A genetic screen for novel genes involved in tracheal development in *Drosophila melanogaster*

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1. Introduction

Many organs of animals, including lung, kidney, blood vessels and most of glands in vertebrates, are built of branched tubular epithelial structures. They enable the transport of gases or liquids in the body. How they form and what controls branching events, direction of growth and tube size are questions that are pertinent to understanding branch morphogenesis. These questions are important not only for biology but also for medicine, because many diseases, such as polycystic kidney disease, are causing plumbing defects. The elucidation of molecular mechanisms of tube formation could lead to new ways of diagnosing and treating such diseases. However, dissection of these processes in mammalian system is rather difficult. Thus the Drosophila tracheal (respiratory) system with its structural simplicity and accessible genetics has become an excellent model in recent years for studying tubulogenesis.

1.1 Development of *Drosophila* tracheal system

The Drosophila tracheal system is a network of single-layered epithelial tubes transporting oxygen and other gases throughout the body. It is comprised of approximately 1600 cells forming ~ 10,000 interconnected tubes in the larval trachea (Ghabrial et al., 2003). They are covered by a luminal cuticular lining, which provides a barrier against dehydration and pathogens. The process of tubulogenesis is based on cell migration, cell shape changes and rearrangements and regulated growth of distinct subcellular domains. The Drosophila trachea arises from ten clusters of ectodermal cells on either side of the embryo, which results in bilateral symmetry and a repeated metameric structure of the network. After two cycles of cell division, ~ 80 cells of each tracheal placode invaginates to form an elongated sac, from which six major buds grow in different directions, giving rise to the different primary branches. The major primary branch is the dorsal trunk (DT) –connecting all tracheal segments along the anterior-posterior axis. On the lateral side this role is played by the lateral trunk (LT). The transverse connective (TC) connects the DT and the LT in each segment. Among the remaining primary branches are the dorsal branches (DB) migrating dorsally, the visceral branches (VB) migrating internally and the ganglionic branches (GB) penetrating the central nervous system. The primary branches later produce secondary branches, with their lumen formed by a single cell. The terminal branches arise from the tips of the secondary branches during stage 16 of embryonic development and their formation last throughout the larval stages. During their development the terminal tracheal cells ramify into extensive arrays that cover and support large area of the target tissue (Manning and Krasnow, 1993). Fig.1.1 depicts the development of the embryonic trachea and the fully formed larval tracheal tree.

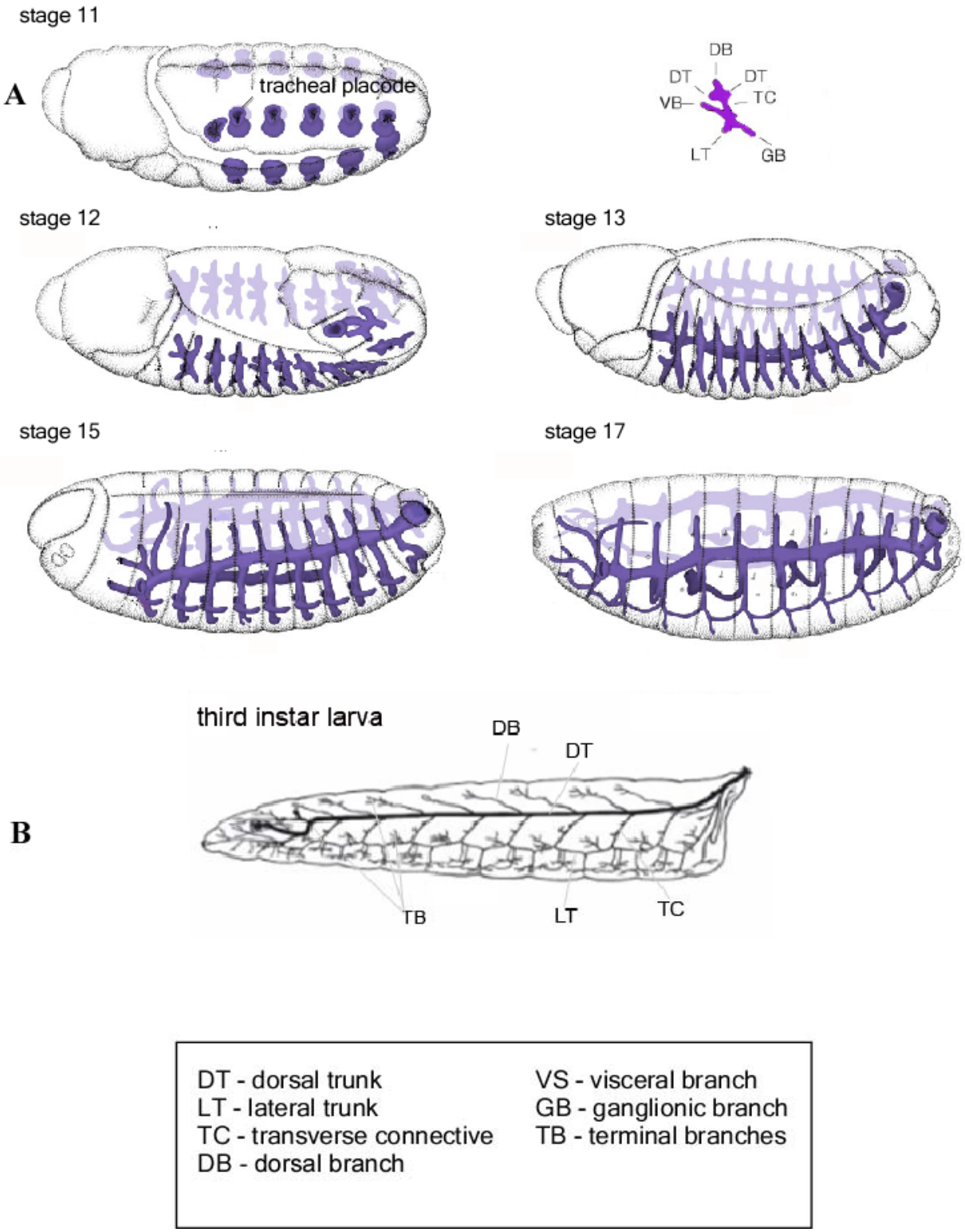

Figure 1.1 Schematic representation highlighting the development of the embryonic trachea (A) and the larval respiratory system (B). After placode invagination at stage 11, cells form elongated sacs from which six major buds grow out giving rise to primary branches (stage 12). One example of tracheal metamere at stage 12 is depicted in the upper right panel. The formation of the buds is followed by cell migration, branch fusions and extensive cell rearrangements which lead to formation of a network supplying all target tissues with oxygen by stage 17. During the larval life terminal branches undergo several ramifications generating variable and complex tracheoles. (Adapted from Hartenstein, 1993 (A) and Ghabrial et al., 2003 (B)).

Proper development of the trachea requires induction and maintenance of expression of the Fibroblast growth factor (FGF) receptor, Breathless (Btl) in each tracheal cell. The FGF pathway plays a major role in the branching process. It is first activated by the FGF-like molecule, Branchless (Bnl), secreted by groups of ectodermal cells surrounding the invaginated placode. Bnl acts as a chemoattractant for the nearest tracheal cells and guides cell migration thereby directing branch outgrowth (Sutherland et al., 1996). This signalling pathway is used in each step of branching, triggering the expression of different genes required for primary, secondary or terminal outgrowth. Although some such genes have been identified, it is still not entirely clear what happens in the cell after Btl activation. The genetic developmental program controlling primary and secondary branching involves other signalling pathways as well, like Epidermal growth factor (EGF), Decapentaplegic (Dpp), Wingless (Wg) etc, responsible for determination of cell fates and branch types. In contrast to stereotyped embryonic development of the trachea, formation of terminal branches in the larva is more flexible and depends more on the physiological needs of the surrounding tissues. (Ghabrial et al., 2003; Kerman et al., 2006; Uv et al., 2003).

1.2 Architecture of tracheal branches

Different branches within each tracheal metamere have a fixed number of cells and a characteristic tube dimensions and structures. They are all made up of polarized epithelial cells, with the apical surface facing the lumen and the basal side facing the surrounding tissue. In late embryonic and larval stages, four distinct types of tubes can be found (Fig. 1.2 and 1.3).

Type-I tubes – DT, part of TC – are multicellular tubes with two to five wedge-shaped cells surrounding the central lumen and connected by intracellular junctions. Their diameter in the embryo ranges from 4 – 5 μm. Type-II tubes are more narrow (1 μm in diameter), formed by interconnected cells lying in a row. They are found in DB, VB, GB, LT and parts of TC. Their lumen is made up of a single, tube-shaped cell sealed by autocellular junctions. To maintain the chain-like assembly of cells, adjacent cells are connected by ring-shaped intercellular junctions. Type–III tubes (fusion anastomoses) interconnect tubes of type I and II (DT, LT and DB) in order to link up neighbouring metamers and form a continuous network. They consist of two single doughnut-shaped cells attached head to head through an intercellular junction. Each of the two cells derive from a different primary branch and they contact each other to mediate lumen fusion. This results in seamless tubes lacking intracellular junctions. The diameter of type-III tubes depends on the type of the connected tubes and varies from ~ 0.5 to 5 μm. Type-IV tubes are formed by terminal cells. They are subcellular seamless capillaries making direct contact with target tissues

and mediating gas exchange. Their diameter is less than 0.5 µm (Kerman et al., 2006; Uv et al., 2003).

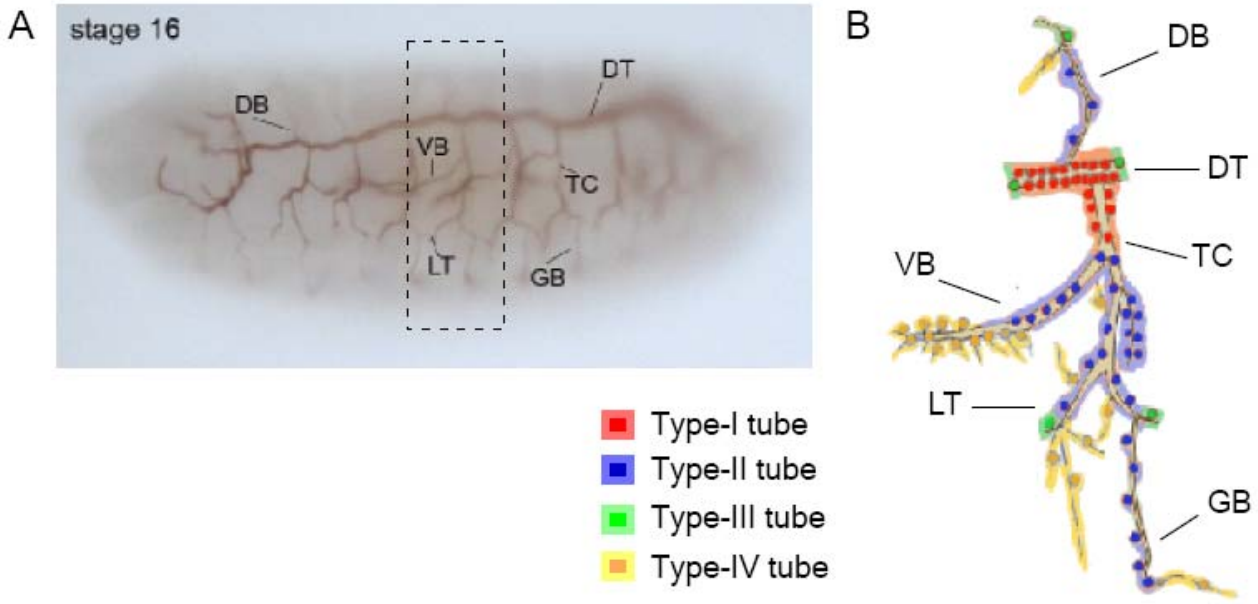

Figure 1.2 Each metamere consists of different types of branches. The tracheal system in a stage 16 embryo, visualised by luminal marker (2A12) (A), and schematic representation of one metamere (B). In the late embryo and in larval stages, four different tube types can be distinguished. Adapted from (Uv et al., 2003).

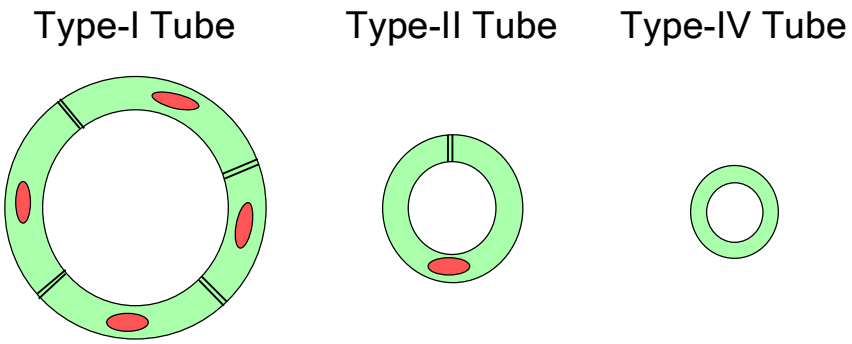

Figure 1.3 Schematic cross sections of the lumen of different tube types. The cell body is marked in green and nuclei in red.

1.3 Morphogenesis of tracheal branches

The formation of elongated tracheal branches with different tube types from the sac-like structure of the invaginated placode requires cell rearrangements and cell shape changes. Since tracheal cells are part of a tightly sealed epithelium, the epithelial adherens junctions, responsible for cell-cell adhesion, also undergo extensive remodeling during formation of branches.

1.3.1 Primary branching

The first branches to form are multicellular type-I tubes, consisting of several cells wrapped around the lumen and held together by intercellular adherens junctions (AJs) established at a subapical position between neighbouring cells. Their formation is based on invagination of the tracheal placode and further migration of cells, without dramatic changes in cell to cell contacts. Throughout tracheal development, only large tubes like DT or more dorsal parts of TC keep this character. Finer secondary branches which arise from primary branches during trachea morphogenesis undergo more drastic rearrangements.

1.3.2 Secondary branching

Transition from type–I tubes to type II, accompanied by tube elongation, can be divided into distinct steps. They were revealed by in vivo analysis of AJ rearrangements during the formation of dorsal branches (Jazwinska et al., 2003; Ribeiro et al., 2004). In the first step referred to as 'pairing', cells are aligned in a pairwise fashion along the lumen, made up of two cells. Afterwards, cells start to intercalate, which requires extensive AJ remodelling. First, two neighbouring cells reach around the lumen, which leads to the formation of the first autocellular AJs as a cell touches its own membrane extension. Both cells reach around the lumen on opposite ends, proximal and distal, along the elongation axis. As the first autocellular contact is established, cells seem to 'zip up' by replacing intercellular AJs with autocellular ones. This step is referred to as 'zipping'. To ensure that cells stay in contact with their neighbours in a head to tail arrangement, the zipping process must be terminated. This results in the presence of small, ring-like intercellular AJs connecting neighbouring cells.

So far not much is known about the molecular events underlying transition from type I to type II tubes. It has been shown that zinc-finger transcription factor Spalt (Sal) is requird for the control of this process, by blocking intercalation (Ribeiro et al., 2004). *Sal* expression is spatially induced by

wingless signalling in dorsal trunk (Chihara and Hayashi, 2000; Llimargas, 2000) and repressed by dpp signalling in cells positioned dorsally and ventrally to the dorsal trunk (Chen et al., 1998). This expression pattern defines branches in which intercalation takes place. However, it is not clear how this happens, since no Sal target genes have been found so far. Little is known also about molecules involved in cell pairing, cells reaching around the lumen or in the zipping process. It is likely that regulation of cell adhesion is required for these steps. DE-cadherin regulation might be important for this process as it is involved in cell adhesion. DE-cadherin is a single–pass transmembrane protein. Its extracellular domain forms homophilic transdimers between adjacent cell membranes. The cytoplasmic domain interacts with p120 catenin, β-catenin and Hakai, an E3-ubiquitin ligase (Fujita et al., 2002). β-catenin binds to α-catenin, which associates with actin filaments. The cell to cell contact based on the strong adhesivness of cadherin is caused probably by anchoring of cadherin-catenin complexes to the cytoskeleton and has to be modulated to allow cell rearrangements. It is possible that regulation takes place at different levels. It could be for example, control of E-cadherin turnover or recycling, modulation of interaction with the cytoskeleton, or regulation of adhesion through inside-out signalling (Neumann and Affolter, 2006). There are several possible candidates shown to be involved in the intercalation process: Src, shown to induce the dissociation of epithelial cells, Hakai, targeting E-cadherin for degradation (Fujita et al., 2002), Arf6, a GTPase mediating E-cadherin internalization (Paterson et al., 2003). There are a number of proteins that might play a role in regulation of the actin cytoskeleton - E-cadherin interaction. These are for example small GTPases like Rho, Rac and Cdc42 and the Ras family member Rap1. Also, components of AJs might be involved in regulation of cell intercalation (Neumann and Affolter, 2006). Some of the genes listed above have been already shown to cause tracheal defects, like Src (Takahashi et al., 2005) or the GTPase Rac1 (Chihara et al., 2003).

Two genes, piopio (pio) and dumpy (dp), have been shown to be required for the termination of cell intercalation, (Jazwinska et al., 2003). In their absence, all fine tubes are transformed into epithelial cysts, disconnected from the remaining multicellular tubes in the embryo. They both encode proteins containing zona pellucida (ZP) domain, they are produced by tracheal cells and secreted into the lumen. It has been proposed that Pio and Dp might form a luminal scaffold that prevents the complete zipping of the autocellular junctions and thus helping to preserve branch integrity.

1.3.3 Fusion cell formation

As the tracheal system develops from 20 independent metameres, branch fusion is needed to form a continuous network. Thus, cells at the tips of the connecting branches undergo a complex process of

partner recognition and formation of an intracellular tube. Fusion type-III tubes are doughnut-shaped and lack autocellular junctions (Samakovlis et al., 1996a). They are formed in a process know as anastomosis in which fusion cells recognize each other, make contact on their basal surface and subsequently generate the apical lumen that bridges the junction between them. The initial lumen is provided by the apical surface of the bordering cells of branches to be fused. It is pulled along or penetrates the fusion cell actively, forming finger-like extensions. The lumen growth inside the fusion cell occurs in a proximo-distal orientation, with an increased number of vesicles containing luminal material at the tip of the growing lumen. This observation made by Samakovlis et al. (1996a) indicates that the intracellular lumen forms by assembly and fusion of vesicles, using the 'finger' tips as nucleation points (Uv et al., 2003).

The first molecular step in a fusion event is the deposition of DE-cadherin at the site of contact on the basal surface of the cells (Tanaka-Matakatsu et al., 1996). This leads to accumulation of several cytoskeleton binding proteins, including α – catenin, β – catenin, short stop, which encods a plakin that binds both F-actin and microtubules and Formin 3, a regulator of several actin-based processes (Tanaka-Matakatsu et al., 1996; Lee and Kolodziej, 2002; Tanaka et al., 2004). These proteins mediate the formation and maintenance of an actin bridge that spans both fusion cells from one initial lumen bud to the other. This bridge serves as a site of fusion of vesicles with luminal material. Subsequently DE-cadherin and associated proteins form a circle perpendicular to the presumptive lumen at the interface between the two fusion cells. After docking and fusion of vesicles lead to the formation of a continuous lumen, the adherens ring expands to the same diameter as the connected tubes and two fusion cells form bicellular anastomosis.

The number of cells undergoing the process of fusion is controled by Notch signalling as well as expression of fusion cells markers such as Escargot, Fusion-2 and Fusion-3. Determination of fusion cell fate is a result of spatial interplay of Dpp, FGF and Wg signalling (Ikeya and Hayashi, 1999; Steneberg et al., 1999).

1.3.4 Terminal branching

The tip cells on the secondary branches that do not form fusion cells form terminal branches. The initially compact cells grow thin cytoplasmic extensions towards the bnl expressing target tissue. The extensions finally come into close contact with the plasma membrane of target cells in order to allow gas diffusion. Their outgrowth is accompanied by repeated events of cytoplasm extension and formation of the lumen within the cell which leads to the creation of a ramified network contacting almost every cell in target tissues (Guillemin et al., 1996).

As terminal cells start to sprout, they also change their shape by extending broad cytoplasmic processes away from the neighbouring stalk cell. The nuclei are initailly moved along and initial branch lumen, possibly provided by neighbouring stalk cells, is acquired. During the further branches outgrowth the nucleus of the terminal cell remains stationary at the cell base. As branching proceeds, first long narrow cytoplasmic protrusions are formed which are later invaded by lumen. Similar to the lumen in fusion cells, it grows in a proximo-distal orientation, possibly by fusion of luminal vesicles (Guillemin et al., 1996). However, the mechanism leading to the formation of the lumen as a junction-less, intracellular, membrane-bound channel is poorly understood.

The first steps of terminal branch formation during late embryogenesis seem to be stereotyped to ensure air supply to all regions of the newly hatched larvae. Larval life however, is dominated by extensive body growth and enhanced oxygen needs, which requires a more flexible system. The terminal branches ramify several times during this period, generating variable and complex trees of tracheoles. However, branching variability does not mean disorganization. Spacing between branching points is regular and branches never cross each other. Sprouting is regulated by oxygen needs of the tissues. Low oxygen (hypoxia) induces terminal branching and high oxygen (hyperoxia) suppresses it via control of branchless expression (Jarecki et al., 1999). It is not fully understood how the Drosophila cells sense low oxygen and how this leads to induction of branchless expression. It has been proposed that the Drosophila homologue of mammalian hypoxia inducible factor (HIF), the transcription factor playing a major role in mammalian hypoxia response, may mediate sensing and response to oxygen levels. In corcondance with this notion branchless has been identified as one of its targets. The other candidate is nitric oxide (NO) signalling, since its perturbation during larval life affects terminal branching in a fashion similar to hypoxia or hyperoxia (Ghabrial et al. 2003). What happens in the terminal cells upon FGF signal activation by Bnl binding to the receptor Btl is also not fully understood, but some players have been identified. One of them is blistered (bs, named also pruned), the Drosophila homologue of mammalian serum response factor (SRF). It is selectively required for terminal branch growth, since in embryos missing DSRF, terminal cells undergo the initial sprouting but fail to develop an intracellular tube distal to the nucleus. It is a transcription factor whose activation in terminal cells by the FGF pathway induces expression of so far unknown target genes required for cytoplasmic outgrowth and terminal branch formation (Affolter et al., 1994; Guillemin et al., 1996). One other identified regulator of terminal outgrowth is sprouty (spry). It acts downstream of FGF signalling, but as a negative regulator, antagonising the pathway and limiting the number of cells producing terminal branches (Hacohen et al., 1998).

