGTTATG GAGTCGCCGT CGCAGTCGAA
 501 GTTGGAGCTG GAGTTGGGGT TGGACTCCTC TTGGGTTTGT TTATAGGCAG
 551 GCGGCGGCGG TCAAGCCGGC GACACCATGG AGCCCGCGAA GAAAAAGAA
 601 AAGAAGAGGA GCAGGAAGAT GAGCGAAATT ATGGGTTTCG CCAAAAACTT
 651 GATCCTGCTG CTGTTGCTGG CGGTGGTGGC GTTGGTGCTC TGCTGCCGAT
 701 GTGCTTTTGC TTTTGCTTGG TGTGAGCGCG TGATCACGTT GATTATAATT
 751 ATATACGAAA GGTGGCAATA CAAATCAGGT GAAACTCAAT CCGCAGTTAA
 801 GGCATATCCT GTGTGTTTCT GGTTGTTTTT CCCCAGTGTT TACACTATTT
 851 TGTTTATTCG CACTGGCACT TGTTTAAGCA TAGAAATATA TGTGCAATTT
 901 CCCATTTAAA TCATTAACTA TAGATTGCCG TTGATGCACT TTTATTATTC
 951 GTTTTTCATT AGTTAACAAT ATTTCAGCTT AGGCACACAG GTTTTTGGTT
1001 GAAACTCGAA AATATTTCAC GCGTAAACAA AATCGACGAT TCTTTGGATT
1051 GTATCTCAAA ATATGGTTTA TTTTTCCAAT CCGGTGGATA CGTCACGACT
1101 CGCGAAGAAC GTATCGACGA ATGTGCAACA GTTGGAAACG CCGCTGCTGG
1151 TAGACCCGAA AAAGAGAGCG CAGAGAGTGC GCTGCTCTCG CAAAGAGAGA
1201 GAGAGTAGAC GGGAAAAACA TGAGAGCGTG GCGGAAAACG CGAGAAAATG
1251 CTGGCAGGCG TTTAAAATCG CGCAACTCAC ACACTTGCTG GCGTTTAAAA
1301 ACGCGCGTGA GGTAAAAATA CTCTCTCTTC TTGGCTCTCT CCCTTTGCAT
1351 TTGTTCTTGT TTTGCCAGCT CTCACTTGAA ATTCAAGTTT TTTTTTCTCA
1401 CAAAGGTGTG TGCGTGTGTA TCTTGATCTT TGAGTTAGCA TCGCAGTGCT
1451 GTAGTTGTTG TTGTTGTTGT TCGTGCTCGT ATTTCTTTAG CTGCTTATTA
1501 TAACTGACAT ATCTAGATGG GGAATACGTA CGTACGTACA TATGGTGGGG
1551 AGGTATCAGG AGAATCGGAA TGGCCTGCAT AATTGCAGGG CCCTTTTGCA
1601 TGCACACTAT GTGCAGGGAA TAATAACAGT TTTGGTCGAT TTTGTAAGCG
1651 AACAAACGTG TGATTAGCAG CAACACCATT GCCAACTAAT AGAACTCCGC
1701 CTCCTGTTCT CCCGAGCGCA TCCACAAGAT AGTATCCCAC CGCCACCGCC
1751 AAACGCATCC CCAAGATATC AGTTTTACAG GCTCTCCGCG GATGGTCTCA
1801 TTAGGCGCAC AGTAGTCGTT GCTAATTATG GCCTAGACTC TGAGGCTTCC
1851 CATTCTTTTA CCTGTGGAAA TCAGAAGCGC TTAGCACTTC GGGTTTGTTC
1901 AGGTAAACTC CTGGCATTAA AAACGCATTT GAACCAGCGG ACGACCTCTG
1951 TAATCCGCGC ATAACTTGCT CATTTCGTCT ATTGCTCGCG ATAACAACCG
2001 TCAGAATCTA TGCCTCCCGA ACTTAAACCT CCACCTTGGC CATATCTTTT
2051 TGGTTTCACA CAAAAAAAAA TTTAACAAAG TTCTCAATAA TCACTAAAAA
2101 TAAATATTTA TATAGAAGAA ATGCCATTTT ATTGCTATAC TATGAATTTT
2151 AAGACCTAGA AATATAAAAA GTATATTTGT ATAAATTCTT CTCAATTTAA
2201 ATCGCATCTT TTTTCATATA ATTCCAAATT TTTCTTCGTG CATTTCCACT
2251 TGAGTTCCAG TGGTGATCTT TCGTTTATTA TCACGCCTGC GGCCATAATT
2301 CATTTCGATC TGGTTCGGGG TCTGCTCTGC TTTTGTCTAG TCAAAATCTC
2351 TGGATTATGC CCCCAGCTCC AGTTCCAGCT GCAGCTCCCT AAATGCCCGG
2401 ATTGTGGATG TGGCTGCTGA GGCTGATGCT GCTGCTGCTG CTGGTGCCTC
2451 CGAGGTGTGA TTTCTCAAGT GTAGGAGTCA TGCGGTGCAC AGCCAGACGC
2501 TGAGAGGTTG CACCAAAGGC AGAGAAATCG AAAAGAAGGC GAAGCGAACC
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2551 AAGAGGGACA GAGAAGAAGA CTGGCAAAGG GACTTGGATA CAATCGGCCA
2601 GAGGGCAACT GTTCGTGTAC CCCTTTCGAT GAGAGGAGCC AAGAAATAAA
2651 GTTGGATCTG GGGATGCCGT CAATTGAGTG TAATGGAAAG CAACATACCA
2701 TTTATATTTA ACTCGTCAGA GGAATTAAAA GAACTTAATA TACCATACGT
2751 TATCTTATCA TTTCTTTTAA GATGGGGTTC TTTAAGCAAA TTTAATATTG
2801 TTTTGTGTTT ATCATTTCTT TTAGGTTCTA TGTGTCTCTC ATTTTTATTC
2851 TGACGATTTT TTAAATATAA ATATGAAGTT GATTCAACCG AAATGTGTAA