Extensive ramification of terminal branches during larval life requires not only proper outgrowth and lumen formation but also its maintenance and stability. A recent study (Levi et al., 2006) revealed that Drosophila Talin, a large cytoskeletal protein that links integrin cell-adhesion molecules to the cytoskeleton, is required for maintenance of the terminal branches. The integrin-talin adhesion complex anchors mature terminal branches to their substrata and maintain luminal organization. If these complexes are absent, the lumen becomes disorganised and retracts from the branch which leads to the degeneration of lumenless branches.

1.4 Control of tracheal tube size

During embryonic and larval development, tracheal tubes not only remodel their structure but also dramatically increase in size and diameter. At the end of larval life, tracheal tubes would have expanded up to 40 times their initial size. Extensive studies of the mechanical and molecular bases of this expansion and its control revealed several aspects of this process.

First insight into tube size changes came with the identification of eight mutations leading to abnormal tube elongation or irregular tube diameter (Beitel and Krasnow, 2000). This study showed that tube size is linked to branch identity and is not controlled by number, size or overall shape of the cells forming the tracheal tubes, but by coordinated regulation of apical surface. Further studies revealed that tube length and diameter are controlled independently. Thus products of genes playing a role in tube size control were divided into two categories: one involved in tube elongation control and second required for control of tube diameter.

1.4.1 Genes involved in tube elongation control

This category consists of genes causing an increase in tube length when mutated. They mostly encode components of pleated septate junctions (SJs), revealing a new role for these cell to cell junctions. The SJs are membrane structures found basal to the adherens junctions in many epithelia. They are functional analogues of vertebrate tight junctions, since they form a diffusion barrier for water and solutes between epithelial cells.

Mutations in *megatrachea* and *sinous* lead to an increased tube size. They encode claudins (Behr et al., 2003; Wu and Beitel, 2004), four transmembranepass proteins whose homolougs form a paracellular barrier in vertabarte tight junctions. Also the *bulbous* (*lachsin*) phenotype is caused by a mutation in a cell adhesion molecule which localises to SJs (Llimargas et al., 2004). *Ecstatic*, which turned out to be a new allele of *nervana2* encodes the β-subunit of a Na^+/K^+ ATPase , which

also localises to SJs. Also other components of SJs, like *coracle*, *neurexin*, gliotactin, and the α – subunit of Na^+/K^+ ATPase show similar defects in tracheal tube size when mutated (Paul et al., 2003). Additionally, this study revealed that the function of SJs in tube size control is separate from the regulation of paracellular diffusion. How the SJs control tube elongation is not clear. It was proposed that a group of SJ genes could regulate relative levels of apical membrane components such as Crumbs and thus affect luminal expansion (Wu and Beitel, 2004). The other model suggests that SJs could regulate the apical extracellular matrix indirectly. The identification and analysis of two luminal chitin-binding proteins Vermiform and Serpentine, which, when mutated leads to tube elongation phenotypes similar to those of mutations in SJs components (Luschnig et al., 2006; Wang et al., 2006) revealed that late modification of the luminal chitin matrix is specifically required for tube length control. Additionally it was shown that SJs are necessary for the proper localisation and apical secretion of Vermiform. These results suggest that the second model involving indirect control of tube size through modifications of apical luminal matrix is more likely.

1.4.2 Genes involved in tube diameter control

The second category of tube size mutations consists of genes affecting tube diameter when mutated. They encode proteins involved in chitin biogenesis, secretion or maturation. The tracheal chitin is synthesized by Chitin synthase 1 (CS 1 – *krotzkopf verkehrt*) and deposited into the lumen where it assembles into a defined transient cable that expands in unison with lumen diameter growth (Tonning et al., 2005). Loss of this transient chitin matrix results in local tube dilation and constriction. Such phenotypes have been observed in *mummy*/*cystic* mutants, in which UDP-*N*-acetylglucosamine diphosphorylase required for chitin synthesis is mutated (Araujo et al., 2005; Devine et al., 2005). Also *knickkopf* (*gnarled*) and *retroactive*, which encode proteins involved in targeting and/or secretion of chitin or chitin synthesis enzymes are necessary for regular tube diameter expansion (Devine et al., 2005; Moussian et al., 2006).

1.4.3 Model of tube size regulation

Recently, Wang et al. (2006) proposed the following model. During tube expansion, assembly and growth of the chitin matrix is required to coordinate uniform radial expansion of the tubes. Subsequent modifications in chitin fibril structure by secreted deacetylases (Vermiform and Serpentine), whose localisation depends on SJ function, instruct the epithelial cells to terminate the tube elongation. Thus dynamic structural changes of the luminal matrix may independently determine diameter growth and tube elongation.

1.5 Phenotypic analysis of mutant clones using the MARCM system.

Investigation of tracheal development during embryogenesis has been mostly based on analyses of homozygous mutant embryos. Such an approach is not always possible for studying tracheal morphogenesis during larval life as mutations in many of the genes involved in this process are embryonic lethal. This could be overcome by generation of genetically mosaic organisms. Induction of mutant clones surrounded by wild type tissue is a very useful tool for analysing the consequences of gene loss of function during animal development. The commonly used FRT/FLP system (Theodosiou and Xu, 1998) allows the generation of mutant clonal cells. Usually, the mutant cells can be distinguished from their twin spot sister cells and wild type cells by the loss of marker gene expression. However, in many cases it is very useful to mark the mutant cells positively through expression of marker. Therefore the MARCM (Mosaic Analysis with a Repressible Cell Marker) system was established in flies (Lee and Luo, 1999). In this system, expression of a GAL4-UAS construct with a reporter gene is suppressed by the presence of the GAL4 repressor GAL80, which is placed on the FRT chromosome whose homolog is carrying a mutation to be analysed. When a FLP dependent mitotic recombination event occurs, the reporter gene is expressed only in clones homozygous for the mutation (fig. 1.4).

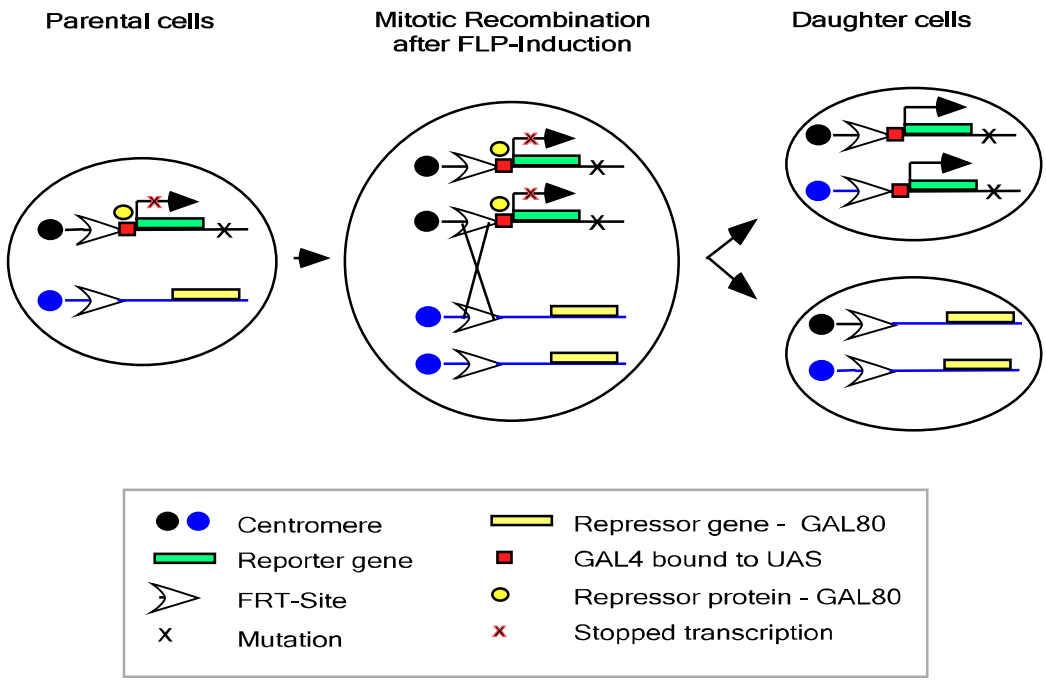

Fig. 1.4 The MARCM system. In parental cells, expression of reporter gene is suppressed by the presence of the GAL4 repressor, GAL 80. The repressor gene is placed distal to the FRT-site on the homologous arm of the chromosome arm with mutation of interest. After FLP induced recombination the cells homozygous for the mutation loose the repressor gene and thus reporter gene can be expressed in these cells (Adapted from Lee and Luo, 1999)

The system has been wildly applied to investigate many cellular and developmental processes, including analysis of tracheal morphogenesis (Cabernard and Affolter, 2005; Ghabrial and Krasnow, 2006; Levi et al., 2006, A.Bilstein, PhD thesis).

1.6 The aim

Several studies on tracheal development in recent years have given an insight into the complexity of the genetic control and cellular processes of tracheal morphogenesis. However there are still many open questions: what are the 'effectors' of signalling pathways controlling tubulogenesis, which molecules are involved in cell remodelling and how do they coordinate extensive cell shape changes and rearrangements. Also, not much is known about the molecular mechanisms responsible for the sprouting of cytoplasmic outgrowth and lumen formation in terminal branching. Since extensive cell remodelling, needed during terminal branching lasts up to larval stages it is important to be able to analyse these processes also at later stages of development. This is not always possible since many genes that might play a role in larval tracheal morphogenesis could be also required during embryogenesis. This disadvantage can be overcome by application of clonal analysis with the MARCM system. It has been successfully applied to analyse role of known genes in the later stages of trachea formation. Thus, it can be also used for identification of new genes involved in tracheal development, a different approach for a better understanding of this process.

The aim of this study was to perform a genetic mosaic screen of the second chromosome, for EMS-induced mutations affecting tracheal morphogenesis. The second chromosome was chosen, because the first and third were simultaneously screened by a different group. To identified new mutants affecting larval tracheal development we applied clonal analysis with the MARCM system and examined mutation effects in third instar larvae.

2. Material and Methods

2.1 Materials

2.1.1 Antibodies

Primary antibodies:

- mouse anti-m2A12 ; 1:20 (against unknown tracheal lumen protein , N. Patel);
- rabbit anti- β – Gal; 1:500 (Cappel);
- mouse anti-arm; 1:3000;
- rabbit anti-GFP; 1:500;

Secondary antibodies:

- goat anti-mouse, biotin; 1:500
- goat anti-rabbit, biotin; 1:500
- goat anti-mouse, Alexa 568; 1:500
- goat anti-rabbit Alexa 488; 1:500

2.1.2 Fly stocks

Stocks listed in tab. 2.1 were used for experiments. TB170 line was used as a wild type control in MARCM analysis. For embryonic analysis w^- flies were used.

Stock number	Genotype	Reference
TB170	$y^{d2}w^{1118}$P{ey-FLP.N}2 P{GMR-lacZ.C(38.1)}TPN1;P{neoFRT}40A	Bloomington #5615
TB114	yw,hsFlp1.22;tub-Gal80,FRT40A;btl-GAL4, UAS-GFP	S.Luschnig (Krasnow lab)
TL521	P{Hs-hid}sp/CyO^{8}ftz lacZ	R. Lehmann
TB136	w^-; If/CyO; btl-Gal4, UAS-βspectrin-PH domain-eGFP-2xHA	A. Bilstein
TMB013	y^{d2} w^{1118} P{ey-FLP.N}2 P{GMR-lacZ.C(38.1)}TPN1**;** P{EP}EP511/CyO	Bloomington #6415
TN044	w^{1118}**; ;** P{UAS-myr-mRFP}2/TM6B**,** Tb^1	Bloomington #7119
F-232	w^-; If/CyO; MKRS/TM6B	F. Sprenger
TMB017	w^-; conv$^{[K6507]}$/CyO^{wgLacZ}	G.Beitel
TMB018	w^-; nrv2[23B]/ CyO^{wgLacZ}	G. Beitel
TMB19	w^*; vari$^{[K5953]}$/CyO	G. Beitel

Tabele 2.1 List of used fly stocks

2.1.3 General reagents and equipment

- general chemicals: Boehringer, Fluka, Gibco, Merck and Sigma
- plastic ware:, Eppendorf, Falcon, Greiner, Sarstedt
- microscopes: Leica Fluorescent-Stereomicroscope MZFLIII (Fluo-Combi)
  Flourescent Microscope – Axioplan 2 imaging - Zeiss
  Light Microsocpe – Axioplan - Zeiss
- cameras: AxioCam MRc5 – Zeiss
  AxioCam HRm - Zeiss

2.2 Methods

2.2.1 Mutagenesis and establishing of the stocks

To establish 5000 lines carrying point mutations on the left arm of the second chromosome chemical mutagenesis was performed. To avoid difficulties of handling such a high numbers of lines at once, they were generated in batches of 1000 lines. In each round three hundreds 2–3 days old males from an isogenised stock, with FRT site on the left arm of the second chromosome ($y^{d2}w^{1118}$P{ey-FLP.N}2 P{GMR-lacZ.C(38.1)}TPN1;P{neoFRT}40A), were mutagenised with 30mM EMS (standard protocol, Grigliatti, 1998). EMS fed males were crossed with hs-Hid/CyO females (P{Hs-hid}sp/CyO^{8}ftz lacZ) (mass cross). To select progeny with required genotype (FRT40A, mutation/CyO) larvae were heat-shocked on day 5 and 6 after egg lay for 2 hours at 38^{O} C (to induce the hsHid transgene expression). Only larvae carrying the mutated FRT chromosome but lacking the one with hsHid transgene, survived this procedure. Next, 1000 males from the progeny were used to establish single lines by crossing them to hsHid/CyO females. Again selection for flies with correct genotype was performed by heat-shock treatment as described above (the crossing scheme is shown on fig.2.1).

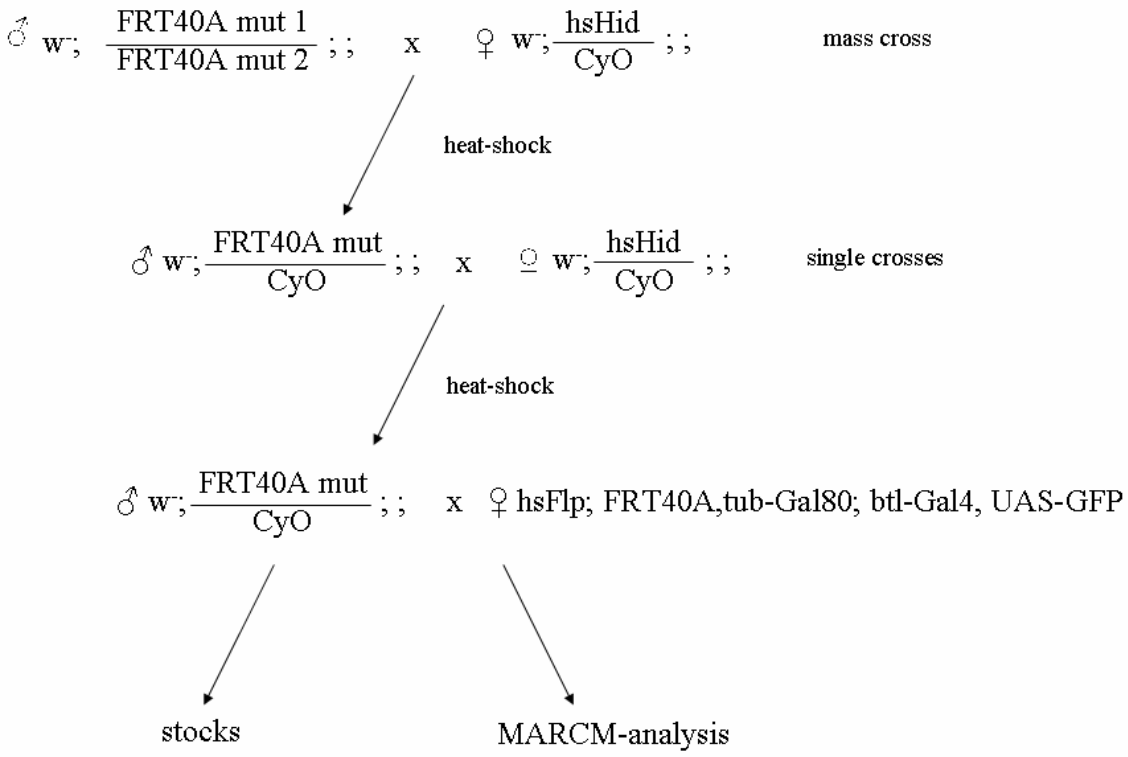

Figure 2.1. Screen crossing scheme: The screen was performed in six batches. In each round 1000 lines were established and analysed in the way shown above.

2.2.2 MARCM analysis

The male progeny from stocks established as depicted in fig.1 were crossed with females carrying all components of the MARCM system (yw,hsFlp1.22;tub-Gal80,FRT40A;btl-GAL4, UAS-GFP). A UAS-GFP construct was used as a reporter gene, driven by the trachea specific btl-GAL4. The eggs were collected for 4 hours at 25^{O}C and heat-shocked for 30 min at 38^{O} C to induce mitotic recombination. Live third instar larvae were observed under a fluorescent-stereomicroscope. For live observation larvae were first immobilised by adding water to the vials and after 1 hour placed on the microscopic slide with plastic grid preventing disturbance of larval structure by cover slip. 8 larvae were analysed per line. Each line showing a defect was retested on the next day with 16 larvae.

2.2.3 Complementation test

In order to perform a complementation test, all the lines were checked for lethality. The homozygous lethal lines were identified by absence of non- CyO flies after 4 generations. Later, these lines were tested against each other within the phenotypic groups. Approximately 2 males and 4 females were taken for each cross. The crosses were kept at 25^{o}C and progeny was scored for presence of non-CyO flies.

2.2.4 Embryonic analysis

2.2.4.1 Embryo collection and fixation

Embryos were collected on apple juice - agar plates for 16 h at 25^{O}C. They were dechoronized by adding bleach (diluted 1:1 with tap water) to the plates for 1,5 min. After washing away the bleach embryos were put into freshly prepared fixation solution (4% formaldehyde, heptan 1:1) and placed on rotor wheel for 30 min at 37^{O}C. Next, aqueous phase was removed and 1 vol. of 100% methanol was added. After vortexing for 1 min. fixative was removed and embryos were quickly washed several times with methanol and store at – 20^{O}C.

2.2.4.2 Immunostaning

Prior to staining procedure methanol was removed from the embryos and replaced by 1xPBST (1xPBS, 0.1% Tween 20). After several quick and three long (15 min) washing steps with PBST, embryos were incubated for 30 min at RT with blocking solution (1x PBST + 1% BSA). Next 300 µl of diluted primary antibody was added (x µl 1^{o} antibody - accordingly to working dilution, 3 µl of normal goat serum (NGS), filled up to 300 µl with blocking solution) and embryos were incubated overnight at 4^{O}C. Removing of primary antibody was followed by several quick and three 15 min long washes with 1xPBST. Afterwards embryos were incubated with corresponding secondary antibody, diluted in 300 µl blocking solution with NGS, for 1-2 hours at RT. Depending on the secondary antibody labelling, the embryos were handle in following way:

Fluorescent labelled antibodies:

Antibody solution was removed and embryos were washed several times with PBST. Next, they were transferred into a drop of Vectashield mounting medium on the microscope slide. Afterwards, cover slip was placed on the top and sealed with nail polish.

Biotin labelled antibodies:

After removing the antibody solution embryos were washed with PBST and incubated for 30 min with ABC-Mix (Vecta Stain) (solution A and B diluted 1:100 in PBST). Incubation was followed by washing step and embryos were transferred to 24-well plates, where after removing of PBST, DAB solution was added to develop the signal (DAB diluted 1:5 in PBST + 1 µl of 0,3% H_2O_2). When staining achieved wished strength, reaction was stopped by removing DAB solution and washing with PBST. Next embryos were dehydrated by series of 10 min washing in increasing ethanol concentrations: 25%, 50%, 75%, 90%, 95% and 100%. The dehydrated embryos were transferred into 100% acetone and incubated for 10 min. Subsequently acetone was replaced with acetone: araldite solution (1:1) and embryos were transferred into the plastic pot filled partially with pure araldite. Embedded embryos were kept at -20^{O}C. For microscopic observation single embryos were transferred form the pot onto microscopic slides.

2.2.5 Immunostaining of third instar larvae.

L3 larvae were filleted open along the ventral midline, dissected in 1x PBS and fixed for 20 min in 4% paraformaldehyde in PBS at room temperature. After 2- 3 10 minutes long washes in 1xPBT (1x PBS + 0.3% TritonX) dissected larvae were incubated for 30 min. in blocking buffer (1xPBTB - 1x PBT with 1% BSA). Fixed larvae were incubated with primary antibody (diluted in 1xPBTB) overnight at 4^{O}C. Prior to incubation with secondary antibody (1.5h, RT) samples were washed twice with 1xPBT. Washing step was repeated after removing secondary antibody and larvae were mounted in Vectashield mounting medium on the microscope slide.

2.2.6 Survival test

To evaluate lethality phase of the mutants embryos were collected on apple juice – agar plates for 2 -3 h at 25^{O}C, and were counted after 3 hours. These plates were kept at 25^{O}C and non-hatched embryos were counted after 24h and 48h. To determinate the developmental stage of these embryos they were covered with Voltalef 3S oil (which makes the chorion transparent).

2.2.7 Mapping of chosen mutants

2.2.7.1 SNP mapping

SNP mapping strategy, described by Berger et al.(2001) is based on sequence polymorphism between the strain used for mutagenesis (with FRT proximal insertion) and reference strain (with distal EP insertion – $y^{d2}w^{1118}$ P{ey-FLP.N}2 P{GMR-lacZ.C(38.1)}TPN1; P{EP}EP511/CyO). The mapping strategy can be divided in two steps: obtaining of the set of FRT and EP recombinant chromosomes with different break points and mapping of the mutation to the genome region by molecular and phenotypic analysis of recombinants.

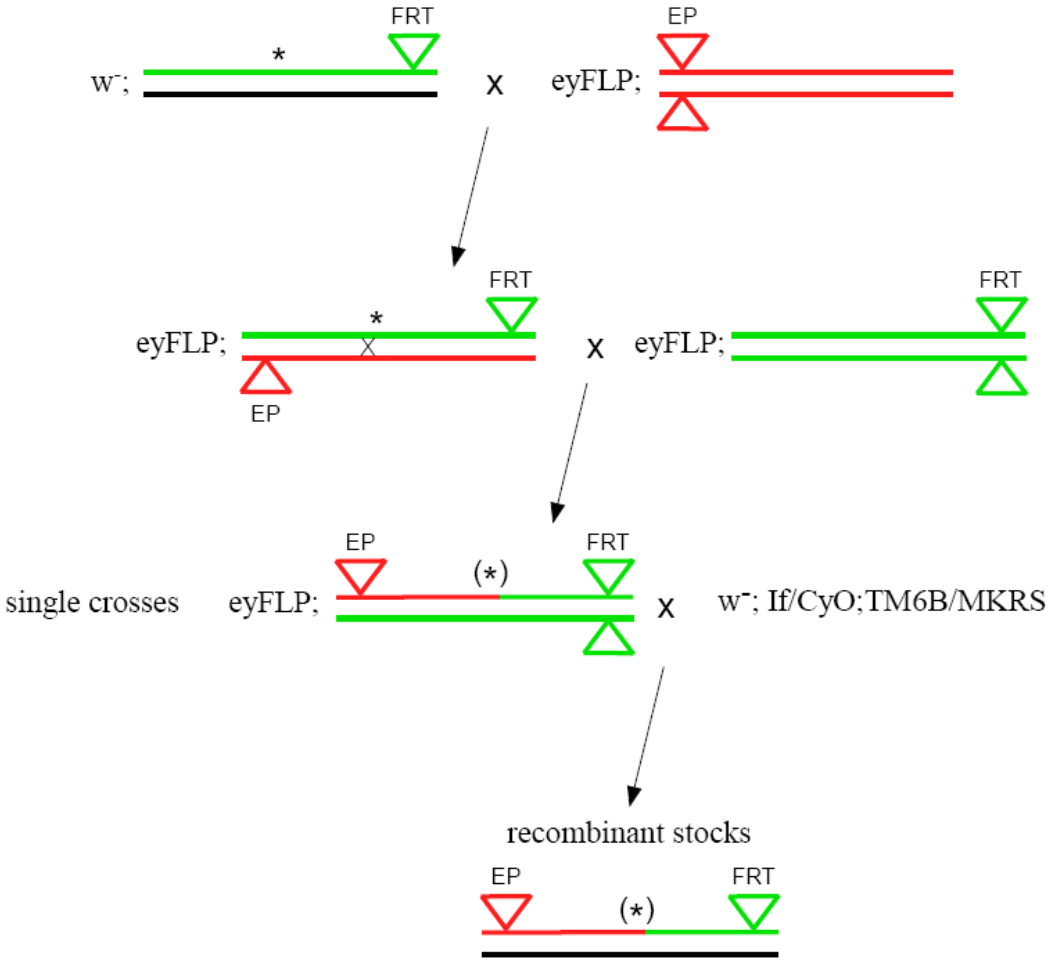

Figure 2.2 Scheme of establishing recombinants stocks. Green line represents FRT chromosome, red EP chromosome, black – balancer chromosome. Triangles represent FRT and EP insertion (green and red respectively). Asterisk – mutation.

Establishing of recombinants stocks

Stocks of different recombinants between mutagenised FRT chromosome and reference line were established (fig. 2.2). To obtain these stocks, males from mutant line of interest were crossed to females from EP line. In next generation females carrying both mutated FRT and EP chromosomes were collected and crossed to males from original FRT line. From their progeny, males carrying recombinant chromosome were collected. The eye-colour mosaicism was used to identify recombinant chromosome with both EP and FRT insertion - due to presence of eyFLP transgene, such a chromosome results in mosaic eyes. In the next step single recombinant males were crossed to double balancer stock in order to establish stable stocks.

Phenotypic analysis of recombinants

All recombinant stocks were analysed for presence or absence of mutant phenotype with MARCM system as described earlier.

Molecular analysis

Genomic DNA was isolated for each recombinant stock according to the large scale DNA preparation protocol (FlySNP Website, http://flysnp.imp.ac.at). From each line, 1-2 males were placed in PCR tubes (0.2ml) and mashed for 5-10 seconds with the tip of a pipet containing 50 µl of Squashing Buffer (10mM Tris-HCl pH8.2, 1mM EDTA, 25mM NaCl, 200µg/ml Proteinase K (stock 20mg/ml, Quiagen)). Then buffer was expelled from the tip and tubes were placed in 37^{O}C for 30 minutes followed by proteinase K inactivation by incubation at 95^{O}C for 5 min. In last step 50 µl of H_2O was added and DNA could be used for PCR reactions.

Randomly chosen DNAs were analysed in Restriction Fragment Length Polymorphism (RLFP) and PCR-product length polymorphism (PLP) assay for different SNP markers (FlySNP Website, http://flysnp.imp.ac.at). Used markers and attributed data are listed in tab.2.2

RFLP assay

PCR mix:

2.5 µl	10x RedTaq Buffer without $MgCl_2$ (Sigma)
2 µl	10mM dNTPs
1.5 µl	$MgCl_2$ (25mM)
13.5 µl	dH_2O
0.5 µl	RedTaq Polymerase (1u/ µl) (Sigma)
1 µl	primer L (10pmol/ µl)

1 µl	primer R (10pmol/ µl)
3 µl	genomic DNA (from 1-2 flies)

PCR programm:

1. Denaturation	5’	$94^{O}C$
2. Denaturation	30’’	$94^{O}C$
3. Annealing	30’’	$62^{O}C$
4. Extension	2’	$72^{O}C$ ($2 \rightarrow 4$ x 40 cycles)
5. Extension	5’	$72^{O}C$
6.	∞	$4^{O}C$

5 µl of PCR product was digested with the appropriate restriction enzyme in 20 µl reaction and separated on 2.5% agarose gel.

PLP assay

PCR mix:

2.5 µl	10x RedTaq buffer without $MgCl_2$ (Sigma)
2 µl	10mM dNTPs
3.5 µl	$MgCl_2$ (25mM)
14.5 µl	dH_2O
0.5 µl	RedTaq Polymerase (1u/ µl) (Sigma)
1 µl	primer L
1 µl	primer R
1 µl	genomic DNA (from 1-2 flies)

PCR programm:

1. 5’ $94^{O}C$
2. 30’’ $94^{O}C$
3. 30’’ $60^{O}C$
4. 1’ $72^{O}C$ ($2 \rightarrow 4$ x 40)
5. 5’ $72^{O}C$
6. ∞ $4^{O}C$

8-10 µl of PCR product were loaded on 2.5% agarose gel.

Marker	Cyt. region	Marker type	Primer L	Primer R	Restriction site	PLP size Δ (bp)
2L015	21F3	RFLP	TCCAAGGGCAGCGGGTTCAATAA	CGAAAACTGTAAGACACGGACACG GAAG	PstI	-
2L030	23F1	PLP	CCCCCTTGAAAACTCCCTTCATTTT	CACTGGGTTTCCCTGGTTTCTGTTT	-	20
2L041	25A2	RFLP	CAGCCCAGTCCGCCAAAGAAACT	TGCCGTTCTAATGCGTCTCGTCCT	ClaI	-
2L057	26A3	RFLP	GCAAAACGAGTAATGGGCTGTAAGCA A	GGATTAGTTTTAGACCAAAATTGG GAGCAAC	-	19
2L063	27B1	RFLP	TCGCCGCTTGCGTTTAAATCGAAAA TAC	GCTCGCCGAAAAATGTGAAAAAGC TACAAAC	DraI	-
2L095	32A5	RFLP	ATATGCGCGAAACCAAAATAAGCGGA CTGATA	CCGGCGAGGCATCTGTTTTATGGA GTG	BglII	-
2L124	34D6	RFLP	CATCCAGCAAACACAAAACGAACCAC	GTTCCACTCATTTCCGCTCCTCACA	SalI	-
2L167	36E2	RFLP	GCCACCAAATACAACCCACACACAA	GCCACCAAATACAACCCACACACA A	PstI	-
2L187	38A2	RFLP	TGGGCGGCACTTGTCATCAGTTT	CGTCTGTGTGTTCGTTTGTGGTGTG TT	HpaI	-

Table 2.2 SNP markers used for molecular analysis. Primers sequence and restriction sites information from The FLYSNP Database (http://flysnp.imp.ac.at/snpdb.php).

Combined information from phenotypic and molecular analysis resulted in placement of the mutation between two markers. Having the region of app. 1-2 Mb determined, all recombinants were screened for informative crossing over event between these two markers. If possible additional marker placed in between was tested to narrow down the region of interests. Recombinants with informative crossing over events were analysed further for more markers in these region.

2.2.7.2 Deficiency mapping

Deficiency mapping was based on lethality complementation between mutants and different sets of deficiencies. Deficiencies stocks are listed in Appendix (tab.A.1). For each cross 4 to 5 females from mutant stock were crossed to 2-3 males from deficiencies stocks. Crosses were kept at 27^{O}C and progeny was screened for absence of non-CyO flies, meaning lack of complementation between crossed lines.

2.2.7.3 Sequencing of candidate genes

DNA preparation from single embryo.

Embryos from original FRT line and respective mutant line were collected for 5 hours and fixed as described earlier. After fixation methanol was removed and embryos were washed 2x in PBST. Mutant embryos were additionally stained for presence of CyO ftz – lacZ chromosome. Single homozygous embryos were placed in PCR 0.2 ml tube and 10 µl of homogenizing buffer (10mM Tris pH 8.3, 0.5% Tween20, 0.5% NP-40, 50 µlM KCl) with 0.2 µl Proteinase K (20mg/ml, Quiagen) were added. Tubes were first kept for 2 hours at -20^{O}C and later for 30 min at 37^{O}C and 3 min. at 95^{O}C. After gentle mixing of the reaction 1 µl was used for PCR.

Amplification of candidate genes

For each candidate gene primers were designed with primer3_www.cgi v 0.2 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). From all genes only exons were amplified and size of the products varies from 400 – 700 bp. Oligos used for PCR are listed in Appendix (tab.A.2).

PCR Mix

5 µl	5x buffer without $MgCl_2$ (Expanded High Fidelity^{PLUS} PCR System, Roche)
1 µl	10mM dNTPs
1.5 µl	25mM $MgCl_2$
0.3 µl	High Fidelity Polymerase (Expanded High Fidelity^{PLUS} PCR System, Roche)
1 µl	primer 1
1 µl	primer 2
1 µl	DNA
16.7 µl	dH_2O

PCR program

1. 5’	94^OC	
2. 30’’	94^OC	
3. 30’’	54^OC	
4. 1’30’’	72^OC	$2 \rightarrow 4$ x 30
5. 5’	72^OC	
6. ∞	4^OC	

5 µl of PCR product were separated on the 1.5% agarose gel to estimate amount of DNA.

Purification and sequencing of PCR product

PCR products were purified from the solution with GFX PCR DNA gel and solution purification kit (Amersham Biosciences) and 1- 6.5 µl was used for sequencing reaction (depending on DNA concentration). Each PCR product was sequenced in both directions with the same primers as used for amplification.

Sequencing Mix

2 µl	Big Dye Terminator v.3.1 (Perlin Elmer)
1 µl	primer (10pmol)
1-6.5 µl	DNA
6-0.5 µl	dH_2O
10 µl	

Sequencing program

1. 5’ $96^{O}C$
2. 30’’ $96^{O}C$
3. 15’’ $50^{O}C$
4. 4’ $60^{O}C$ $2 \rightarrow 4$ 25x
5. ∞ $4^{O}C$

The volume of sequencing reaction was made up to 20 µl before the sequencing of amplified product, obtained sequences were analysed using Vector NTI software (Invitrogene).

3. Results

3.1 MARCM screen

To identify novel genes involved in tracheal morphogenesis we performed a genetic screen, based on EMS (Ethyl Methane Sulfonate) mutagenesis. We were interested in late events of tracheal formation thus to overcome potential early lethality caused by induced mutations, we applied as a screen tool the MARCM system allowing analysis of homozygous mutant clonal cells in heterozygous background.

Previous analysis of clonal cells in trachea of wild type third instar larvae and a pilot screen (see A. Bilstein, PhD thesis) led to determination of wild type features and to establishment of screening criteria. In larvae with wild type clonal cells, number of clonal cells varied from 30 to 80 per larva. They were distributed randomly in all types of branches. Usually the number of clonal cells was slightly higher in anterior part of the larva. The clonal cells on the dorsal trunk were large and hexagonal, except for fusion cells which had a ring-like shape. Cells on secondary branches were elongated and varying in size depending on position. The number and lengths of branches in clonal terminal cells were highly variable with higher branching rate in the anterior part of the animal. A properly formed lumen filled with gas can be found in all clonal cells. Additionally, due to *breathless* expression in the epidermis, a number of GFP positive epidermis cells could be observed. Also two GFP marked cells were often found in the heart.

After inducing mutations with EMS in males we set up approximately 6000 single crosses and were able to establish 4779 stocks carrying mutated chromosomes. The loss of lines occurred at two steps of the procedure. 16% of single crosses did not give any progeny and 6% did not survive the heat-shock. We screened 4779 mutated lines analyzing the number, size, shape and distribution of clonal cells. Also the shape and formation of the lumen within clonal cells were taken into account. We observed the wild type phenotype in 4452 lines, whereas in 344 we detected defects in one or more of the analysed criteria. We classified the mutations into the five phenotypic groups as listed in table 3.1 and described in detail below. Five mutations showed phenotypes that did not fall into any of the proposed phenotypic classes. These were:

- 2L0419 – mutant tracheal histoblasts were smaller; line was homozygous lethal
- 2L0445 - abnormally shaped and too small clonal cells in the dorsal trunk and secondary branches (e.g. transverse connective); line was homozygous viable
- 2L1281, 2L1296 – clonal cells absent in the epidermis; 2L1281 was homozygous lethal, 2L1296 viable;

- 2L3574 – clonal cells present only in the epidermis but not in the trachea; line was homozygous lethal.

All mutant lines are listed in table A3 (Appendix).

GROUP	PHENOTYPE	N^{O} of LINES	SUB-GROUP	SUBGROUP PHENOTYPE	N^{O} of LINES (in subgroup)
A	no clones - cell lethal mutations	163	-	-	-
B	low number of clonal cells	20	0	low number	11
			I	extremely low number	9
C	small clonal cells	47	0	small clonal cells	20
			I	extremely small clonal cells	7
			II	low number and small clonal cells	20
D	dorsal trunk	28	0	not sub-classified	6
			I	DT bendings	10
			II	clonal cells only in DT	4
			III	small clonal cells in DT	5
			IV	low n^{o} / no clonal cells in DT	3
E	terminal branching	81	0	not sub-classified	10
			I	TB crossing/fusion	24
			II	less or no branches	11
			III	problems with lumen formation	19
			IV	expanded/elongated TB	4
			V	no/less clones in TB	13

Table 3.1. Classification of identified mutants. Additionally, 5 lines showed phenotypes that did fall into any of these groups (see above). (DT – dorsal trunk, TB – terminal branches)

3.2 Phenotypic classes

The phenotypes observed in the mutants of the five phenotypic classes are described in detail in the following sections.

3.2.1 Group A – no clones

The phenotype characteristic for this class was the absence of clonal cells. This was the largest group, consisting of 163 lines. The class was not analysed any further.

3.2.2 Group B - low number of clonal cells

The 20 lines classified into group B displayed a reduced number of clonal cells (fig. 3.1). We observed two phenotypic subgroups; one with number of clonal cells varying from 10 to 30 per larva (10 lines –subgroup B 0, low number) and one with less than 10 clonal cells per larva (10 lines - subgroup B I, extremely low number).

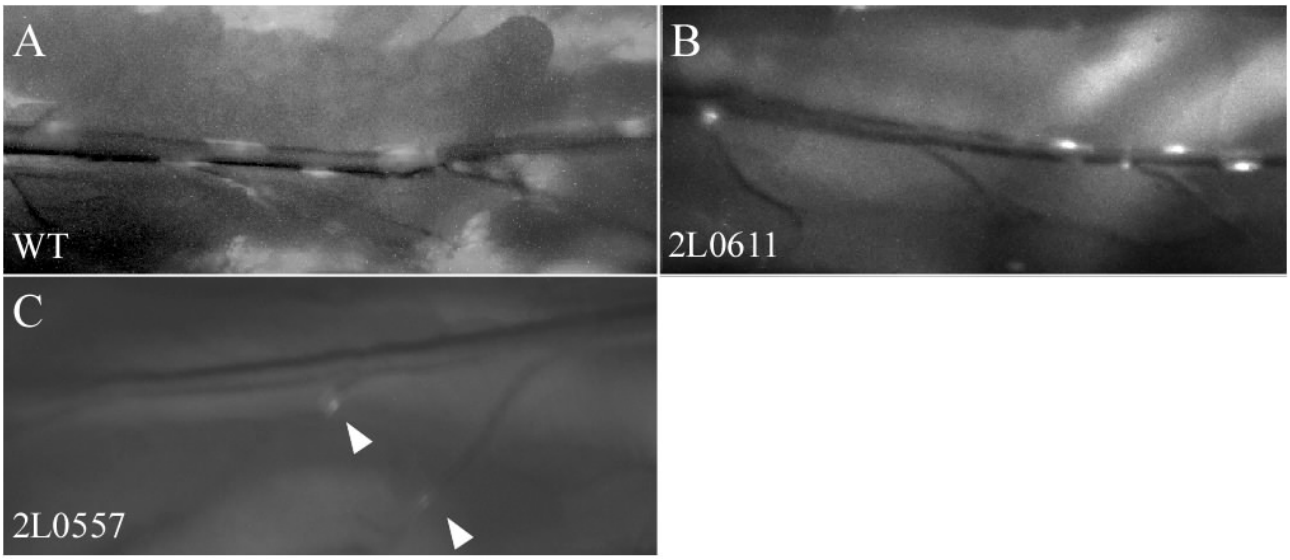

Figure 3.1. The phenotypes of group B – low number of clonal cells. Dorsal trunk of wt (A) and two mutant lines (B and C). Lines classified into this group were divided into two sub-groups: with clone number between 10 and 30 (B 0 – B) and below 10 (B I – C) Arrowheads indicate clones in transverse connectives.

3.2.3 Group C – small clonal cells

Group C consists of 47 lines with clonal cells of reduced size (fig 3.2). Here we also distinguished more than one type of defect. In 7 lines the size reduction was very strong (subgroup C I, extremely

small clonal cells) and in 20 lines the small size of the clonal cells was combined with their low number (subgroup C II, low number and small clonal cells). In addition to reduced size clonal cells often had irregular shapes, especially in the dorsal trunk (fig. 3.2 C).

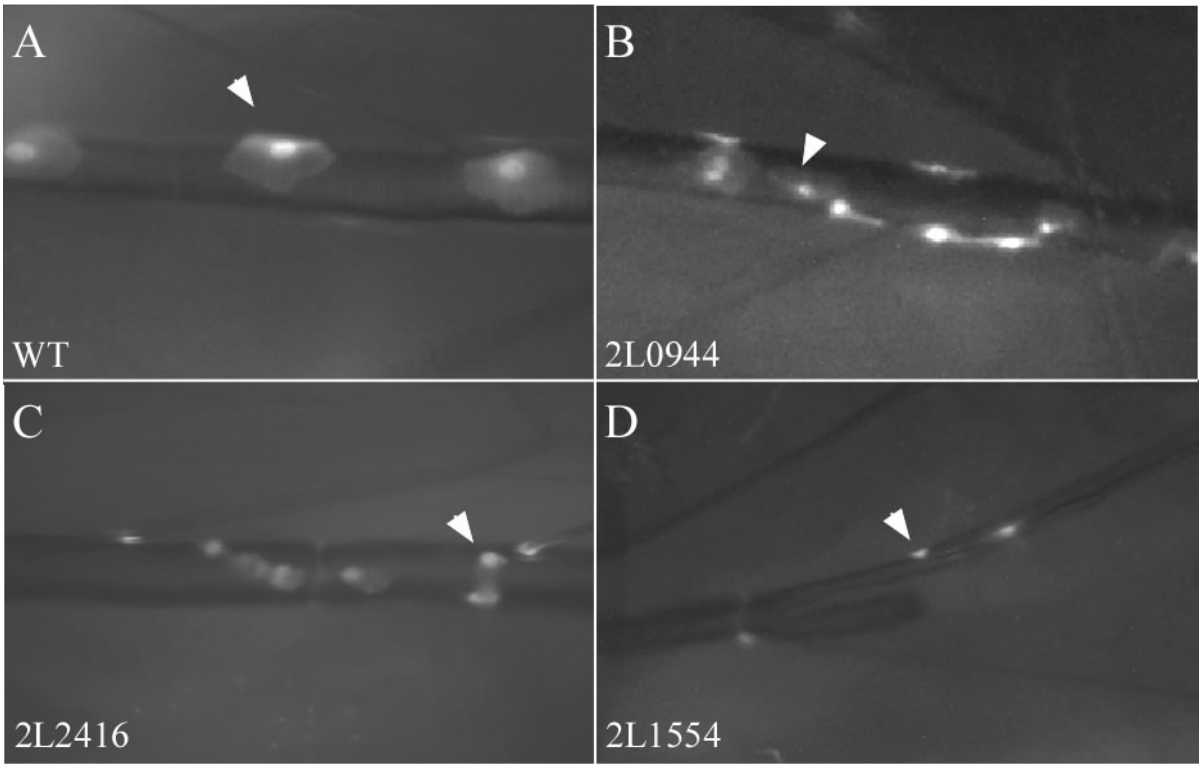

Figure 3.2. The phenotypes of group C – small clonal cells. Dorsal trunk with wt (A) and mutant (B-D) clonal cells (arrowheads). Lines representing three sub-groups are shown, B – small clonal cells (C 0), C – extremely small clonal cells (C I) and D – low number and small clonal cells (C II). Clonal cells in the mutant line in C did not show the hexagonal shape typical for wild type dorsal trunk cells.

3.2.4 Group D – dorsal trunk defects

The 28 lines from group D showed defects in the dorsal trunk (DT). We classified them into five subgroups.