2901 GGTAAGAAGA ATTGCATTGA TCGTTTTTCC AAATTTATAT CATTAATACA
2951 ATATACTGTG GCGGCTATAA ATCTAATTAT TTTTTTAGAC TTAGTTTTTT
3001 TTAGACCATG TTAAAAAACC AATTTATCAT CTTTTCACTG GTCCGCTACG
3051 CTTGAGAAAT TGACAGTTTT GCAGTTAAGT GGAATGTGCA GAAGGCATGA
3101 CTTGTGGATC GCAATTGATT AGAGGCAGAG TTCTGAAAGT ATCTCTAGCG
3151 CGAAATATCC GAACGATCCG TGCTAATTCT CGCAATAGGA GAACGCGATT
3201 ATTTGCTGCA TTGTCCAATT AGCACCTGTG CTAAAAACCT TTCGATTTGT
3251 TCGTTCCATT AACACTGCAC TGTTCCCCTC GAAATTGTTT GCTCTTTCGC
3301 TCGAGTGGAA ATGCGGGGGC TAAAGGTTGA TTCTAGCCAT ACATACATAT
3351 TTACTTTGAA ATCCAGACAA TAGAAAATCC ATGTTGGATT TTCGAGTATT
3401 TCGTGTGAAA AACGCTGGAG GGCAGGGGAA CACTATTCCA TCCATACAAT
3451 AAACACAAAA CGGGGCCGAC ACTCCCAGGG AAAAGGCCAG ACAAGCTGGC
3501 GGGCCGAATG TATCTGTATC TGTAACGAAT CCGACTGTAT CTCTGGAAAT
3551 ATTGTTAGAA CTGAGCATTA GATACACAAA GCTGCAGAGA GGTGAACCCC
3601 GGGTCACTGC AACATGGCCA ACAGATGTGC GCAACAGGCT CCCAATCAAC
3651 GAAATCACCA GAAATCAGAC AAAAGACGAA GGTCTCGGAA AAGGTCCAAG
3701 ATGTCACTTG GAGTGTGGCT GGGCCCTAAA TCAAAGTCAG GCAATGCTAC
3751 CCGATTGTTC TTGGTCTTTT TTTTCCTTTT GCTCCGTCAG TCTTTTGGTC
3801 GGTAGCACTG TCCAGCATCT CATGGATACC AGTCTGCGTA TCTGTATCTC
3851 TGCAGCATGG AAATTTTCGT ATTGTACCAG AAATTTGTGT CAACGTGGGC
3901 GACACTTTAT TGTGTGCCCG TGTTGTTGGG TGTGTGTAAA AATAAACCAA
3951 AGACATTTCA TGTTGTCAGC AACATGTGGA AAAATGCTCG GAATTCGTTT
4001 CGTTCTTGCA CACTTTTGCA ATGAATTAAA ATCTGATAGA TACACACATG
4051 AAAAACCGAA AAACAGATCA CCCTGCCACC TCTTCATTAA CACCACTAAA
4101 TCTCCTCTTC GTCAAAAACT GAAGTGCAAT TAACCAGGCA CCAACTCATA
4151 TTCGATTCTA TCTCAGGCTC CTTTTCGTTC TGCTGCACTT CACGGTCCCA
4201 AGGTGAGGTG TTAAAGTTTT ATGCAATTTC AACAACGATA AATTATAATT
4251 ACACGCAGTG CTGGGCATAA ATCTACCCAC ACAATCACGA GGTTATGAAG
4301 CACACAGATA CTCCTCTGCC ACATTGCGAG TTCAGGCGCG TCAAACATAA
4351 TTTGTAAGAG CATTAAGTAT TCCCCCCATT AACACCTTTC GAGGGTGACT
4401 CCAGCCGATC GCCTCTATTA ATAAATTTCC AAACTGAACT TAATGCCAAA
4451 GGAGGGAAGG TCTTCCACTG ATGGAATCCA ACCGAAGTCT AACGAGAATC
4501 GTATGCCACA TTTGGCGAGT GTCGTTCACC GTCCTCGATA GTATAGTGGT
4551 TAGTATCCCC GCCTGTCACG CGGGAGACCG GTGTTCAATT CCCCGTCGGG
4601 GAGATGCGAG AGCATTTTTT CACAATATAT TTTTCCAGTT AATAACAATA
4651 ACAATAATTT TATACATTTT TGAAGCTTTT ATTTTTGTTA GCTTTTATGT
4701 AGCACTTCTT TCATATGTTG AAACAGATGT ATTGTTTTGT TTAACAAACT
4751 AAATTCTTCC GTACCCAGGC AATTATTAAA TTTAACTGTT AAATAATATA
4801 TAAATTTCGA AAATTAAAAA GTAACAACAT AAATTAAATA TCGAACCAAA
4851 AAAAAAAAAT TATTTACACA TCTCCCCGAC GGGGAATTGA ACCCCGGTCT
4901 CCCGCGTGAC AGGCGGGGAT ACTAACCACT ATACTATCGA GGATGTTGAA
4951 CTTAGGTCGC CAAATTCAAA ATATAAACTT GCTGTTGATC TATACATCCG
5001 AGTGTTATGA TCATAAGTTA TATTAATCCG AACTAATATT ATATATAAGA
5051 TAAAATAAGA TAAGATATGC AAAATATTCG ATGCTGATTT ATAACACGCT
5101 TTTTTAAATC ATTTTATTAA GATGGTAGAC TCACCATTTT CCACGGTCTT
5151 GCTTTTCTCG TTGTCGGCAT CCTCATA
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Fragment	lenght	Oligos	Oligo Sequence	Chr.location	TBE sequence
tkv C [3.9Kb]	3925 bp	974 tkv1052for	CCGAAGGCGTG TGAATATCAAT	2L: 52500	TTTCACACCA

		975 tkv4955rev	CCGAAGGCGTG TGAATATCAAT	01-52560 00	AGGTGTGAAA
tkv D [3.4Kb]	3363 bp	976 tkv171for	GAGACTCGCGA AGACTGTCAAT	2L: 52570 01-52610 00	AGGTGTGAAG
		977 tkv3512rev	GCAGCGCGTGT AACGATGTAAT		

Table 14. Information about the *tkv* C [3.9Kb] and *tkv* D [3.4Kb] subcloned enhancer fragments. Sequence length, oligonucleotides used for its amplification and the encompassed TBE sequences.

5.3.4 hh enhancer fragments (*hh [3.1Kb]- TBE3, hh* [2.4Kb]- TBE5)

Fragment	lenght	Oligos	Oligo Sequence	TBE Chromosomal location	TBE sequence
hh [3.1Kb]- TBE3	3086 bp	1014 hh5115for	TGGGAACTGATC GACGAATCTG	18.956 .417	TGGTGTGAAA
		1296 hh-3311rev	TGGGTGGGCCTG GTCAGTTAGC		
hh [2.4Kb]- TBE5	2355 bp	1297 hh-3913for	GTTAATAGCCAG ACGCCATGTT	18.960 .798	AGGTGTGAAC
		1015 hh11360rev	TGCGCACTTGAA TTATGTCGTT		

Table 15. Information about the *hh* [3.1Kb]- TBE3 and *hh* [2.4Kb]- TBE5 subcloned enhancer fragments. Sequence length, oligonucleotides used for its amplification and the encompassed TBE sequences.