The first – D 0 - consists of 6 lines with defects not observed in any other subgroup.

- 2L0128 – clonal cells in DT larger than wild type (fig 3.3 B)
- 2L0196 - DT narrowed within clonal cell
- 2L0372 – smaller lumen and irregular shape of clonal cells in DT
- 2L3090 – rounded and smaller clonal cells in DT and secondary branches (fig 3.3 D)
- 2L3191 – irregular lumen in DT at clonal position
- 2L4771 – bendings in fusion cells of DT if clonal, no clonal cells in terminal branches and no clonal cells in other fusion cells

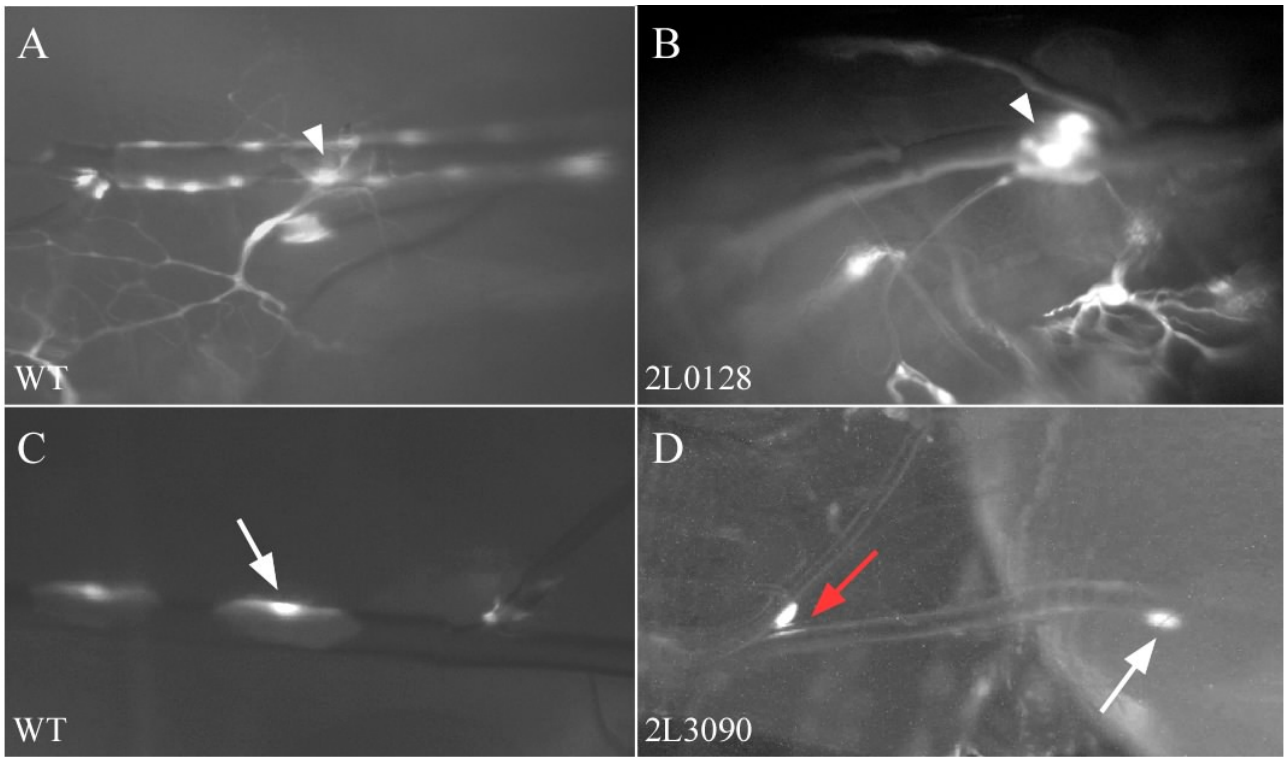

Figure 3.3 Phenotypes of group D 0. In line 2L0128 (B) clonal cells on dorsal trunk (DT) - arrowhead - are larger than in wt (A). Clonal cell in dorsal trunk and transverse connective (TC) of line 2L3090 (D, white arrow DT, red TC) are smaller and rounded up comparing to wt (C).

The second subgroup – (D I – dorsal trunk bending) consists of 10 lines. They exhibited bendings of the dorsal trunk at the position of clonal cell (fig.3.4) whereas other branches were unaffected.

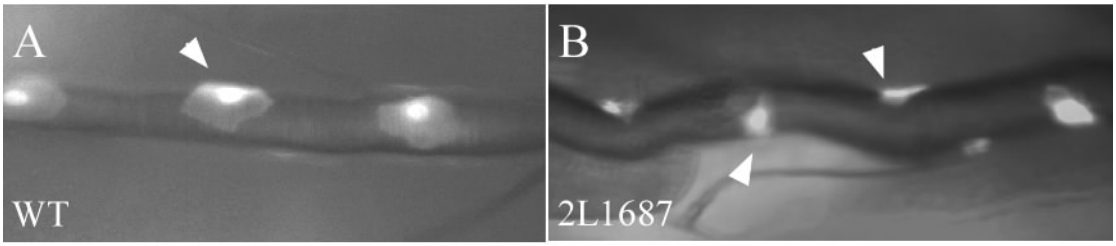

Figure 3.4 Group D I – DT bendings – in the mutant (B) dorsal trunk bends at position of clonal cells (arrowheads) what is not observed in wild type (A)

In four lines belonging to subgroup D II clonal cells were present only in the dorsal trunk (data not shown).

The subgroup D III consisting of five lines had small clonal cells in the dorsal trunk (fig. 3.5). The defect in the size of clonal cell was restricted to the dorsal trunk. The clonal cells on other branches were like in wild type.

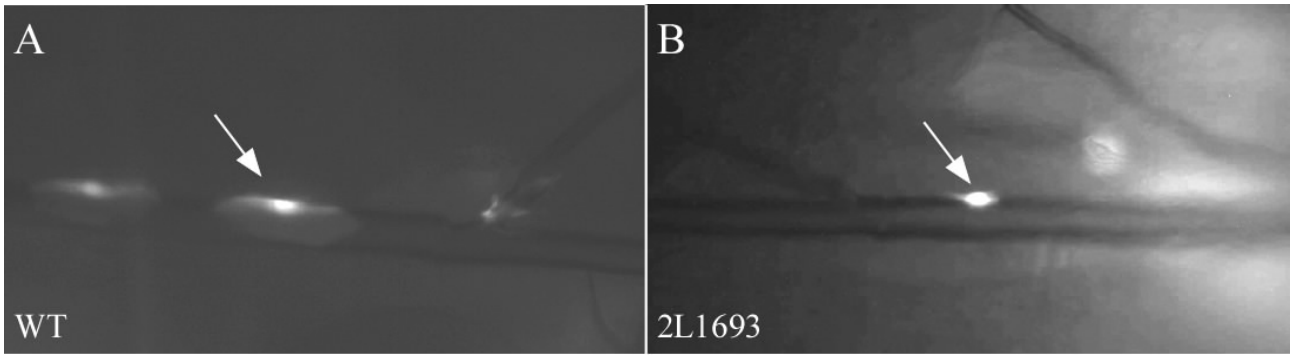

Figure. 3.5 Group D III – small clonal cells in DT. Clonal cells in DT (arrows) of mutant line (B) are reduce in size comparing to wt (A)

The last subgroup, D IV (low number/no clonal cells in the dorsal trunk; data not shown), was characterized by a low number or complete lack of clonal cells in the dorsal trunk and harboured three lines.

3.2.5 Group E – terminal branching phenotypes

The last class, consisting of 81 lines with terminal branching phenotypes, was divided into six subgroups.

The subgroup E 0 includes 10 lines with complex defects.

- abnormal formation of terminal branches (2L1668, 2L1923, 2L2806, 2L3047, 2L3193, 2L3393; fig. 3.6, B-D)
- larger diameter of terminal branches (2L2084, 2L2944, 2L3807; not shown)
- smaller diameter of terminal branches (2L4105, 2L4515; fig 3.6 E)
- abnormal ratio of clonal cells in terminal branches and other types of branches (2L4155, 2L3807; not shown)
- fewer branches in terminal cells (2L4155, 2L2944; fig 3.6 F)
- no lumen in terminal branches (2L4155; fig 3.6 F)

The subgroup E I (terminal branches crossing/fusion) is characterized by 'terminal branch crossing' (fig. 3.7). Two types of this defect can be distinguished: crossing of branches of single terminal cell and crossing of branches of two different cells. As shown in fig.3.7 A-B, in wild type the branches of neighbouring terminal cells, even if growing into the same region of tissue, never crossed the 'border' present between them and thus did not cross each other. In mutants such a restriction was not observed. Also the phenomenon of 'crossing' or fusion between branches of single terminal cell found in these mutants (fig. 3.7 C-F) did not occur in the wild type larvae. We identified 24 lines with such a phenotype.

The subgroup E II (less or no branches) consists of eleven lines exhibiting reduced or completely absent branching of terminal cells. The strength of this phenotype differs between the lines as depicted in fig 3.8. In some lines terminal cell formed a proper tree, but the cytoplasmic extensions were shorter (fig 3.8 B). In other branching did not occur at all (fig 3.8 C) or was strongly reduced (fig 3.8 D). Finally, in a few lines branching stopped after the first ramification (fig 3.8 E and F).

In four lines from subgroup E IV (expanded/elongated terminal branches) the opposite phenotype - excessive branching - was observed. Lines from this subgroup are shown in fig 3.9. In all lines, terminal cells were strongly ramified compared to wild type cells at similar positions (dorsal terminal cells).

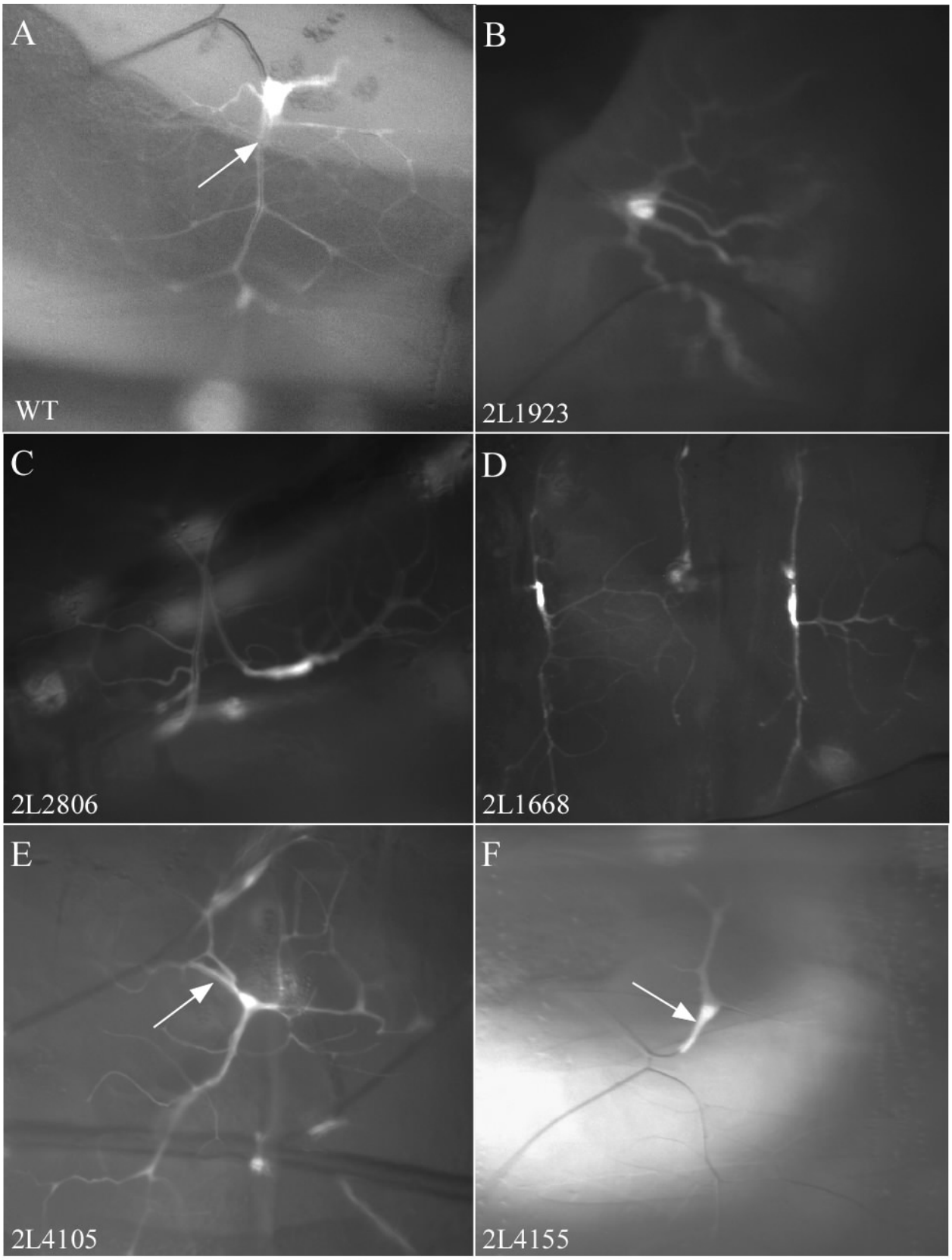

Figure 3.6 Phenotypes of group E 0. A – wt terminal clonal cell – branches were growing in all direction and formed extended trees. Gas filled lumen could be visible (arrow). B-D – different examples of abnormal TB formation. E – diameter of the lumen was smaller than in wt (arrow). F – no gas filled lumen could be seen in terminal cell (arrow), very few branches could be found.

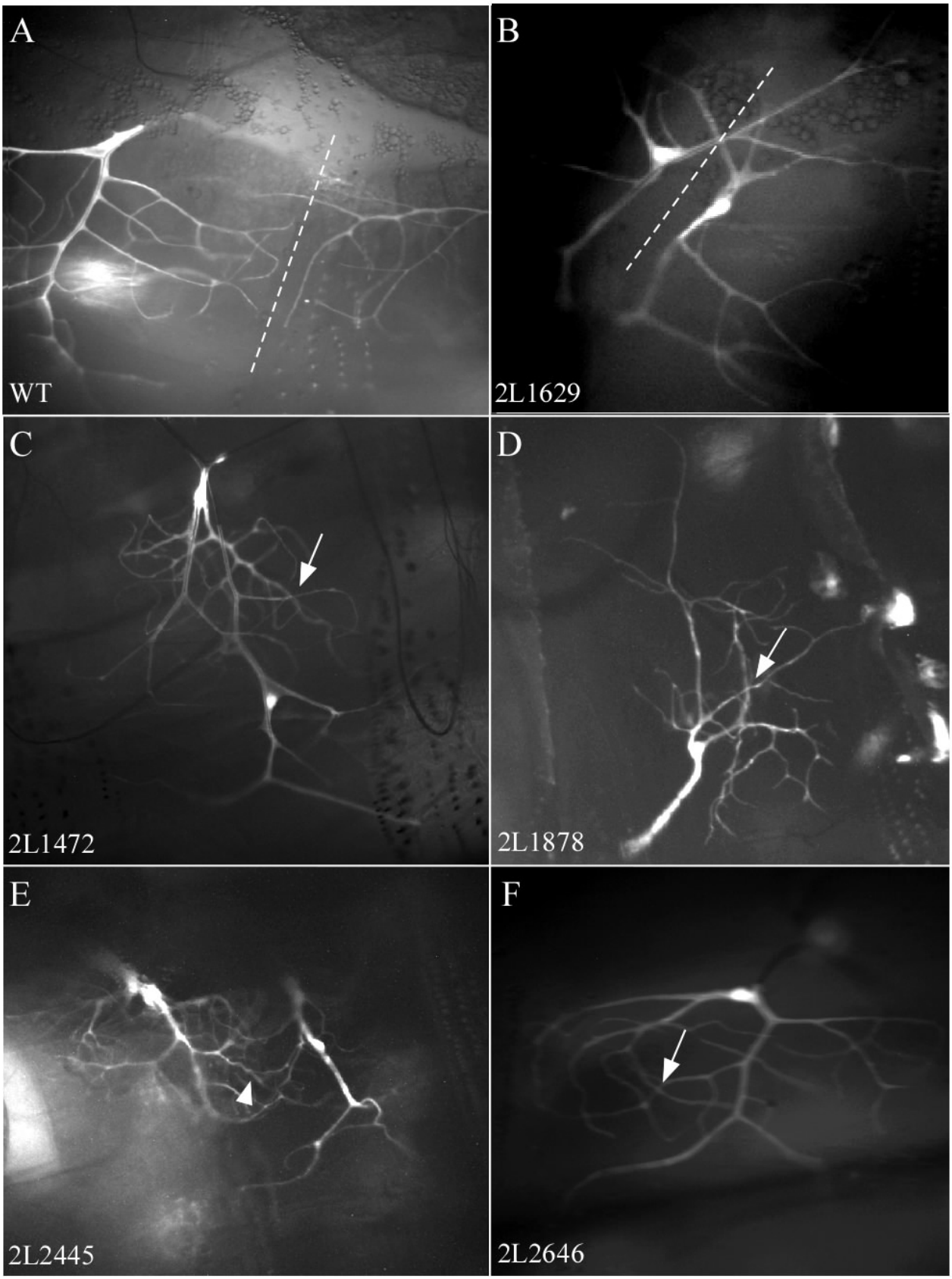

Figure 3.7 Phenotypes of group E I –'terminal branches crossing/fusion'. In wt (A) two neighbouring terminal cells populating the same region of target tissue do not cross each other and do not cross 'border' between their regions (dotted line). Also branches of individual cell do not cross each other or fuse. In mutant lines neighbouring cells cross 'the border' (dotted line in B) and branches of other cells (C, arrow). Also within single cell 'crossing' can be observed (arrow in D and F). Additionally in few lines (i.e. 2L2445) branches fuse (E, arrowhead)

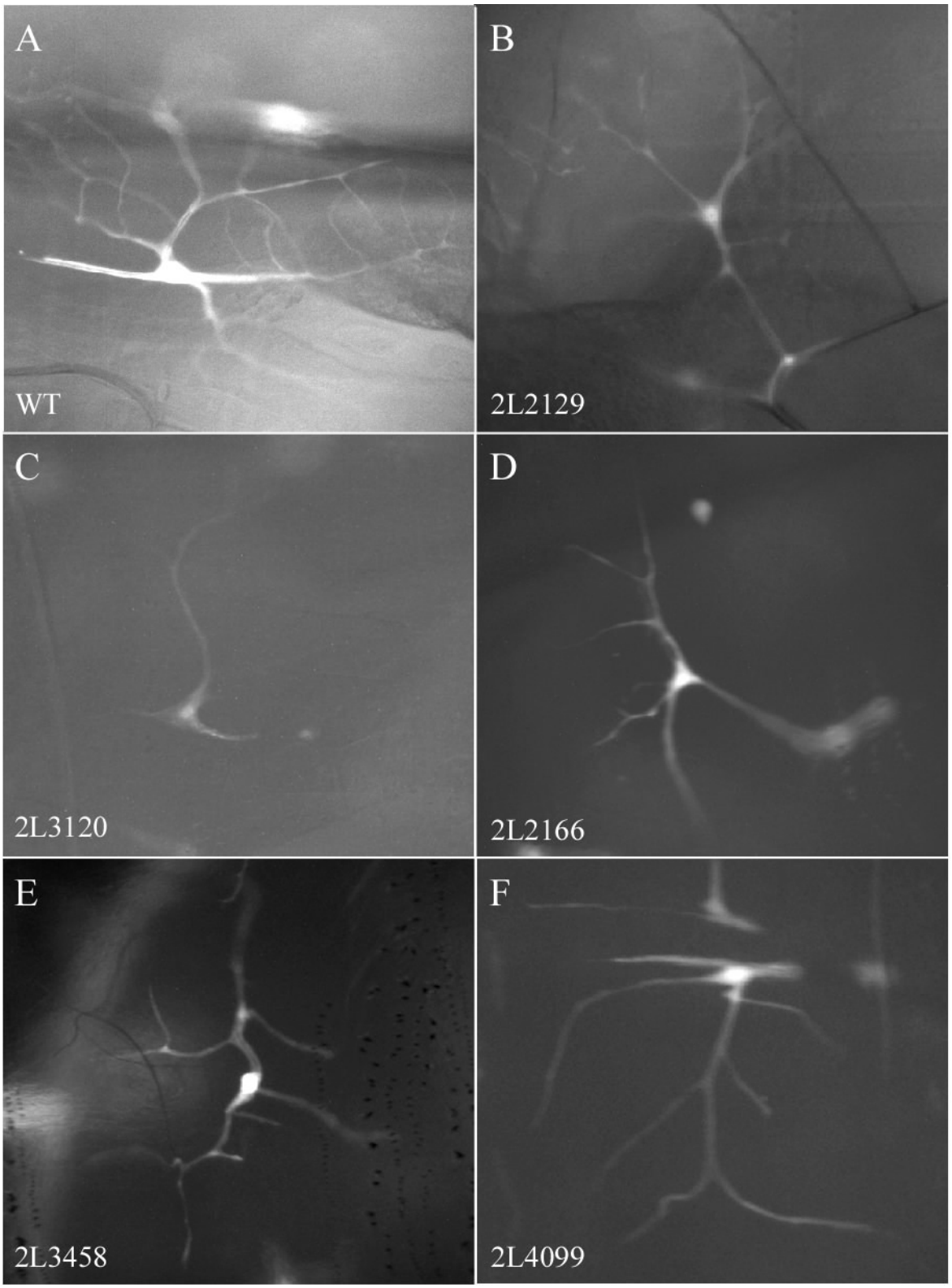

Figure 3.8 Phenotypes of group E II – less or no branches. A - wt terminal cell. B-F - examples of mutant cells with different strength of phenotype. B – weak - tree is properly formed but branches are shorter; C – strong – branching almost completely abolished; D – branching strongly reduced; E-F – mild – branching stops after first ramification.