hh [3.1Kb]- TBE3, (oligos , TBE3)

```
   1 tgggaactga tcgacgaatc tgcaagtagg agaggaaaga atttggttag
  51 aaaattggat tttaaataaa tataaggtgc tatttaaata ttcatagaaa
 101 gttataataa atattcgtat ccagagtagt tacacgatta ctttaaaggc
 151 ttgcatgggt gttcatgtat tttacacaat ctaaaatctg atctgcagat
 201 tgcttgtttg gttactgaga gccgcccagg taatggcatt agtttgctaa
 251 tctaatttgg cgttaagcat ccaattgcca agcccaacaa gtcaaaaagg
 301 ttttcacaga tccggggctg gcccgttgca gcccaatgtg aaacaatgga
 351 ctcgaaagga ctgaaaggac tgccttttgc ggctcgccaa caagccagga
 401 acctaatgcg atcgttgaac agagatccga gatcggagat cagcaatctg
 451 cgatctgcca ggcatatcaa taacaagcgg tcgacgtgtt tttgtttcgg
 501 cttgcacaaa aattgtgatc ttgttttccc tgcaagatta gccgtgggat
 551 cagcatcagc cgcatgcgaa gccgaactcg agccaaagcc gcttagaaat
 601 cccaaggaaa aagaaatgca aaggaataaa atgagtagaa caaaaaaggg
 651 ggatgtgcca ggtagcagct tgtggtttgt tttcccaacc atttttggca
 701 acctgttggc aattaccgaa catgctgcaa cccacctaaa caataatcga
 751 agtgcaggcc acattaatca tcattaatgg gtatcggatt gcagggaaca
 801 ggcccaccca gatgcatctg gaatatatcg agatgcactc tatagaactt
 851 tctctggggg ccttcacact ccacactcga agcgccgatg ctgcttgtaa
 901 aattcaaatt aaaaattcca gaccaaatgc gaccactaac cgcgcgcttt
 951 attataatga gcggccataa agtggccttg ttgccgctgc tgctgccgct
1001 gctgctgcca tggaattcca aagaaaatgc accttcccag tggccagccc
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1051 gaaattccca ttgatgcatt ttggtaaaca gagacaggcg gacacacaaa
1101 agtgtagaaa caataaaaat cggattaaat acatatggct ggcggtctat
1151 aaaacccata atcgatgtgg atggacgaaa ggggaatggg atcggaaaga
1201 gaataggaat cggaatcgaa atcgggccca aaagaacaca cgaaatggac
1251 aaaagattct ggggagatgg agagaaggaa gcgcggtagt aacaagaaaa
1301 attggtgtga aatctacctt ctaccaaaac acggcactga aaccgaaaca
1351 gaaacagaaa ccaaaaataa aaaataaaaa cgagggacaa gaaaaatctc
1401 aggccgtctt cgttgttggc ggtgagtgga aagtgaagaa cttgaagggt
1451 cataaattaa taaaccatta aagtggttaa aattatcgtt attttatctt
1501 tgttgtgaag actaaactga taaattatta caaaacagag gacagccgag
1551 ctggtgctgc tgctgttgtt gttgtttttg ttgctgctgt tgttggcttg
1601 gtgactgatc gtttggacag cattaacatg aacatgcaac agcaacaaca
1651 acaacaacaa caacacgcgc ttgggggctt taatgcgcgc gcgcgacaca
1701 ccagcaacaa caacaaaaga cagcagcaac attggtaaga gcgaaacaaa
1751 aaaaaaaact gaaaaaaaaa ttagtgtcga caaaaaggtt tacagccatt
1801 actcttcgtc gtcgtgctgc tgctgttgct gttgttgctt ttgcttttgc
1851 ttttgctgtt gctcttgctg ctgccgttta ggcccctaca aacgaaagac
1901 ttaggcagcg aactcgaggc acagcgaatg ctgtgccaaa actgtcataa
1951 gcccgtggtt tatgtgcagc ggcctatcag caatcaatcg ccccgtaatt
2001 tattgatagc tagcagcagc actagcctca ctagcaccac tggcaacaca
2051 tggctttcag gacccgctgg accacacaca cacacatgtt gccgaacggg
2101 caatggtatg acctgcttgc cacttgttgt gcgtcggctg acctttgat
2151 attgcccaag gagaccaagt tggagtgggg gaggatgaca gcaggatcaa
2201 ggttcatagc ctggcggctc ctttcataag caattagaat caatgagcaa
2251 ggacactcgg ctccgtttcc tttccatcgc ccttttgtcc tgccgcaatc
2301 tttatgggcc gccaccaaaa gtcacaatat tgtgactcga ctgcgtgtgt
2351 gggaaagagg cgtggcatct tgtaattgta gatggaaatt aaatgaacaa
2401 ctgacagccg gtcgcaccca aagtgtctat ctgtctgtat gtgtgggaaa
2451 cttgaaaggg aaaatccctc tagagccgct aaagacatgc aaaaaaaaaa
2501 aaaacatgaa catgaagaaa atgaagtatg aaaaaggaac cgaagagaga
2551 gagcaaatct tggcaagaaa acttaattgt cttggcccgt tttggttggc
2601 tgatcgggcc aaaagcattt tcttctcggt ctttggggca gtttcgttat
2651 gtttcgggga aaactggttg ctggccataa tggcagctca cggatacaca
2701 ctttgacggc caggcttgtc ttaactgatg atgctaactt gctggccgtt
2751 cagtgttttt ttttttttc ttttttcaa ttaggcccgc gctcaggttt
2801 tagcccggca tttaattcat ctttgccagt ttgcctgttg cccgtttgcc
2851 tgcttctcca acagtctcgg ccagtttgtg ctgcctgaga aacgctttgg
2901 gtgtcgtatc atcaattcgt ttaatttgac aggcgcaaac ctaaacatgt
2951 caaaacaaca gccatgcggt tagtagtggt ggttgccgcc tctggagccg
3001 acttttcggg ttccagccca gtcttcagtc ctccgtcccc agttcccagt
3051 tcccagtcct cgtaccctca gaccatccac atgcacagtc cggttttctg
3101 actttgttgc cgtttttctt cctctttcgg atcttgagag tcgtgtcttt
3151 taactggcac tggcactggt actgggcact gcagccgtgg ccaattcggc
3201 ggatgcgaag tgcagggcga gctgcgagcg gctgaaattt gccaacgcct
3251 gtcagcttag cgatcgccag tacgaaacat gcgaaattac gcttgttagt
3301 gtcacttctg gctaactgac caggcccacc catcagccag cacctaggcc
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TBE3 conservation_alignment (18955948 - 18956665), (**TBE3**)

hh [2.4Kb]- TBE5, (oligos , TBE5)

```
3901 cgattaggta ttgttaatag ccagacgcca tgtttaaaga cacgcagcac
3951 gcagcacgca cgagggagcg ggatggctac actgctgtcg gcgggggcgt
4001 ggcatgcggg cgttcatttc ggttgcccaa agggcccaat gcgttcattt
4051 tgcccactta gtgcactcaa caaaattatt gagcatgttg atatggagat
4101 aatctaattt tttaaataag aactgaagga atattatcaa tcaaattctc
4151 tatgaaaatc attaacttta taattaacaa gactatagct agatctgaaa
4201 aacaaatgac tagccagagg aggttctact tttttccgag tgcttctacc
4251 tctttgcgct ctgtgagtct gccctttttg gccgcatcct ttgaacctgt
4301 tgctgcaacc tcaacaaaca ggcgatctta tttgcccagt tccctttttgg
4351 ccaaaggctt atttgcccat gcttacgtgc agttcaaaca ctgaccctcg
4401 acgatggcga tggtaatggt gaccctaacc cacgctcgca gatagacctt
4451 ttatttttctc ccttttcact ctttgttttt cctgtcgata atatgaaaat
4501 gcttacgctt tgtttgccaa tgtggtgggt gctcatccgt ttgtcggctc
4551 gttttcattt gatttttta gtggatagaa ctggcccggc ctggagaaat
4601 gccagccaag cagaaaagga ttcgaacaag tctatgcaaa tatttgagat
4651 cccaaaattc aaaattcgaa atacgaaaag gaaaaaaaaa acgtgtggac
4701 tcaggcgggg caacaagaga gtgtcttaaa gattgagcca gccacaaaat
4751 caattatcaa cggactgagt ccgggggtga caggatgttg cctttgtggg
4801 caaacagaat gcgagccgta aaatcaattt gcgacacgaa gatccttttc
4851 ttctttttta gttgatggca aaccacagcg atcagttgct gttgcctttt
4901 cgggcttatg ttttgatgag gtgtggatct gtagcgttca ttgtttgcta
4951 ttatttggcc aaaacaggac aaccgaattt gtcacacagt cgtggccctg
5001 tggcaaattg cgatggcagg ccgcattccg agaagagttc aatgatattt
5051 gcccaacgcg ctgcaacttt gctttattaa aaggcttaac cagtcgagag
5101 ttgagactta agaggcgaga gccaagtcgt gctaatgagc acaaaagcgt
5151 aacgacaaac agaggatcgc tgccaacgag tgtgtatatc catatccatg
5201 gagccataag ttggggtaaa gacaaacgct tttgtcttaa gcactgccaa
5251 cgatgtttac ccaacatgct aatgcattga gctcgaattc gaatcgatgc
5301 gctggacgcg caatgaacaa acaacggccc gtctcagatc tcggatctga
5351 gatctgggat ctgagttctt tgggatctct cgagactggg atctctcctc
5401 gcagatccca tattccactg cactctccgc ccaacttcgt ttgccgacgg
5451 gtgcgtaagg taaacattta cgtatacacg gcgtcggcgg cccaaaaaga
5501 tggctaaata atgtgtgtgc cttgtaagtg gccgttagac aacagcgtca
5551 agtgtaaata agcccgtgcc ctggcaactc ggggtatcgg aaaatcccaa
5601 tacccatatt aatacgtatt atacccatag ccatagccat accatctccc
5651 cctaggaaag ctgcattaat tagacattca acaaggtgtg aactttgcct
5701 tttcttcagc cattaccccg aaaaaggcgg aaaactcaaa tcttcgaaaa
5751 atccgaccac gccccattgt gcatttttgt ttatatgcgt gtatggaggg
5801 gggggggggg gcgactgcat ttgattttgg ctatttcggt ttctgttttt
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5851 ttttttattc ctcacccatt ttattcgcct ctgatgcgtt tttaatggac
5901 gcgcgactgc agttttattg atttaaattg tctgactggc tggctgccaa
5951 tttgacgctc ttaatggtcg ggcgaaatta ttatggcacg aagattatta
6001 ggcactcatc atcattagcc aagtcaggac gcgagttggc caagtcaggt
6051 cgagttaata gcccgctctc gaccagagac aagttcgggg gctttgtttg
6101 ttttgcccat cccaaaccca agtccaggcc ataccatacc ataccatgta
6151 ccatctccca tctgtgggct tttttttgc gagggtctcg gattacttgt
6201 cttgggtacc ccagggaaac cgacgattag gaaacgacac aatttaacga
6251 cataattcaa gtgcgca
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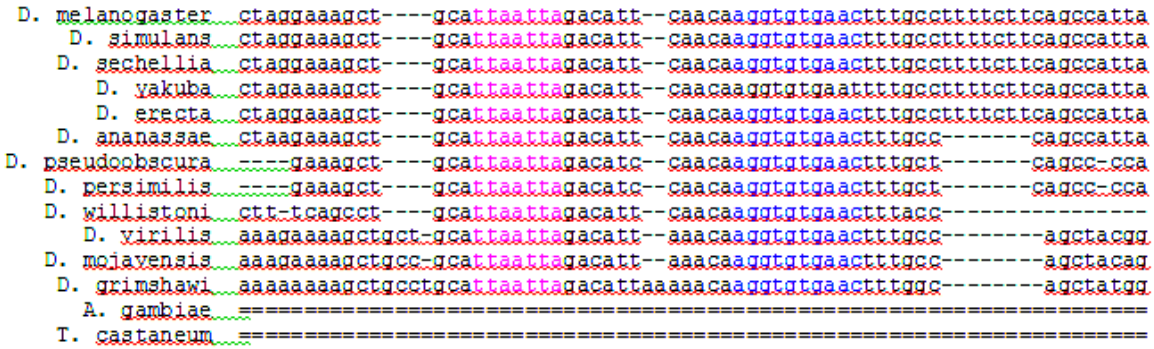
TBE5 conservation_alignment (18960534 - 18961394), (**TBE5, potential homeodomain**)