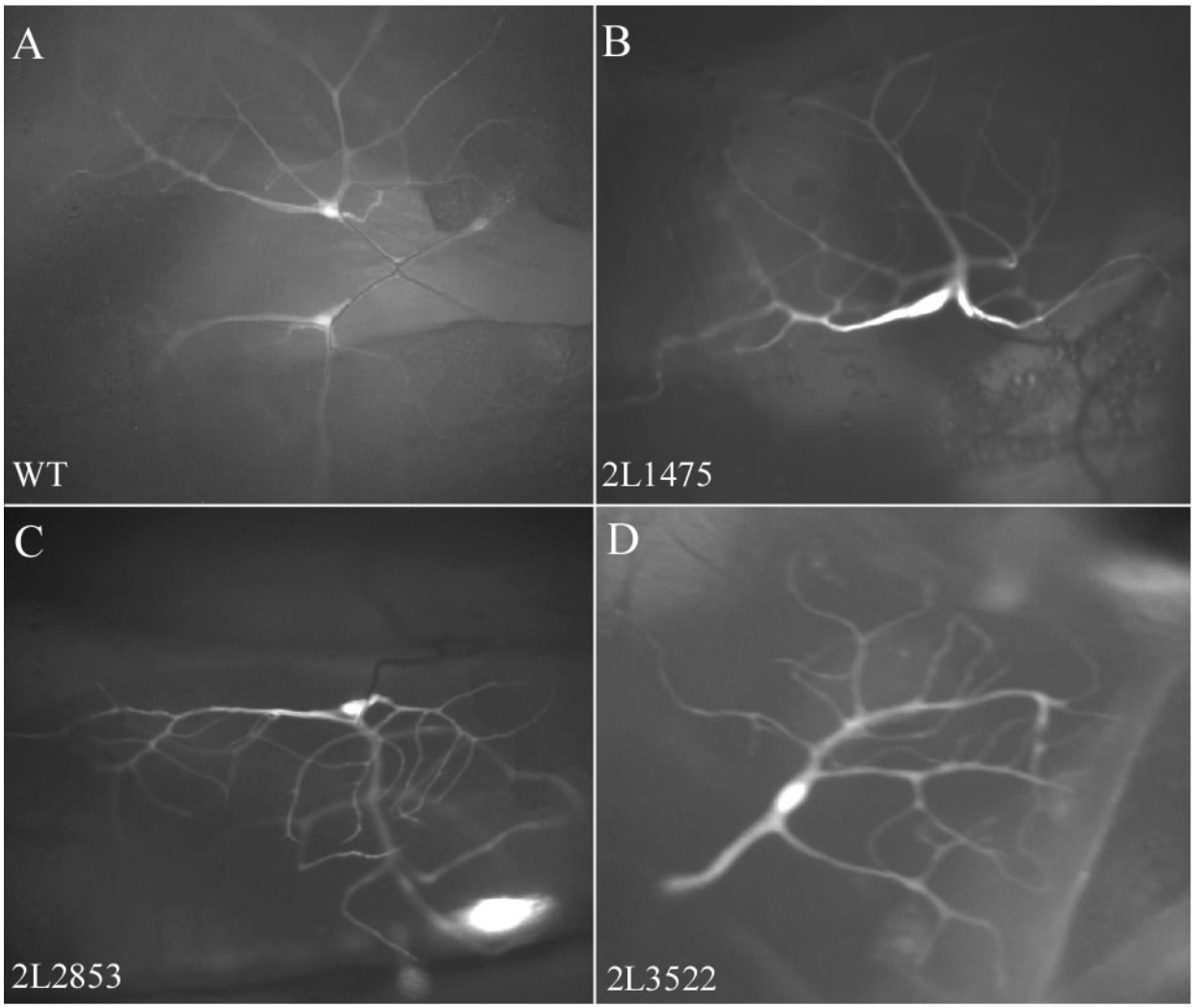

Fig. 3.9 Group E IV – expanded terminal branches. Dorsal branches of mutant lines (B-D) are more ramified than the wt (A)

The subgroup E III exhibited problems with lumen formation within the terminal branches. The strongest phenotype in this group was a complete abolition of lumen formation within the branch as depicted in fig. 3.10 G-H (6 lines). Also milder phenotypes with partially formed lumen were found (10 lines fig 3.10 C-D). In three cases we observed properly formed but mispositioned lumen. In the wild type, the lumen was placed centrally in the cell whereas in these lines it was on the side and in one case lumen seemed to 'turn back' within the cell (fig. 3.10 E-F). Most of the 19 lines showed reduced number and length of branches. Additionally two lines were lacking lumen in clonal cells in secondary branches.

The subgroup E V, the last class with terminal branching defects, consists of 13 lines in which no clones could be observed in terminal cells or their number was strongly reduced in comparison to the number of clones in other branches (data not shown).

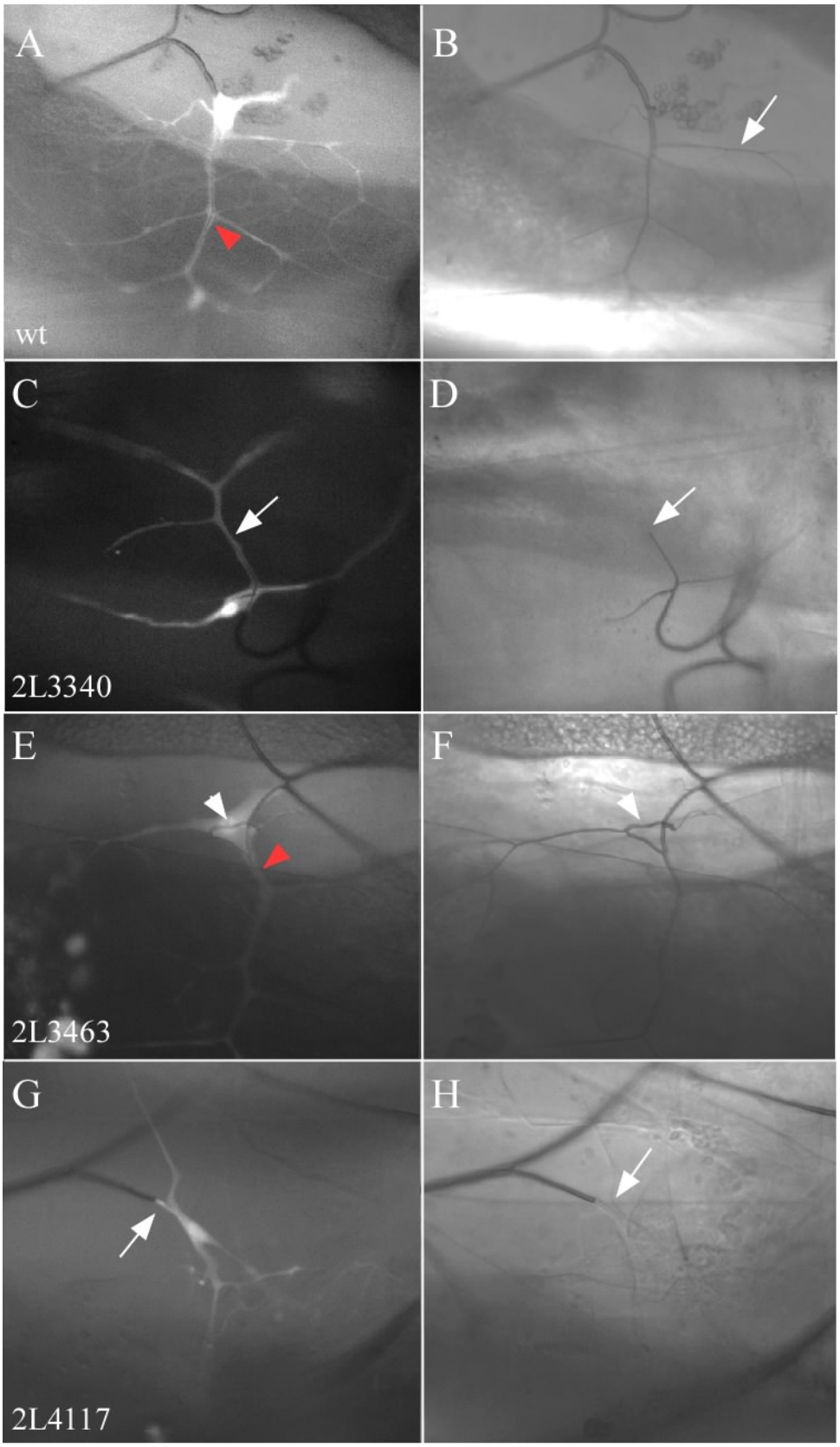

Figure 3.10 Group E III – lumen formation problems. Right panel: – GFP; left panel: bright field images of the same cells. A- wild type terminal cell of dorsal branch. White arrow indicates presence of gas filled lumen, red arrowhead shows centrally placed lumen within the cell. In line 2L3340 (C-D) terminal cells do branch, but gas filled lumen is only partially formed (arrows). White arrowheads in E and F mark branch 'turning back' within the cell. The lumen of this cell is misplaced to the lateral side of the branch (red arrowhead). G and H: complete abolishment of lumen formation within clonal cell (arrow).

3.3 Complementation test

To evaluate if any of the observed phenotypes are caused by mutations in the same gene we performed complementation tests. Phenotypes of mutant lines can be observed only in clonal analysis which requires crossing in the MARCM system components. Thus it was impossible to complement the phenotypes of different lines. Hence we decided to complement mutant lines for lethality.

First we checked all the lines except the 'no clones' group if they were homozygous lethal or not. We found 78% of lines from groups B-E to be homozygous lethal. We tested these lines within each phenotypic group for absence of complementation. We did not cross lines from different groups, except for subgroup C II (low number and small clonal cells) and group B.

Group	N° of lines		Lethality %	N° of complementation groups	Complementation groups	Group members	N° of single hits
	In total	lethals					
A	163	-	-	-	-	-	-
B	20	13	65	2	B1	2L4332/2L4449	10
					B/C1	2L3456(CII)/2L4332	
C	47	41	87.2%	16	C1	2L2416/2L2648/2L3301	14
					C2	2L3301/2L3918/2L3937	
					C3	2L4081/2L1749/2L2953	
					C4	2L4081/2L2816	
					C5	2L2816/2L3547	
					C6	2L3311/2L3500/2L3908	
					C7	2L3908/2L3642	
					C8	2L1663/2L3642/2L3390	
					C9	2L1663/2L2572	
					C10	2L1554/2L2572	
					C11	2L1554/2L2269	
					C12	2L2269/2L2526	
					C13	2L3569/2L3948	
					C14	2L1360/2L4774	
					C15	2L0944/2L3651/2L4508	
					C16	2L4508/2L3456	
D	28	24	85.7	3	D1	2L0439/2L1506/2L1687 2L2181/2L3179/ 2L3696/2L3910/2L4333	10
					D2	2L0028/2L1693/2L3707	
					D3	2L2676/2L2769/2L3090	
E	81	58	71.6	3	E1	2L1923/2L3244	52
					E2	2L3637/2L4501	
					E3	2L2218/2L3443	

Table 3.2: Results of complementation test.

In total we have identified 24 complementation groups (tab. 3.2):
Group B: 2 groups with 2 members each;
Group C: 16 groups: 6 with 3 members each and 10 with 2 members each;
Group D: 3 groups: 1 with 8 members and 2 with 3 members;
Group E: 3 groups with 2 members each;
The eight lines from group C belong to more than one complementation group. In case of one group from class D (D3) all three lines not complementing each other show different phenotypes.

3.3.1 Complementation test for 'no clones' class

When we analysed lines identified in the pilot screen for defects in the development of embryonic trachea (A. Bilstien, PhD thesis) we observed slightly convoluted dorsal trunks in line 2L0058, a member of the 'no clones' class (fig 3.11 C). This phenotype is characteristic for mutations in genes involved in control of tube size and diameter (reviewed in Wu and Beitel, 2004). There are three known genes on the left arm of the second chromosome showing such defects when mutated: *varicose* (*vari*), *convoluted* and *nrv2*, and there was a possibility that line 2L0058 carried a mutation in one of these genes. To test this we checked line 2L0058 for complementation of lethal alleles of these three genes and found it to complement all three. However, the observation that a line showing no clones in MARCM system exhibited convolution of dorsal trunk in the embryo indicated that other lines from the 'no clones' class might also have such a phenotype and be allelic to one of genes mentioned above. Thus we performed complementation tests between all 'no clones' lines and 2L0058, *vari* and *convoluted*. *Nrv2* has not been tested due to stock contamination.
Two complementation groups were identified:
A1) 2L0058, 2L1963, 2L3002, 2L3794, 2L4745 and 2L3215;
A2) *vari*, 2L1623 and 2L3215;
Line 2L3215 did not complement members of either.
Lines from group A1, except for 2L0058, did not show any defect in embryonic trachea. Both lines from group A2 exhibit the *vari* phenotype (fig.3.11), although the phenotype in line 2L3215 seemed to be weaker.

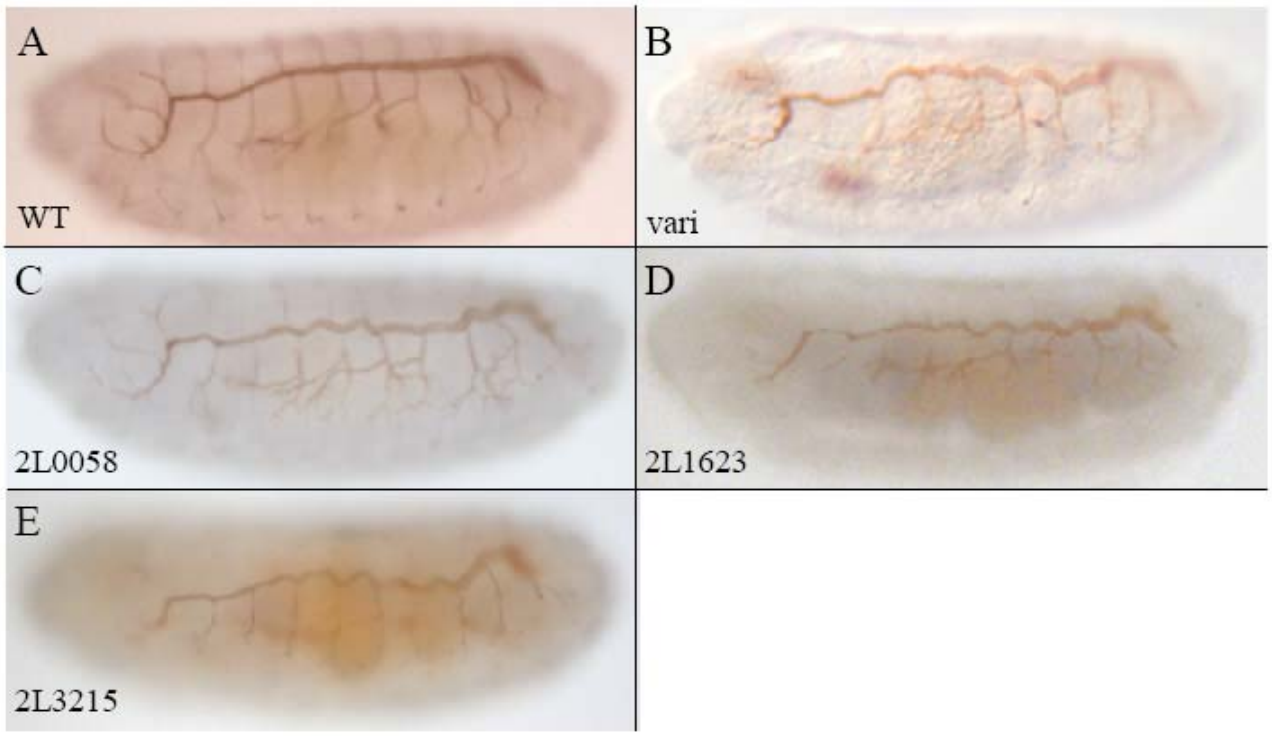

Figure 3.11 Embryonic trachea in homozygous embryos (stage 15/16) visualised by luminal marker 2A12. A – wt embryo, B,D-E embryos from complementation group A2, C – embryo from line 2L0058. All mutant lines show dorsal trunk convolution phenotype. Anterior to the left, dorsal up.

3.4 Analysis and mapping of selected candidate lines.

After the preliminary description of mutant lines the next steps was the characterisation of observed phenotypes in detail and mapping of the mutation. However, handling all lines simultaneously was difficult. So I decided to concentrate on three groups of lines. Selection criteria were high number of alleles or interesting phenotype. The first criterion was fulfilled by complementation group D1. The eight lines from this group showed defects in the dorsal trunk. Additionally I found defects in lumen formation in terminal branching very interesting and thus analysed further two complementation groups found within this phenotypic class. These were E2 and E3, consisting of two lines each. Details of analysis and mapping on all three groups are presented in next sections.

3.4.1 Group D1-analysis and mapping.

The group D1 consists of eight lines from class D I – dorsal trunk bendings. They did not complement each other, showed the same phenotype and thus could be considered as allelic. These were: 2L0439, 2L1506, 2L1687, 2L2181, 2L3179, 2L3910, 2L3696 and 2L4333.

3.4.1.1 Phenotype description and analysis

As described in section 3.2.3, in these lines the dorsal trunk bended at the position of the clonal cell. The strength of the bending differed between the mutants (fig. 3.12). The weakest defect was observed in lines 2L3179 and 2L4333 (fig. 3.12 D and F) and the strongest in lines 2L1506 and 2L3696 (fig. 3.12 B and I). Additionally clonal cells in the dorsal trunk looked smaller than wild type clonal cells and their shape was irregular. The total numbers of clonal cells as well as the average amount of clonal cells in the dorsal trunk were not affected in mutant larvae (tab. 3.3).
The observed phenotype was not fully penetrant. The lowest number of clonal cells showing the defect (55.87%) was found in line 2L3696 and highest in line 2L4333 (80%; tab. 3.3). Interestingly line 2L3696 showed very strong bendings, whereas defect in line 2L4333 was weak. However such correlation could be only found for these two lines.

Line	Counted larvae	Counted clones	Average clone n° per larvae	Clones in DT		DT clones with phenotype	% of clones showing defect
				In total	average per larvae		
FRT40A	15	455	30.3 ± 8.8	191	12.7 ± 5.5	0	0%
2L0439	12	322	27.7 ± 8.9	130	10.8 ± 3.2	86	68%
2L1506	15	356	23.7 ± 6.7	144	9.6 ± 3.2	103	72%
2L1687	17	472	27.8 ± 9.1	212	12.5 ± 6.5	149	71.82%
2L2181	15	469	31.3 ± 8.7	186	12.4 ± 8.4	125	72%
2L3179	10	276	27.6 ± 6.3	118	11.8 ± 4.2	94	78.88%
2L3696	10	274	27.4 ± 10.9	82	8.2 ± 4.7	44	55.87%
2L3910	19	542	28.5 ± 10.4	198	10.4 ± 5.1	146	72%
2L4333	14	480	34.3 ± 10.1	195	11.1 ± 3.7	155	80%

Tabel 3.3 Numbers of clonal cells in the alleles from group D1 and phenotypic penetrance.

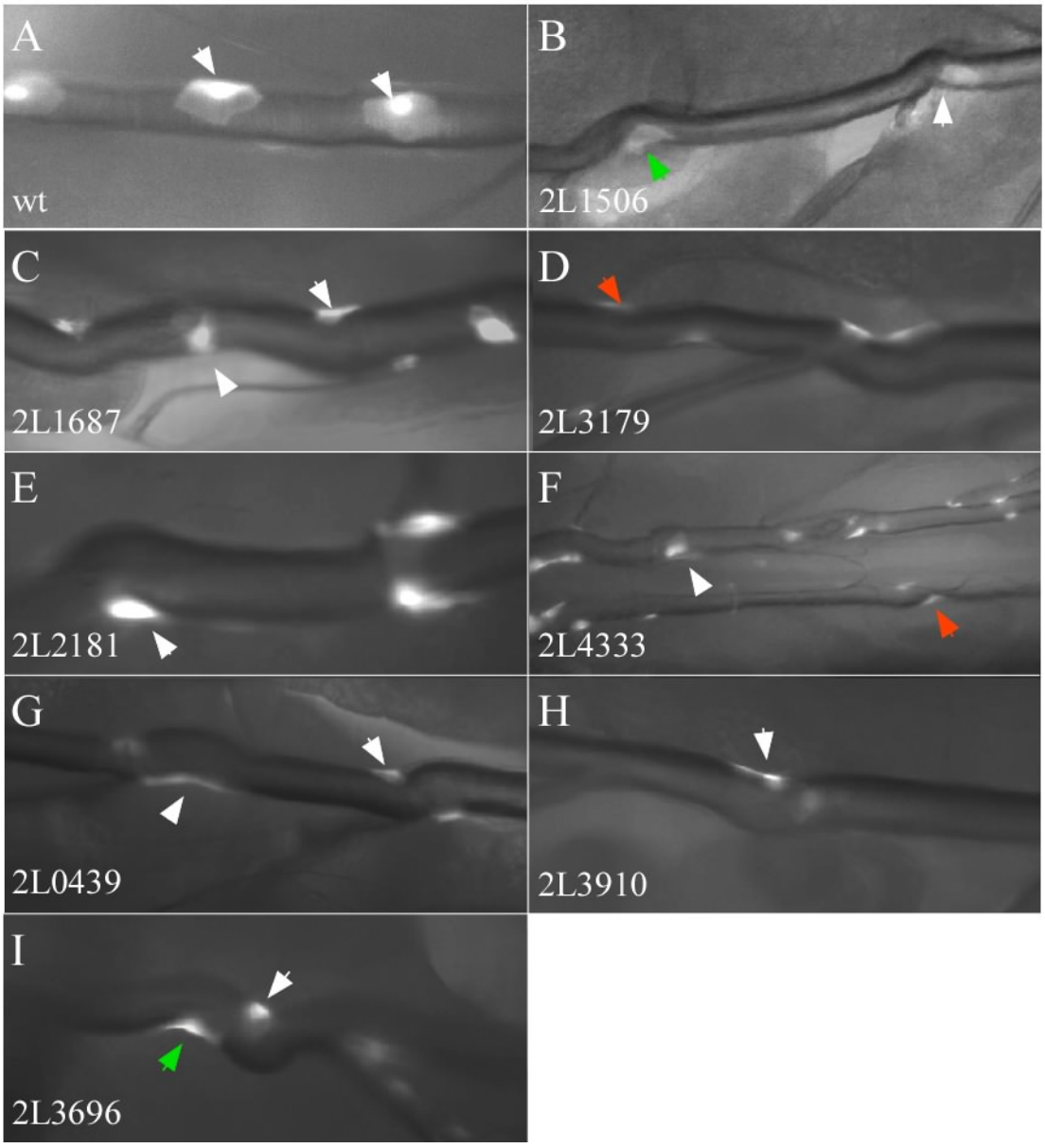

Figure 3.12. Group D1. In all 8 lines (B-I) DT bends at clonal cell position (white arrowheads), what is not observed in wt clonal cells (A). The strength of the bending varies between the mutant lines. Lines 2L3179 (D) and 2L4333 (F) exhibit weakest defect (red arrowheads) whereas lines 2L1506 (B) and 2L3696 (I) strongest (green arrowheads).