5.3.5 Subcloned *inv* enhancer fragments

5.3.5.1 *inv* enhancer fragment (inv [2Kb])

Fragment	lenght	Oligos	Oligo Sequence	Binding site sequence
inv [2Kb]	1966 bp	1414 inv1998for	GATTTAACATGCC ATACACCC	TBE1: GTTCACACCT TBE2: AGGTGTGAGA CiBS: CGGGTGGTC
		1415 inv3943rev	CTCTATCAGTGC GGTGGTTAG	

Table 16. Information about the *inv* subcloned enhancer fragment (*inv [2Kb]).*
Sequence length, oligonucleotides used for its amplification and the encompassed TBE sequences.

***inv* [2Kb], (oligos , TBE2, TBE1, CiBS site)**

```
   1 gatttaacat gccatacacc ctaccacata aaagccaagg cattggggac
  51 ttggcttaaa gcttattaag aaatttcatt aagtaattca cgtaaaagta
 101 aagtgcgaat caaaaaatca ggtcaacttt tatgttttaa aattaatgca
 151 atttcgaagt taatttaccc cgttaatatt tggttgtgca gggcactgtc
 201 taaaccactc caacttgacc aaaaaatggg ctaaacgaag agatctggga
 251 ccttcagtcg gagccctgca tctgggctcg agtgggtttc gttcagatct
 301 tctgcctcac tgcctttccc caccagcatc atcagcaaga tcatcatcgc
 351 gattgggatc gtgatcatgg acctgaagac gatcatcgca gatgaggagg
 401 acctttggtg tggtccagtc tcctttgcga ttggtttgca tctcggtgat
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 451 ccgtggaaga ttgatgttaa tgtcggcatc gccctggtgt gaacacactt
 501 attcgcatat ttgttttgca acccagcgat ttggccattt ggtcatttgg
 551 ccacacacga cgctgatggt ccatcgccgc taatagagcc ataaagcgaa
 601 catttattga tctacaaatt atttgggcgc tgccgctggg gctcaactcg
 651 gttttttggc tgttggatgc tgctttcgtg ggctatctaa atattcatta
 701 gtgtctgggc ggcttgattg gtcggtcgat cgcctcagag atcgcagggt
 751 tcttcgattt aattttattt ttatagcccc cccaaagaac ccgctgacta
 801 ccgcaaccga caaaaaagca aataaacaaa cactcttccg ataccaaagc
 851 gataccttтc gtttcgggtg gtccacgag atcagcgcct ctgatgtgat
 901 cattagggtg aaagtaactc ccggcctgcc agactttgac tgatctaatt
 951 ggagaaactg gagctgagat tgtgatcgaa atcgagactg agtgcctgag
1001 actgaggatt gcgttaattg agaggccata aaagtgcact aatttagtcg
1051 aaatatccat agaagcgtaa aatgttcaca cctcaattgc acaaaggcaa
1101 agcaacgtgt ctattaaggc gcgctaagct cgcgatacat ctacatttac
1151 agttgcagat tcagatacag ctacagatac tctggccgcc catatcggtg
1201 tcgcctgtac ttgaggcctc tggcaatcgc agcaacagtt ccaatcccaa
1251 tcccaatccc agttgcagtg gcagtggcag gccacctgat gctcaacact
1301 gccggattga ttacagtttc ttaatgaaat tgtttgctaa tatcagcgga
1351 aaatgcggtt tgcaatttgt caaaccagtt gcggctgcct catggaccca
1401 atcaatgctg taggtgtaag cgatatctcg gacagggtat taaatcgcca
1451 aaggtgtgag agcgcgtaaa aggttgcctc ggaatttgct gggatcattg
1501 aggaatctcc tcctggccgg cacttttcac cgcgataatc tcaaacgagc
1551 catatttaat gttatagtta tgcgtggctt aaataaccta agagatagtt
1601 aggatggctt gctggctata aacctaacac atggaattcc agttttaatt
1651 tgctttggat tttacattta taacccgaac tgctgatttt ctgatggtgt
1701 aattatagta gttgtcaaga agtttagctc acctttttcta ctttcatttg
1751 ccgccttcaa atcgcgtttg aagcaacatg ttttgacacc tccacctcgc
1801 ccatggagca tattcaatga agcgccctgc gataaaagca ccgcgaggac
1851 aactagtgtg cccctccagc aatttcatat gcaaagccgg caagggctat
1901 gcaaattagc cagcggcagt ggcattggca gcaccgaaca aatagctaac
1951 caccgcactg atagag
```

5.3.5.2 inv enhancer fragment TBE1 mutagenesis (inv [2Kb] with TBE mutagenesis)

Fragment	lenght	Oligos	Oligo Sequence	Binding site sequence
inv [2Kb] with TBE mutagenesis	1966 bp	1418 invTBE1mutfor	CCATAGAAGC GTAAAATGTTC AGGCCTCAAT TGCACAAAGG	**TBE1 5-3'** GTTCACACCT Mutagenesis GTTCAGGCCT
		1419 invTBE1mutrev	CCTTTGTGCA ATTGAGGCCT GAACATTTTAC GCTTCTATGG	

Table 17. Information about the *inv* enhancer subfragment with TBE1 mutagenesis (*inv* [2Kb]^{TBEMUT}). Sequence length, oligonucleotides used for its amplification and the mutated encompassed TBE1 sequence (the mutated nucleotides are unterlined).