In order to analyse clonal cells and the surrounding cells in more detail staining for armadillo (arm), *Drosophila* β-catenin, was performed. Arm is a component of adherens junctions which localises apically in the cells and is involved in cell adhesion (Lecuit, 2005). Thus it is a useful marker for both apical membranes and junctions. If cell to cell contact of mutant clonal cell should be affected due to defects in adherens junction/cell adhesion, one could expect changes in arm localisation. Also any changes in cell shape or size should be revealed by arm staining. In the mutant clonal cells no difference in arm localisation was observed (fig. 3.13), but labelling of cell membranes confirmed a reduction in the size of clonal cells as well as their abnormal shape. As depicted in fig 3.13 D-I, clonal cell were much smaller than neighbouring cells and they lost hexagonal shape typical for cells in dorsal trunk.

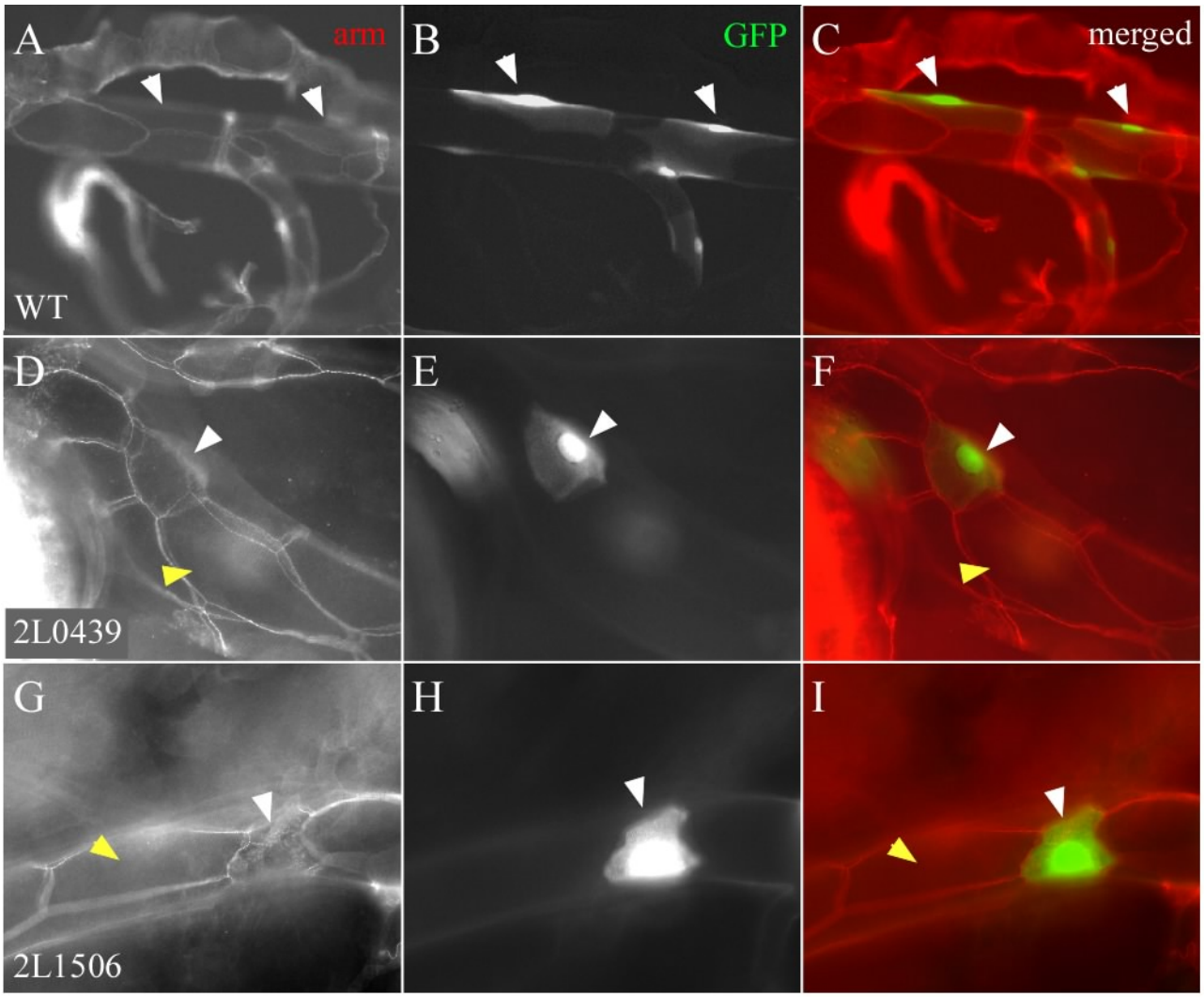

Figure 3.13 Armadillo staining in the DT of larvae with wt (A-C) and mutant (D-I) clones. In wt clonal cells no difference between clonal (white arrowheads) and neighbouring cells could be observed. In the mutant, clonal cells were smaller than neighbouring wild type cells (yellow arrowheads) and irregular in shape.

3.4.1.2 Analysis of embryonic phenotype

For most of the genes known to be involved in tracheal development embryonic phenotypes have been described. So it was interesting to analyse if the alleles from group D1 showed any defect in the development of embryonic trachea when homozygous. That being the case, comparison of observed phenotype with defects in mutants of known genes could help in identification of the gene responsible for clonal phenotype.

The embryonic tracheal development was examined in all eight alleles. To distinguish mutant homozygous embryos from heterozygous or CyO homozygotes, CyO chromosome with ftz-LacZ insertion was used. This insertion leads to expression of β-Galactosidase in the pattern of *fushi tarazu* in the embryos carrying CyO chromosome. By anti-β-Gal staining mutant homozygous embryos could be thus identified. Staining for luminal marker 2A12 revealed that all but one line showed wild type tracheae. In line 2L0439 breaks in the dorsal trunk were observed. As can be seen in fig. 3.14, lumen in the dorsal trunk stayed discontinuous throughout development. In few embryos a milder phenotype showing just narrowed lumen was also found (fig.3.14 F).

Next tracheal cells of mutant embryos were marked with UAS-β spectrin-GFP expressed under control of btl-GAL4 driver in order to verify if absence of 2A12 staining was due to break of the trunk or lack of the lumen. If observed breaks were caused by absence of the lumen, α-GFP antibody staining should showed the intact dorsal trunk and breaks in the α-2A12 staining. If gaps were due to breaks of the dorsal trunk then they should been also obvious by GFP staining. The double staining for GFP and 2A12 revealed that the second scenario is more likely because gaps in luminal staining were accompanied by strong reduction of diameter of surrounding tracheal cells (fig 3.15). The presence of defects in the embryonic tracheae in only one line suggests it is caused by additional mutation on second chromosome in line 2L0439 and the phenotype is not related to defect observed in clonal analysis in larvae.

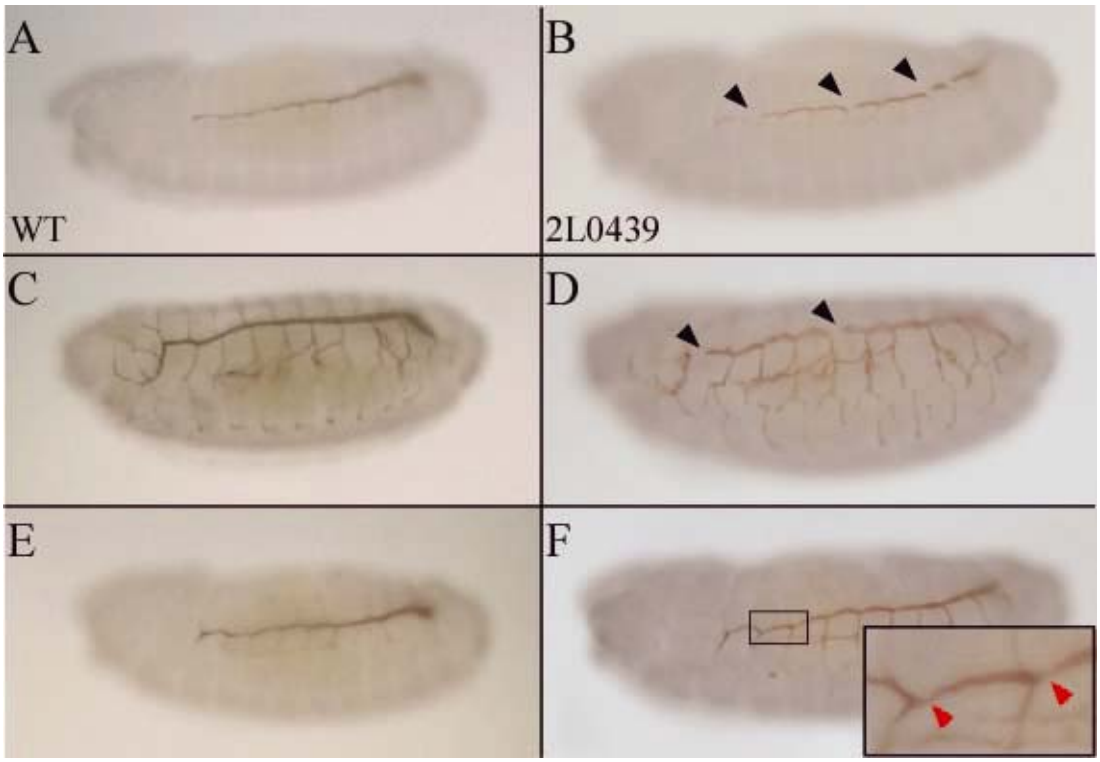

Figure 3.14 Embryonic trachea marked by luminal (2A12) staining of wt (A, C, E) and 2L0439 homozygous embryos (B, D, F). In mutant embryos lumen in DT is discontinuous both in early (st. 14, B) and later (st 16, D) stages (black arrowheads). In some embryos, although no breaks were found, diameter of the lumen was strongly reduced (F red arrowheads, insert show magnification of region marked by rectangle). Lateral view, anterior to the left, dorsal up.

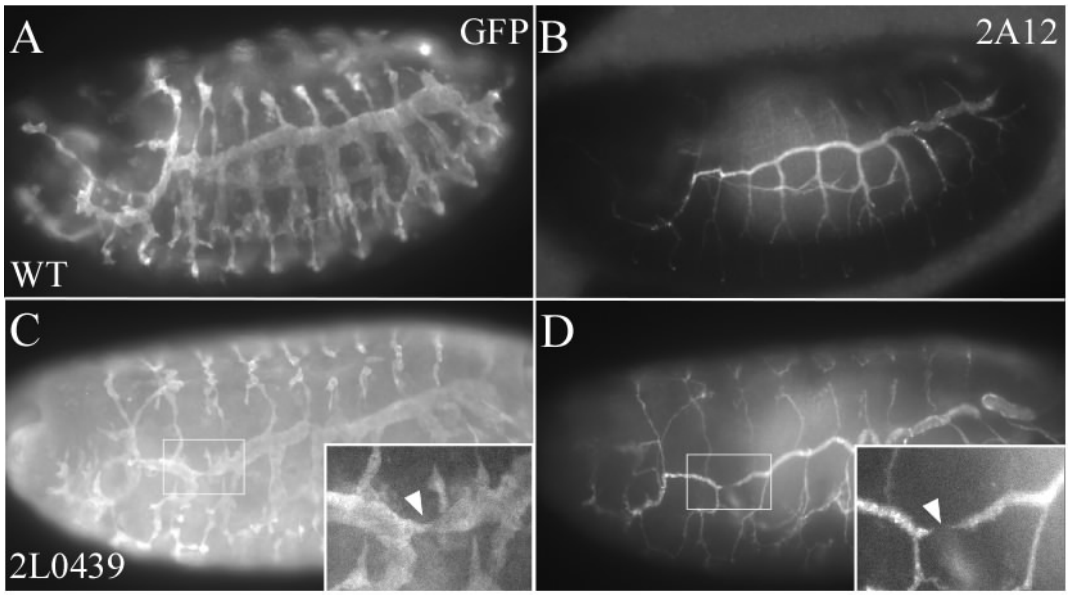

Figure 3.15 Wt and 2L0439 embryos with GFP labelled tracheal cells (A,C) stained for luminal marker 2A12 (B,D). In mutant embryos break in 2A12 staining (insert in D, arrowhead) is accompanied by reduction in diameter of DT (insert in C, arrowhead). Lateral view, anterior to the left, dorsal up.

3.4.1.3 Survival test

The next question to ask was at what stage the analysed mutations cause lethality. Hence a survival test was performed. First, lethality stage of CyO/CyO animals had to be determined. It is known that original CyO balancer leads to larval lethality if homozygous. However chromosome used to balance the mutations has additionally ftz-LacZ insertion and it was not clear if animals heterozygous for this balancer hatch. Thus embryos from FRT40A/ $CyO_{ftz\ LacZ}$ flies were analysed for survival and consider later as a wild type reference. Embryos were collected and left to develop. After 24 and 48 hours non-hatched embryos were counted. 25% of reference embryos did not hatch, which suggests that $CyO_{ftz\ LacZ}$ chromosome is embryonic lethal if homozygous.
After establishing the controls, embryos from each mutant line were tested as described above. If mutations in lines of interest were embryonic lethal 50% of embryos would be expected to hatch. It was the case only for lines 2L0439 and 2L3179 (tab. 3.4). In two lines, 2L2181 and 2L3910, around 40% of embryos did not hatch. The last four lines 2L1506, 2L1687, 2L3696 and 2L4333 seemed not to be embryonic lethal. In all lines a delay in development could be observed (compare number of non-hatched embryos after 24 and 48h in tab. 3.4).

Line n^o	Counted embryos	Non-hatched embryos after 24h	Non-hatched embryos after 48h	Expected n^o of CyO/CyO or mutation/mutation embryos (25%)	% of non-hatched embryos
FRT40A/CyO	187	54	46	47	25%
2L0439	173	91	85	43	49%
2L1506	158	64	47	39	30%
2L1687	154	60	39	38	25%
2L2181	132	82	57	33	43%
2L3179	167	93	90	42	54%
2L3696	172	74	55	43	32%
2L3910	104	68	42	26	40%
2L4333	141	44	20	35	14%

Table 3.4 Survival test summary.

3.4.1.4 Mapping of complementation group D1

The eight lines showing the same phenotype and not complementing each others lethality suggests that mutation causing the phenotype is also the cause for lethality. Thus mapping of lethality should lead to identification of the gene responsible for the phenotype. Therefore the lethality based deficiency mapping was chosen to map the observed phenotype. Two members of the group were crossed to set of deficiencies covering left arm of the second chromosome (stocks listed in

Appendix, table A.1 B - Bloomington Dfs kit). The progeny from these crosses was checked for lethality complementation. Both analysed lines did not complement deficiency Df(2L)spd[j2] which deletes fragment of the chromosome corresponding to region 27C1-2; 28A. To confirm this result, remaining members of the D1 group were crossed to this deficiency, and none complemented. By using smaller deficiencies, partially overlapping with Df(2L)spd[j2], depicted in fig 3.16, the region of the mutation was narrowed down to two intervals of 57kb (A) and 141kb (B). Eighteen genes are predicted in these regions. They are listed together with predicted function in table 3.5.

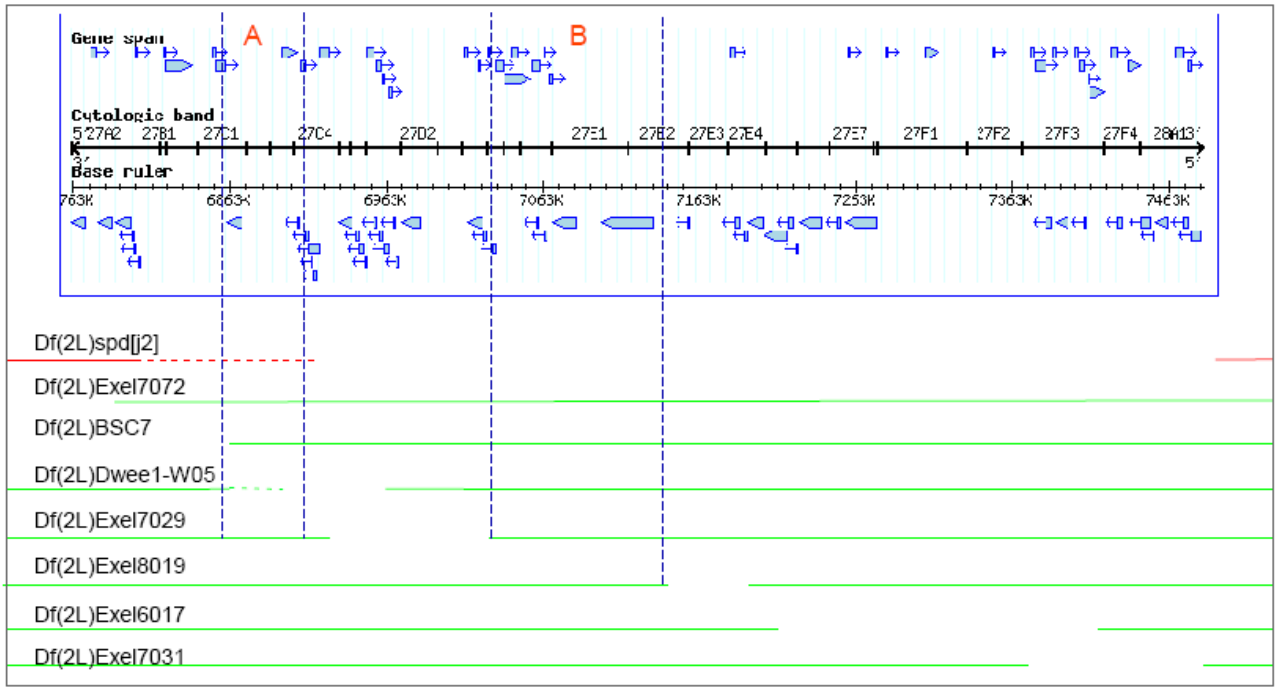

Figure 3.16 Deficiency fine mapping for members of DT bendings complementation group. Solid lines represent undeleted fragments, dashed lines – unclear regions, deleted fragments are shown as blank. Green indicates complementation, red – non-complementation. Horizontal blue dashed lines mark regions A and B uncovered by tested deficiency to which mutation maps. In genomic region representation (FlyBase-http://flybase.bio.indiana.edu) cytologic regions and genes (in blue) are depicted.

Region	Gene	Function
A	CG10805	unknown
A	CG10806	sodium-hydrogen antiporter, regulation of pH
A	neuroligin	Receptor binding protein, involved in development of ectoderm and nervous system
A	CG13773	DNA-directed RNA polymerase activity, involved in transcription
A	CG10354	5'-3' exoribonuclease activity, DNA meatbolism
B	CG31908	unknown
B	CG3476	carnitine transporter activity, lipid metaboslim
B	Rab30	GTPase activity, endocytosis/exocytosis/intracellular transport/small GTPases mediated signal transduction
B	CG11266	spliceosome complex
B	milton	kinesin-associated mitochondrial adaptor, axon transport of mitochondrion
B	CG31630	unknown
B	Mnn1	GTPase activity, JNK cascade
B	CG31907	Calponin – like actin binding protein, localised to microtubule cytoskeleton
B	CG13779	peptidase activity, proteosome complex
B	CG8902	GTPase activity, immune response
B	CG11289	ATPase activity/glucuronosyltransferase activity, defence response/polysaccharide metabolism
B	Pvf2	PDGF and VEGF related factor 2, hemocyte migration, proliferation
B	Pvf3	PDGF and VEGF related factor 3, hemocyte migration

Tabel 3.5 Genes in the region to which mutation in D1 group maps. All information about function is adapted from FlyBase (http://flybase.bio.indiana.edu).

3.4.2 Group E2 - analysis and mapping

Group E2 was chosen for further analysis due to interesting phenotype – defects in lumen formation in terminal branches (class E III). It consists of two lines 2L3637 and 2L4501 which show similar phenotype and do not complement each other.

3.4.2.1 Phenotype analysis

As mentioned before, in wild type terminal cells the lumen can be visualised by bright filed microscopy since it is filled with gas. In most clonal terminal cells in these lines no gas filled lumen could be observed (fig 3.17). There were also few cells with single branches partially filled with gas (data not shown) but no terminal branches completely filled with gas could be seen (tab. 3.6) Additionally gas filling or lumen formation defects were found in clonal cells on secondary branches in which the lumen is made by single cell. As depicted in fig 3.18, there were gaps in gas filling within clonal cells. They spread across whole lengths of the cell (fig. 3.18 D-F) or just part of it (G-H) and the phenotype was not fully penetrant (tab. 3.7). Clonal cells in DT or transverse connectives were like wild type.

Line	Counted larvae	Counted clones	Average clone n^o per larvae	Clones in TB		TB with phenotype	% of clones showing defect
				In total	Av. Per larvae		
FRT40A	16	493	30.8 ±11.9	82	4 ±3.1	0	0%
2L3637	26	628	24.2 ± 10.7	86	3.4 ± 1.6	86	100%
2L4501	24	638	26.6 ± 9.7	68	2.8 ± 1.7	67	99%

Table 3.6 Penetrance of phenotype in terminal branches.

Line	Counted larvae	Counted clones	Average clone n^o per larvae	Clones in secondary branches		Clones in secondary branches with phenotype	% of clones showing defect
				In total	Av. per larvae		
FRT40A	16	493	30.8 ± 11.9	82	5.1 ±4.8	0	0%
2L3637	26	628	24.2 ± 10.7	103	4 ± 2.5	69	74%
2L4501	24	638	26.6 ± 9.7	77	3.2 ± 2.1	56	70%

Table 3.7 Pentrance of secondary branch phenotype.