5.3.5.3 *inv* enhancer fragment with CiBS mutagenesis (inv [2Kb] with Ci binding site mutagenesis)

Fragment	Sequence lenght	Oligos used	Oligo Sequence	Binding site sequence
inv [2Kb] with Ci binding site mutagenesis mutagenesis	1966 bp	1416 invCiBSmutfor	CGCTGATCTC GTGGGACATT CGAAACGAAA GGTATCG	**CiBS 5-3‘** CGGGTGGTC **3-5’** GACCACCCG 3-5’ mutagenesis GACCATTCG
		1417 invCiBSmutrev	CGATACCTTT CGTTTCGAAT GGTCCCACGA GATCAGCG	

Table 18. Information about the *inv* enhancer subfragment with Ci binding site mutagenesis (*inv* [2Kb]^{CiBSMUT}). Sequence length, oligonucleotides used for its amplification and the mutated encompassed CiBS sequence (the mutated nucleotides are unterlined).

5.4 Enhancer trap/reporter lines detailed list with *l(1)omb* crosses number

Gene	PZ Insertion	Chromosomal locatio	Stock	Genotype	l(1)omb cross
apterous (ap)	Dmel\P{PZ}ap^{rK568}	2R (41F8)	Bloomington 6374	P{ry[+t7.2]=PZ}ap[rK568]/ CyO	52
argos (aos)	Dmel\P{lwB}aos^{W11}	3L (73A2)	Bloomington 2513	w[8]; P{w[+mW.hs]=lwB}argos[W11]/TM3, Sb[1]	19
	Dmel\P{PZ}aos^{05845}	3L (73A2)	Bloomington 11674		
abrupt (ab)	Dmel\P{lwB}ab^{1D}	2L (32E1-32E2)	Bloomington 9586	w[*]; P{w[+mW.hs]=lwB}ab[1D] /CyO	71 A, B
brinker (brk)	D298.1= y w hs-flp; Sp/CyO; BM14/TM6B	X	GOP66		
				y w hs-flp; Sp/CyO; BM14/TM6B	96
bunched (bun)	Dmel\P{PZ}bun^{00255}	2L (33E5-33E9)	Bloomington 10936	P{ry[+t7.2]=PZ}bun[00255] cn[1]/CyO; ry[506]	53

dachshund (dac)	Dmel\P{PZ}dac^{P}	2L (36A1)	Bloomington 12047	P{ry[+t7.2]=PZ}dac[P]/CyO; ry[506]	26
Distaless (Dll)	Dmel\P{PZ}Dll^{01092}	2R (60E3)	Bloomington 10981	P{ry[+t7.2]=PZ}Dll[01092] cn[1]/CyO; ry[506]	119
dachsous (ds)	Dmel\P{PZ}ds^{05142}	2L (21E2)	Bloomington 11394	P{ry[+t7.2]=PZ}ds[05142] cn[1]/CyO; ry[506]	109
defective proventriculus (dve)	Dmel\P{PZ}dve^{01738}	2R (58D2)	Bloomington 11073	cn[1] P{ry[+t7.2]=PZ}dve[01738]/CyO; ry[506]	39, 16
grain (grn)	y1 M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP'}ZH-		Bloomington 24481	y[1] M{vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP'}ZH-22A	120
	w; grn-hinge #8/TM6B			w; grn-hinge #8/TM6B	98, 14
homothorax (hth)	Dmel\P{PZ}hth^{05745}	3R (86C2)	Bloomington 11670		117
neuralized (neur)	Dmel\P{lArB}neur^{A101}	3R (85C2-85C3)	Bloomington 4369	P{ry[+t7.2]=lArB}neur[A101] ry[506]/TM3, ry[RK] Sb[1] Ser[1]	116
retained (retn)	Dmel\P{PZ}retn^{02535}	2R (59F5)	Bloomington 11200	cn[1] P{ry[+t7.2]=PZ}retn[02535]/CyO; ry[506]	1
Syndecan (Ddc)	Dmel\P{PZ}Sdc^{10608}	2R (57E5)		P{ry[+t7.2]=PZ}Sdc[10608] cn[1]/CyO; ry[506]	108
sloppy paired 2 (slp)	w; slp-lacZ; UAS:omb/TM3, Sb		M Porsch, 2002 doctoral thesis	w; slp2-lacZ; UAS-omb/TM3, Sb	73
Signal-transducer and activator of transcription protein at 92E (Stat92E)	Dmel\P{PZ}Stat92E^{06346}	3R (92F1)	Bloomington 11681	ry[506] P{ry[+t7.2]=PZ}Stat92E[06346]/TM3, ry[RK] Sb[1] Ser[1]	7
wingless (wg)	wg-lacZ (CyO)/l(2)		Koni Basler, Zürich	wg-lacZ (CyO)/l(2)	112, 113
tkv	Dmel\P{lacW}tkv^{k16713}	2L (25D1)	Bloomington 11191	y[1] w[67c23]; P{w[+mC]=lacW}tkv[k16713]/CyO	80

Gene	PZ Insertion	Stock	Genotype	l(1)omb cross number

spalt major	sal[10.2S/C]	GOP 522	P{salm10.2S/C-lacZ}	70
	sal PS18	GOP 980	SphI – PvuII, insertion 18	41,42
	sal PS19	GOP 981	SphI – PvuII, insertion 19	43, 44
	salm-lacZ	Bloomington 11340	P{ry[+t7.2]=PZ}salm[03602] cn[1]/CyO; ry[506]	55

Cloned Fragment- Enhancer reporter Line		Stock	Genotype	l(1)omb cross number
vestigial boundary enhancer	vgBE	GOP 314	multiple inserions	29
vestigial wt boundary enhancer fragment without TBE mutagenesis	vg BE $[1.1Kb]^{wt}$ (generated 03.2012)	Line did not survive	58A insertion	255
vestigial boundary TBE-MUT enhancer fragment with TBE mutagenesis	vg BE $[1.1Kb]^{TBEMUT}$ (generated 03.2012)	Line did not survive	58A insertion	254
vvl1+2 enhancer fragment	vvl1+2 [1Kb] (generated 03.2012)	Line did not survive	58A insertion	256
tkvA enhancer fragment	tkv A [5.2Kb] (generated 03.2012)	Line did not survive	58A insertion	289
inv enhancer subfragment	inv [2Kb]	Line did not survive	58A insertion	
inv enhancer subfragment with TBE mutagenesis	inv $[2Kb]^{TBEMUT}$	Line did not survive	58A insertion	
inv enhancer subfragment with Ci binding site mutagenesis	inv $[2Kb]^{CiBSMUT}$	Line in GOP stock	58A insertion	X-12H 28.3.13

Table Group 19. Detailed list of all the enhancer trap/reporter lines used in this study along with *l(1)omb* crosses reference. First table: Fly strains from Bloomington used during this study. Second table: Additional fly strains used during this study. Third table: Fly strains generated and used during this study.

5.5 Subcloned enhancers detailed list

Subcloned Fragment	Sequence name	Sequence lenght	Oligos used	Oligo sequence
hh subfragment including TBE3	hh [3.1Kb]-TBE3	3086 bp	1014 hh5115for	TGGGAACTGATCGACGAATCTG
			1296 hh-3311rev	TGGGTGGGCCTGGTCAGTTAGC
hh subfragment including TBE5	hh [2.4Kb]-TBE5	2355 bp	1297 hh-3913for	GTTAATAGCCAGACGCCATGTT
			1015 hh11360rev	TGCGCACTTGAATTATGTCGTT
vvl1+2 enhancer fragment	vvl1+2 [1Kb]	1000 bp	1333 vvl-1334for	TAATTGCGTCCGAAGCGAACC
			1334 vvl-2313rev	GGAGAGGGCCAGCATGTAGAT
vg boundary enhancer fragment	vg BE [1.1Kb] wt	1055 bp	1159 vgBE-1534for	AATTTGTTTGAACGGGCTTGG
			1160 vgBE-2568rev	TATTAGCTGCAGGCGATATTT
vg boundary enhancer fragment with TBE mutagenesis	vg BE [1.1Kb] TBEMUT	1055 bp	1161 vgBEmut-for	CGTCCGCCCTTTCGAAACTTTGGCCAGCCAGC
			1162 vgBEmut-rev	GCTGGCTGGCCAAAGTTTCGAAAGGGCGGACG
tkv enhancer subfragment A	tkv A [5.2Kb]	5177 bp	970 tkvfor235	CTAATATGCCCCCAATAGAATG
			971 tkvrev5390	TATGAGGATGCCGACAACGAGA
tkv enhancer subfragment C	tkv C [3.9Kb]	3925 bp	974 tkv1052for	CCGAAGGCGTGTGAATATCAAT
			975 tkv4955rev	CCGAAGGCGTGTGAATATCAAT
tkv enhancer subfragment D	tkv D [3.4Kb]	3363 bp	976 tkv171for	GAGACTCGCGAAGACTGTCAAT
			977 tkv3512rev	GCAGCGCGTGTAACGATGTAAT
inv enhancer subfragment	inv [2Kb]	1966 bp	1414 inv1998for	GATTTAACATGCCATACACCC
			1415 inv3943rev	CTCTATCAGTGCGGTGGTTAG
inv enhancer subfragment with TBE mutagenesis	inv [2Kb] TBEMUT	1966 bp	1418 invTBE1mutfor	CCATAGAAGCGTAAAATGTTCAGGCCTCAATTGCACAAAGG
			1419 invTBE1mutrev	CCTTTGTGCAATTGAGGCCTGAACATTTTACGCTTCTATGG

inv enhancer subfragment with Ci binding site mutagenesis	inv [2Kb] CiBSMUT	1966 bp	1416 invCiBSmutfor	CGCTGATCTCGTGGGA CCATTCGAAACGAAAG GTATCG
			1417 invCiBSmutrev	CGATACCTTTCGTTTC GAATGGTCCCACGAGA TCAGCG

Table 20. Detailed list of all the enhancers subcloned during this study.

5.6 Figures List

5.7 Tables List

6 Literature references

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