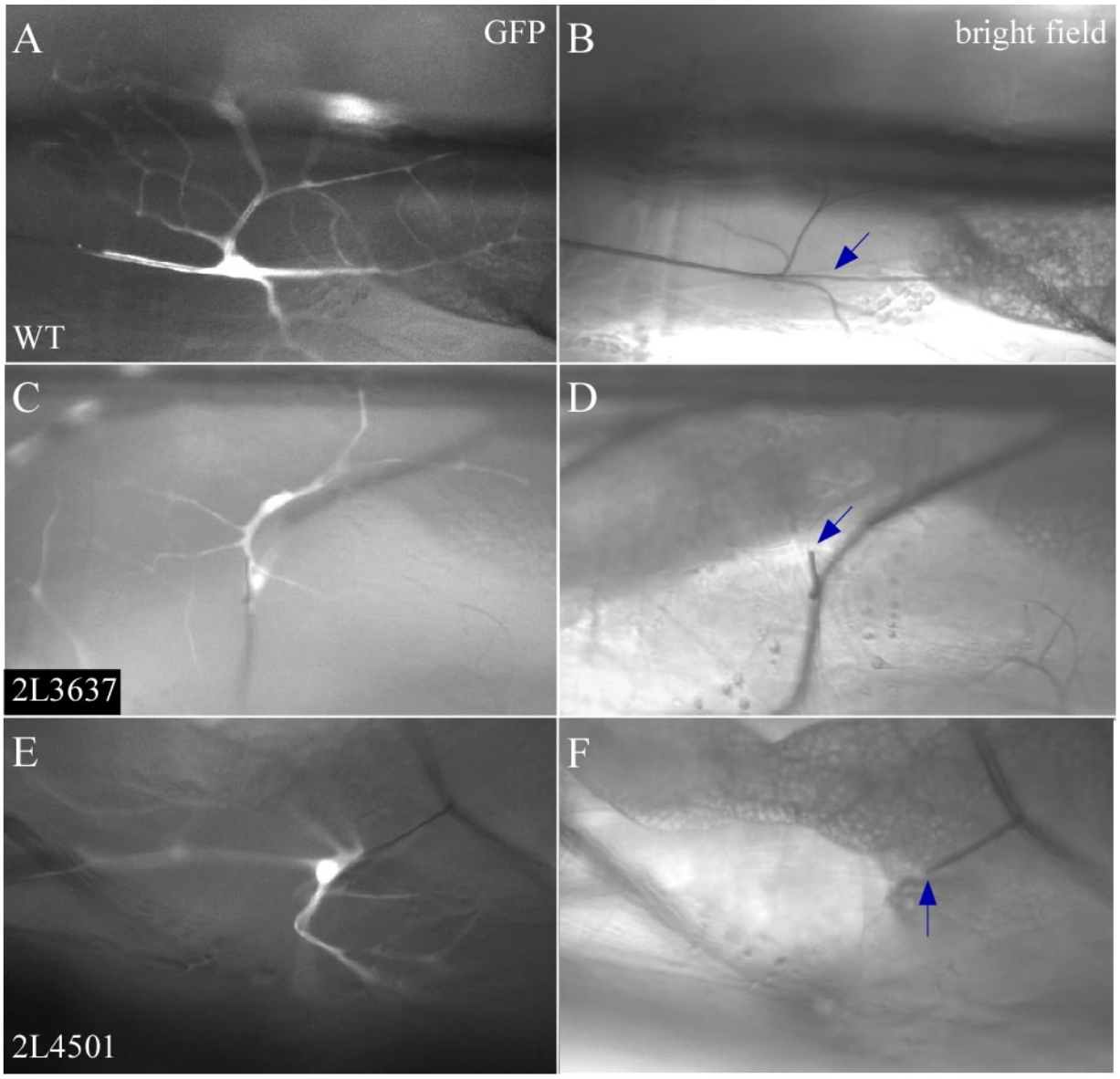

Figure 3.17 Lumen of terminal branches in lines 2L3637 and 2L4501 is not filled with gas. In wt (A, B), lumen of TB can be visualised in bright filed because of gas filling (blue arrow). In both mutant lines no gas filled lumen could be observed. Left panel: GFP channel, right panel: bright field image of the same terminal cells.

To study whether gaps in clonal cells were due to absence of lumen or lack of gas filling, I decided to analyse the membranes of clonal cells. If the lumen was formed in these cells outer basal and inner apical membranes should be detectable. If clonal cells failed to form the lumen only outer basal membrane would be present. In order to test this, chromosome with membrane targeted RFP (UAS-myrRFP) was crossed into the mutant so that clones would be marked with cytoplasmic GFP and membrane localised RFP. If the lumen in clonal cell was formed but not filled with gas no difference in RFP localisation between wild type and mutant clones should be found. If gaps were caused by the absence of the lumen such a difference should be observed. As depicted in fig 3.19, localisation of myr-RFP differed in wild type and mutant clonal cells on secondary branches at the positions where no gas filled lumen was visible in bright field view. In gaps in both mutant lines the

GFP and RFP signals were even whereas in parts of clonal cells with lumen as well as wild type clones, the signal was absent in the lumen. In few cases the lumen was detectable by absence of GFP and RFP signal, but no gas filling could be observed (fig 3.19 I-L and 3.20 I-L). Also in terminal branches the absence of lumen was confirmed (fig 3.20).

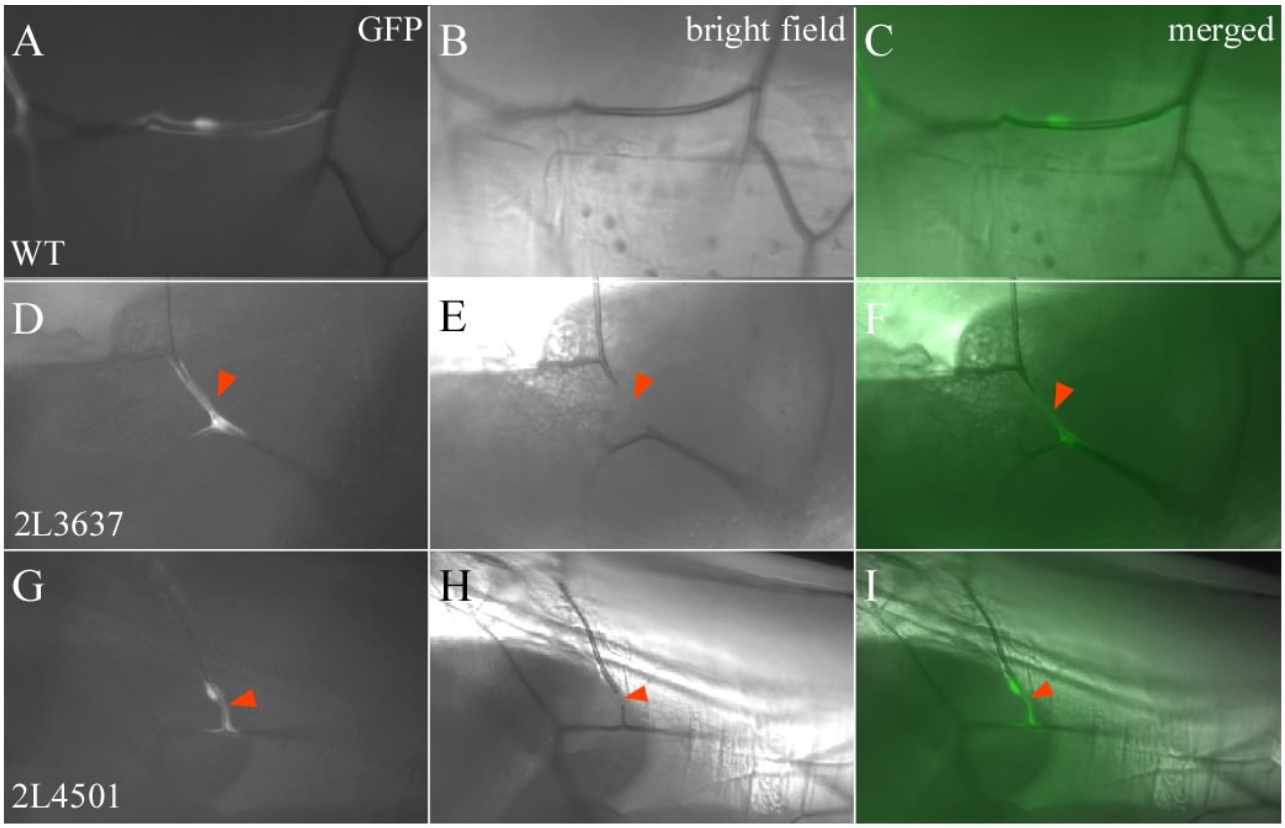

Figure 3.18 Clonal cells in secondary branches of wild type (A-C) and mutant lines (D-I). Lumen in mutant clonal cells is absent or unfilled with gas (red arrowheads)

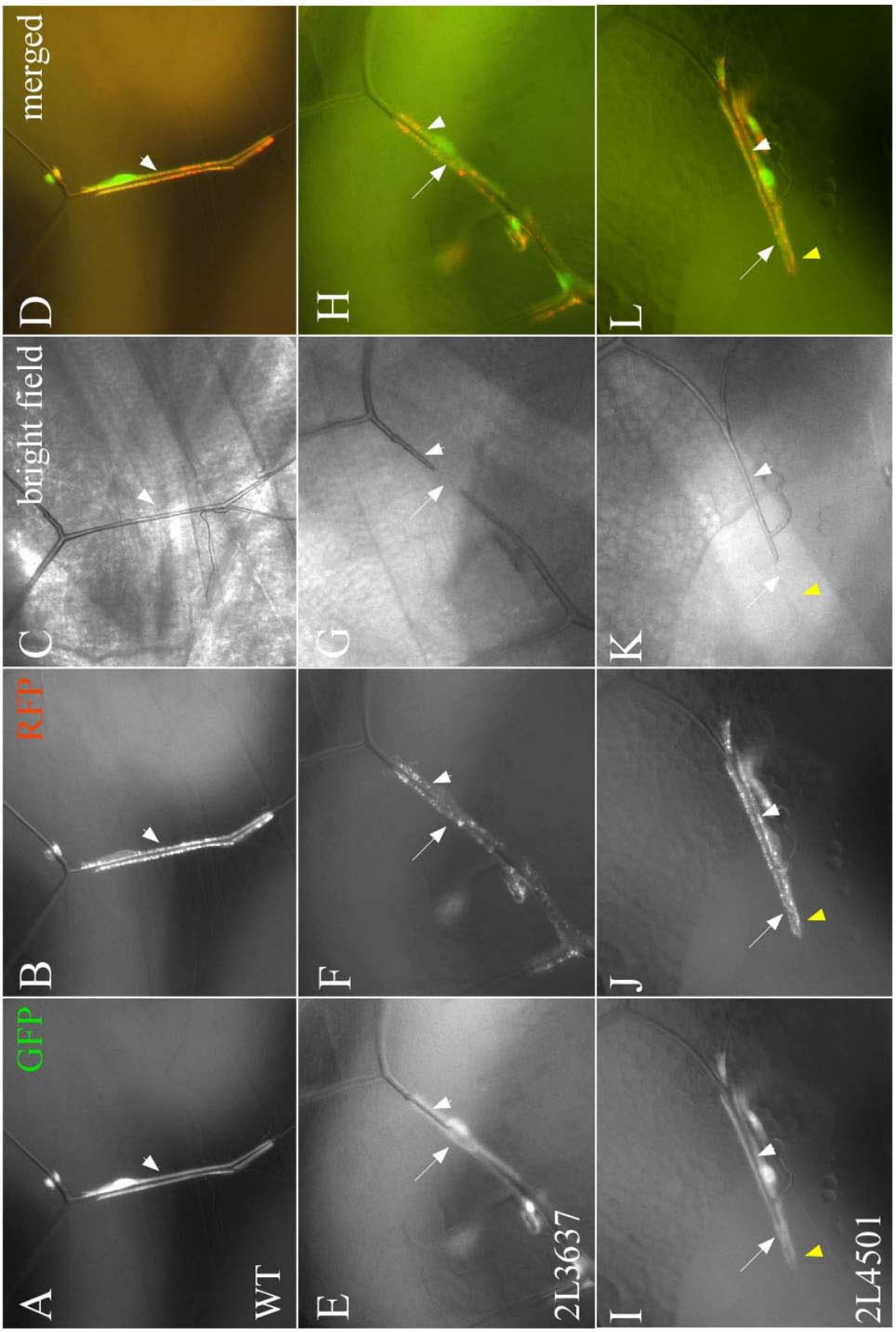

Figure 3.19 Clonal cells on secondary branches in wt (A-D) and mutant (E-L), marked with cytoplasmic GFP and myrRFP. In wt and part of the mutant cell in which lumen is still present, signal is absent in middle of the cell (white arrowheads), whereas in parts missing the gas filled lumen no gaps in signal could be observed (white arrows). Yellow arrowheads indicate part of the cell in which GFP and RFP signal is absent in the middle of the cell, although no gas filled lumen could be seen in bright field.

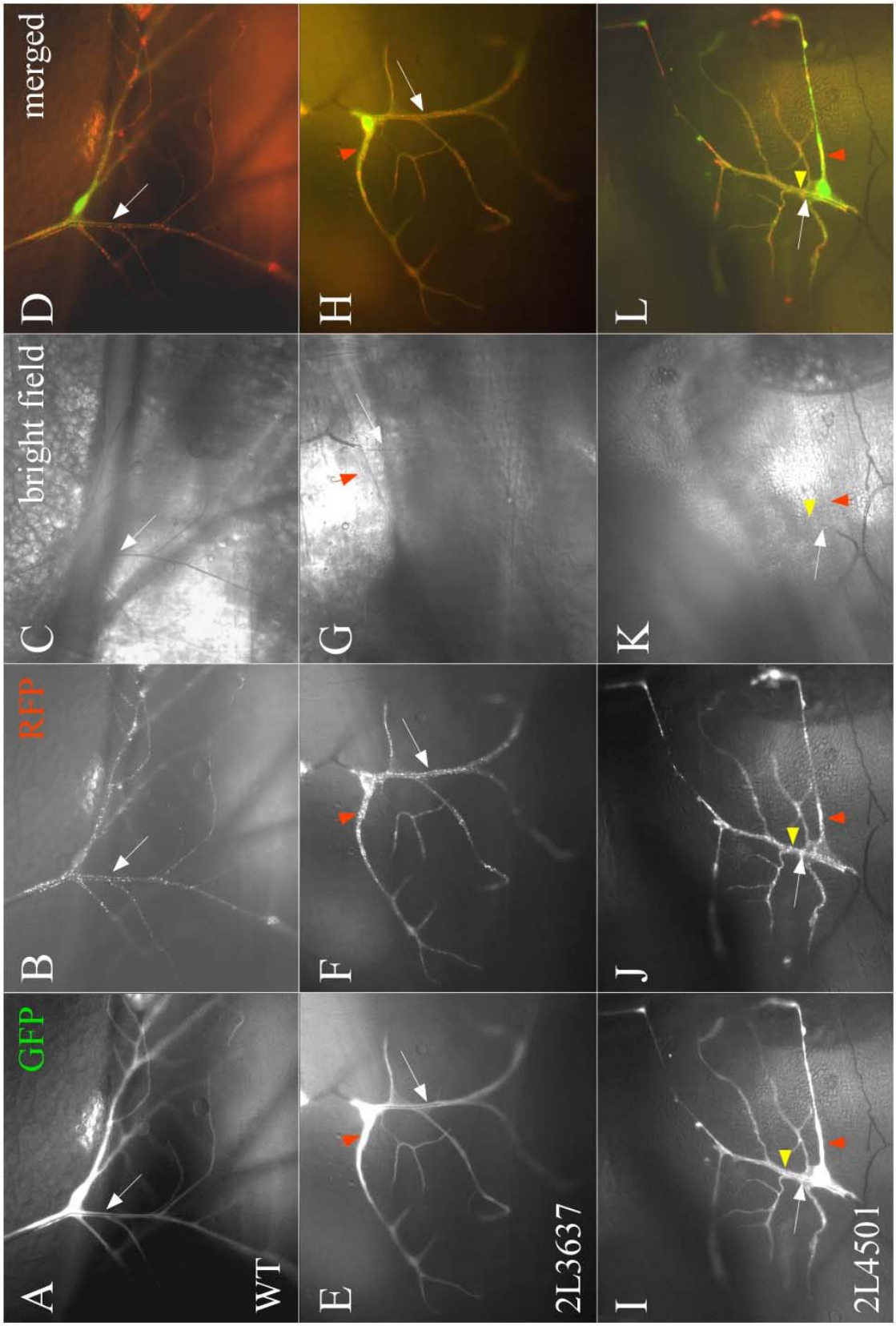

Figure 3.20 Clonal cells in terminal branches in wt (A-D) and mutant (E-L), marked with cytoplasmic GFP and myrRFP. In wt and in part of the mutant cell in which lumen is still present, signal is absent in middle of the cell (white arrows), whereas in parts missing the gas filled lumen no gaps in signal could be observed (red arrows). Yellow arrowheads indicate part of the cell in which GFP and RFP signal is absent in the middle of the cell, although no gas filled lumen could be seen in bright field.

3.4.2.2 Analysis of embryonic phenotype

The fact that gaps in lumen in clonal cells on secondary branches were not always spanning the entire cell length suggested that mutant cells are able to form the lumen. In order to confirm, that in mutant lines formation of a lumen made by a single cell is possible, homozygous embryos from both lines were stained for the luminal marker - 2A12. The absence of staining in dorsal branches or lateral trunk would suggest that formation of the lumen by single cells is impaired. No such defects were found (fig 3.21), thus the lumen in the embryonic trachea is formed normally.

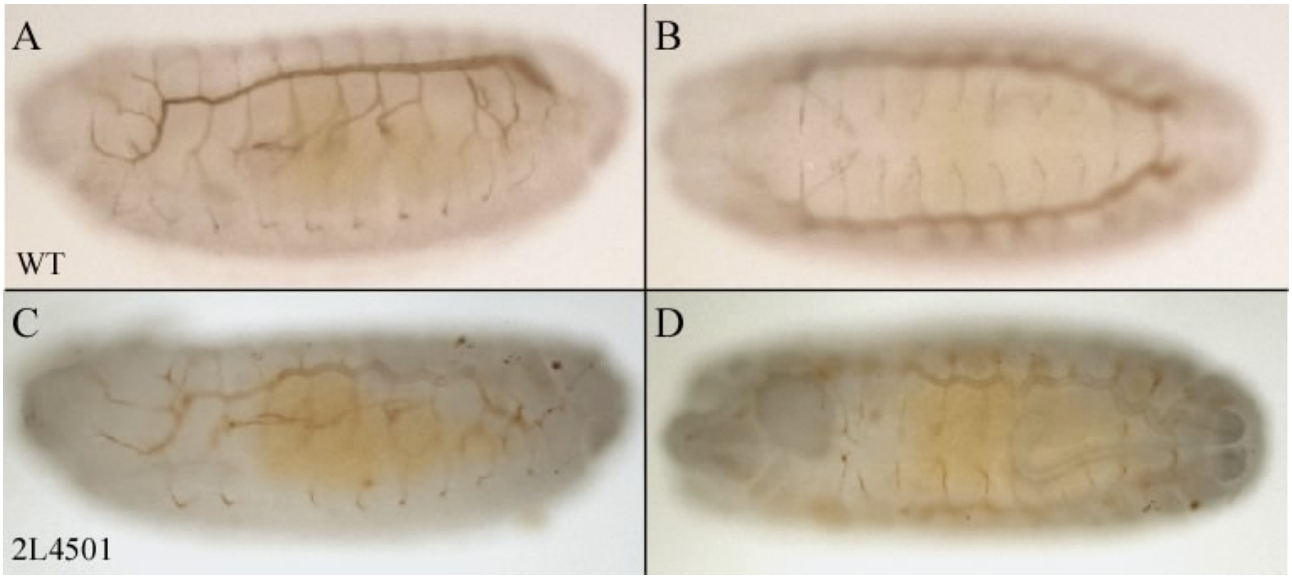

Figure 3.21 Embryonic tracheae stained with luminal marker 2A12 in wild type (A, B) and 2L4501 homozygous embryos (C, D). No defects in lumen formation were found. Embryos stage 15/16 A, C lateral view, anterior to the left dorsal up; B, D dorsal view, anterior to the left.

3.4.2.3 Survival test

To determine at what stage mutations induced lethality, survival test was performed. Embryos from both lines were collected and left to develop. Non-hatched embryos were counted after 24 and 48 hours. As mentioned in section 3.4.1.3, $CyO_{ftz\text{-}LacZ}$ homozygous animals die as embryo. Table 3.8 shows that 34.1% of 2L3637 and 54.6% of 2L4501 embryos did not hatch. It indicates that mutation in line 2L4501 is embryonic lethal whereas line 2L3637 is semi-lethal and some embryos develop to the larval stages.

Line	Counted embryos	Non-hatched embryos after 24h	Non-hatched embryos after 48h	Expected n^{o} of CyO/CyO or mutation/mutation embryos (25%)	% of non-hatched embryos
FRT40A/CyO	187	54	46	46-47	24.6%
2L3637	454	169	155	113	34.1%
2L4501	237	130	120	59	54.6%

Table 3.8 Survival test for lines 2L3637 and 2L4501, FRT40A/CyO flies were used as a reference to determine the number of homozygous CyO embryos that do not hatch.

3.4.2.4 Mapping of group E2

Although this group consists only of two lines, mapping approach based on complementation of lethality by set of deficiencies was chosen. The observed phenotypes of both lines are very likely to be lethal. Additionally probability that two different genes are mutated in both lines is rather low. Thus mapping of lethality should lead to identification of the gene responsible for observed phenotypes.

Both lines were crossed to set of deficiencies covering left arm of second chromosome. These deficiencies, established by Exelixis Inc., have moleculary mapped break-point what makes them more accurate for mapping (stocks listed in Appendix table A.1 C Exelixis Df Kit). The progeny from the crosses were screened for lethality complementation. Both lines complemented lethality of all deficiencies, which indicates that mutation causing the phenotype is in the gene uncovered by this set.

3.4.3 Group E3 –analysis and mapping

Group E3 was chosen for further analysis also due to interesting phenotype and because it consists of two lines, 2L2218 and 2L3443 non-complementing the lethality. They were classified to subgroup E III and show defects with lumen formation.

3.4.3.1 Analysis of the phenotype

In both lines the lumen in terminal branches was partially or completely absent. Additionally, in line 2L2218 number of clonal cells was strongly reduced and very few larvae with clonal cells could be found. Due to difficulties in obtaining larvae with clones in the line 2L2218 as well as sickness of this stock, most of the analysis were performed only with line 2L3443.
As depicted in fig 3.22, the strength of the phenotype differed between the larvae and also between the branches in one animal. In some terminal cells part of the cellular extension still had a gas filled lumen (fig. 3.22, C,D) whereas in others the lumen was present only in the non-ramified part of the cell close to a neighbouring secondary cell (E,F) and finally in some it was completely absent in terminal cells (G,H). Independent of the presence or absence of lumen in these cells the number of branches was reduced in all. However only ~ 50% of terminal cells in line 2L3443 showed the phenotype (tab. 3.9). The penetrance of the phenotype in line 2L2218 could not be evaluated due to low number of analysed larvae.

Line	Counted larvae	Counted clones	Average clone n° per larvae	Clones in TB		TB with phenotype	% of clones showing defect
				In total	Av. per larvae		
TB170	16	435	27.2 ± 10	67	4.2 ± 2.7	0	0%
2L3443	34	615	18.5 ± 7.7	124	3.9 ± 1.8	63	51%
2L2218	2	8	4 ± 0	5	2.5 ± 0.7	5	100%

Table 3.9 Penetrance of the phenotype in E3 complementation group. The low number of analysed larvae in line 2L2218 was caused by difficulties in obtaining larvae with clones (only two out of 191 analysed larvae had clonal cells).

Also the development of embryonic trachea in line 2L3443 was analysed, but no defects were found (data not shown).

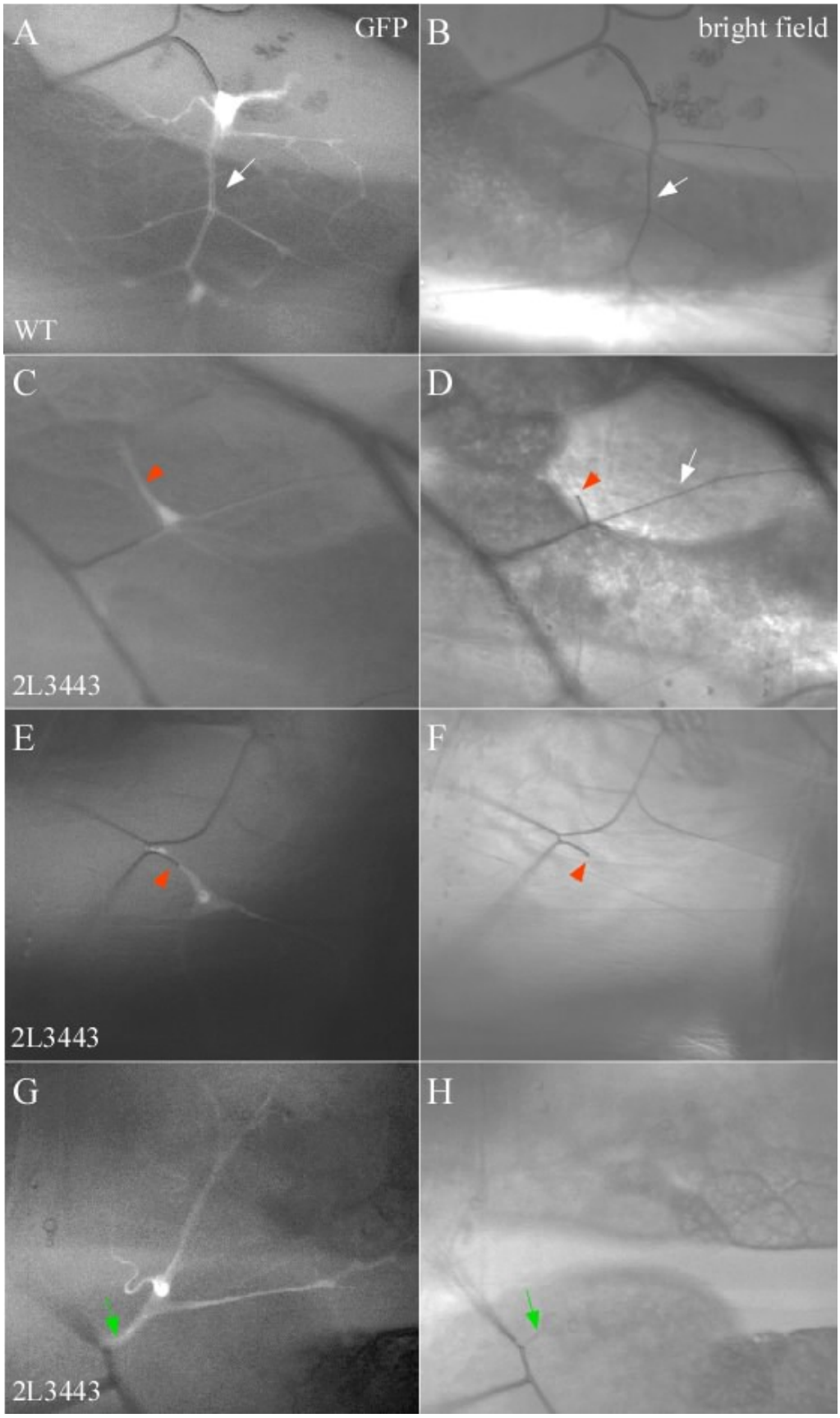

Figure 3.22 Clonal terminal cells in wt (A, B) and mutant (C-H). In wt all branches of terminal cell have gas filled lumen (white arrow) In mutant cell, the lumen is present in part of the branches (white arrow in C and D) or stops within the branch (red arrowheads in C –F) or is completely absent (green arrows in G, H). Right panel shows bright field image of cells from left panel.

3.4.3.2 Survival test

In order to find out at what stage mutation in line 2L3443 is causing lethality, survival test was performed as described for other two groups. 33.3% of the embryos from line 2L3443 did not hatch which suggests that the mutation is embryonic semi-lethal. The development of all mutant embryos was slowed down as 50% of embryos were still in embryo case after 24 hours, whereas after 48 hours only 33.3% remained unhatched. For line 2L2218 it was not possible to collect enough embryos to perform the test.

Line	Counted embryos	Non-hatched embryos after 24h	Non-hatched embryos after 48h	Expected n^{o} of CyO/CyO or mutation/mutation embryos (25%)	% of non-hatched embryos
FRT40A/CyO	187	54	46	46-47	24.6%
2L3443	186	94	62	46-47	33.3%
2L2218	-	-	-	-	-

Table 3.10 Survival test for line 2L3443. FRT40A/CyO flies were used as a control, to estimate embryonic lethality caused by CyO chromosome. For line 2L2218 test was not performed.

3.4.3.3 Mapping of group E3

Although group E3 consists of two allelic lines only line 2L3443 could be used for mapping, due to the sickness of line 2L2218. I decided to map the phenotype not the lethality. The strategy using Single Nucleotide Polymorphism (SNP) makers (Berger, 2001) was chosen as a suitable tool. This method is based on sequence polymorphism between the strain used for mutagenesis and reference strain. It required generation of set of recombinants between mutant line and reference line spanning the whole chromosome arm. The recombinants were then analysed for presence of mutant phenotype and their chromosomes break points were mapped by molecular means. The combined results lead to mapping of the phenotype to the specific genomic region (see Material and Methods, section 2.2.7.1).

69 recombinants between reference line (EP 511) and mutant line were generated and 52 of them were established as stable stocks. It is important to mention that all recombinant chromosomes had the distal tip from EP line and proximal part from 2L3443 line. Next, DNA was isolated from all recombinant lines. DNAs from 12 randomly chosen lines (line 5, 6, 12, 18, 24, 29, 30, 39, 45, 56, 63 and 65) were tested by PLP and RFLP for 8 SNP markers distributed along the chromosome arm. Results are depicted in fig. 3.23 A. Simultaneously all recombinant lines were analysed for

presence of the mutant phenotype by MARCM analysis. Comparison of results from the molecular and phenotypic analysis led to placement of the mutation between markers 2L041 and 2L063. Next DNAs from all the recombinant lines were genotyped for these two markers as well as one localized between them (2L057). The obtained results (fig. 3.23 B) were than combined with information from phenotypic analysis and the mutation was mapped to the region of 850 kb between markers 2L057 and 2L063 corresponding to the region 26A3-27B1 on the cytogenetic map. Several deficiencies covering the region (listed in Appendix tab. A.1 A) were crossed to both mutant lines and analysed for lethality complementation. As shown in fig 3.24, mutant lines did not complement Df(2L)BSC7 and Df(2L)BSC6 which narrowed down the region to 160kb (26D10 – 26F3).

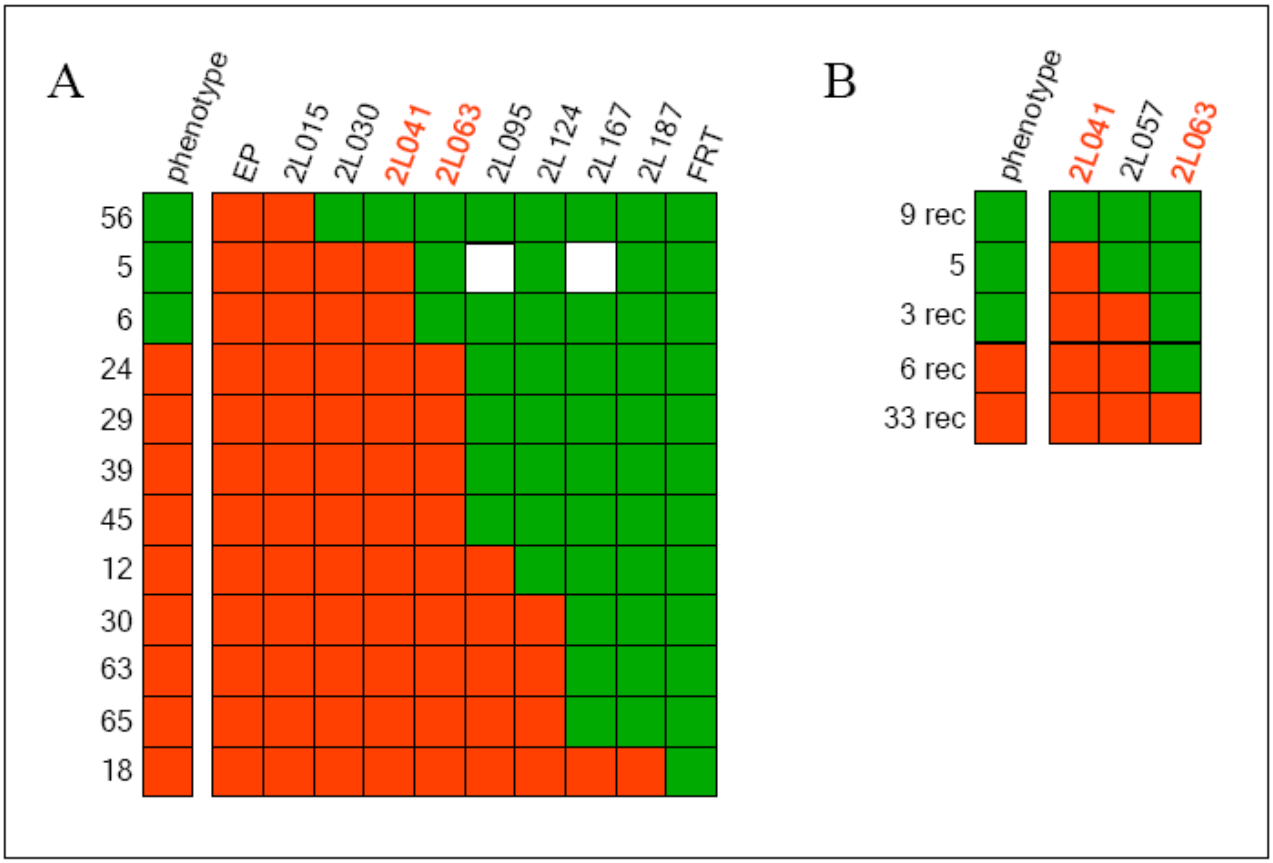

Figure 3.23 Summarised results of SNP mapping of mutation in line 2L3443. A –left column represents presence (green) or absence (red) of mutant phenotype of 12 randomly chosen recombinant lines, listed next to it; right column shows genotyping results for 8 PLP and RFLP markers (numbers above) in these recombinant lines, red indicates EP allele and green FRT; white – unclear results; phenotype maps to region between marker 2L041 and 2L063; B – left column - summarised phenotypic data of all recombinants; right column represents corresponding genotyping results for markers 2L041, 2L057 and 2L063; colour code as in A; phenotype maps between markers 2L057 and 2L063.

There are 21 genes predicted in this region. They are listed in tab 3.11. Homozygous lethal mutants were available only for two of them, namely *eyes absent* and *cup*. For the remaining genes I decided

to sequence their coding region and search for sequence alteration in comparison to wild type allele of the respective gene on the FRT40A chromosome.

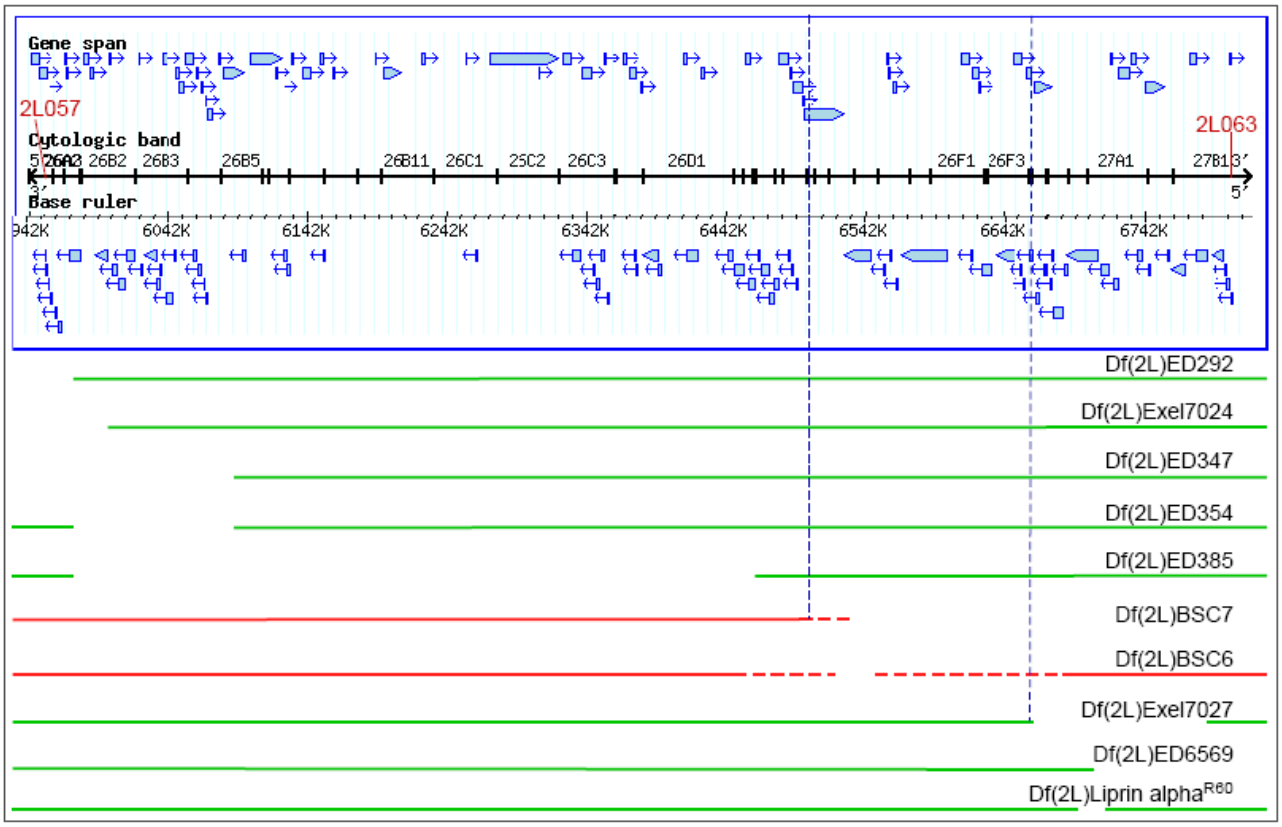

Figure 3.24 Fine deficiency mapping of mutation in line 2L3443. Horizontal lines represent chromosomes in deficiencies: - solid lines - non-deleted fragments of chromosome, dotted lines - unclear regions and blank - deleted parts. Green indicates complementation, red non-complementation. Vertical blue dotted lines mark region to which mutation was mapped. Markers 2L057 and 2L063 are indicated in red in graphic representation of genomic region (FlyBase; http://flybase.bio.indiana.edu). Genes are marked in blue.

Primers were designed to amplify exons of the genes. After amplification PCR fragments were sequenced and compared with corresponding sequences obtained from original FRT40A stock. Additionally sequences were compared with genomic sequence of the genes obtained from 'A Database of *Drosophila* Genes and Genomes' (FlyBase; http://flybase.bio.indiana.edu). So far the following genes have been analysed: Osm-6, CG9596, CG11043, CG13766, CG11320, CG34009, CG11053 and CG31633. No mutations were found in the sequences analysed so far.

Gene	Function
CG31637	carbohydrate metabolism
eyes absent (eya)	eye development (Bolwig's organ morphogenesis)
Osm-6	microtubule based movement
CG11015	mitochondrial electron transport, cytochrome c to oxygen
CG9596	protein biosynthesis and metabolism
CG11043	mitochondrial electron transport, cytochrome c to oxygen
Ent2	nucleobase, nucleoside, nucleotide and nucleic acid meatbolism
CG13766	unknown
CG11319	cell surface receptor linked signal transduction
CG11050	unknown
CG11320	cell adhesion, signal transduction
CG34009	unknown
CG11053	peptidyl - proline hydroxylation to 4-hydroxy L - proline
Oatp26F	organic anion transport
CG31635	cell proliferation
CG11098	unknown
CG31633	unknown
CG31636	coenzyme meatbolism
CG11070	defence response, ubiquitin ligase complex
CG13771	lipid metabolism
cup	chromosome organisation and biogenesis, involved in oogenesis

Table 3.11 Predicted genes in region to which mutation in 2L3443 line was mapped. Function information from FlyBase (http://flybase.bio.indiana.edu).

4. Discussion

The development of the Drosophila tracheal system has been extensively studied in the last decade as it is a perfect model to analyse tubulogenesis. Several screens performed in the past (Samakovlis et al., 1996; Beitel and Krasnow, 2000; Cela and Llimargas, 2006; Hemphala et al., 2003), application of advanced imaging techniques (Ribeiro et al., 2002; Jazwinska et al., 2003; Ribeiro et al., 2004) and detailed analysis of known components of different signalling pathways gave an insight into the genetic control and cellular processes involved in trachea morphogenesis. However, many open questions remain: what are the downstream effectors of signalling cascades, which molecules are involved in cell remodelling and how do they orchestrate extensive cell shape changes and rearrangements. Since most of the studies concentrated on tracheal development in the embryo, not much is known about later stages, especially the molecular bases of terminal branching events during larval life. However, the analysis of tracheal development in the larva is hampered by a possible earlier requirement of the genes during embryogenesis. Thus, the genetic dissection of larval tracheal development requires different approaches, i.e. clonal analysis with MARCM system. This system allows to study the function of known genes but it can also be adopted to identify involvement of new genes in tracheal formation. The results obtain from the genetic screen presented here show that the MARCM system is a useful tool to identify novel genes involved in tracheal development.

4.1 The MARCM screen

The screen was designed to identify genes on the second chromosome affecting tracheal development. We chose the second chromosome because the first and the third chromosomes were screened by a similar approach in the group of M. Krasnow (personal communication). In total, we screened 4779 mutagenised lines and 344 of them did not fulfill wild type criteria (7.2% of analysed lines). Defects observed in 230 lines do not seem to be trachea specific (4.8% of analysed lines) whereas in the remaining 114 they do (2.4%). Among the lines with trachea specific phenotypes, a large number (81) show terminal branching defects which proves that the chosen approach is suitable for revealing late involvement in the trachea morphogenesis.

In 78% of lines, the mutations led to homozygous lethality. The lethality could be due to the mutation causing the clonal phenotype but it cannot be excluded that the lethality is caused by additional mutation or mutations on the second chromosome. Complementation tests based on lethality led to identification of 26 complementation groups, indicating that some genes were hit

more than once. Of these, one complementation group consists of eight members, another of six, eight of three and 16 of two. Most of the complementation groups consist of lines showing non-tracheal specific defects (20). There are two within the the dorsal trunk class and three in the terminal branching group. Additionally, nine lines failed to complement members of more than one group. In the case of one group, all the members showed different tracheal phenotypes. These results indicate that some of the mutagenised lines carry more than one mutation and that the lethality is not necessarily associated with the tracheal defect.

4.2 The mutant phenotypes

The phenotypes observed in the 344 mutant lines were divided into five classes: A - no clones, B - low number of clonal cells, C – small clonal cells, D - dorsal trunk phenotypes and E – terminal branching phenotypes, and are discussed below.

In the class A, no clonal cells were found. The absence of clonal cells could result either from cell lethal mutations or defects in the FRT site. Mutations leading to cell lethality are most likely found in house keeping genes and thus not affecting tracheal development specifically. However, the discovery of two new alleles of *varicose* (involved in tracheal tube size control; Beitel and Krasnow, 2000) within this group indicates that some of the 'no clones' lines might have mutations affecting tracheal development.
The lines from group B (low number of clonal cells) and C (small clonal cells) are also likely to carry mutations in genes not specifically required for tracheal morphogenesis. They could affect survival of the cells (group B) or the regulation of cell growth (group C).

The remaining 144 lines belonging to group D (dorsal trunk defects) and E (terminal branching defects) show trachea specific phenotypes.
The phenotypes observed in group D were: dorsal trunk bendings (fig. 3.12), presence of clonal cells only in dorsal trunk, reduced size of clonal cells in dorsal trunk (fig. 3.5) and absence or decreased number of clonal cells in dorsal trunk. The presence or absence of clonal cells in specific branches might be caused by mutations in genes involved in the establishment of cell fate or branch identity. Three signalling pathways have been implicated to play a role in these processes: the Decapentaplegic (Dpp), EGF and Wingless (Wg) pathways. Dpp signalling is required for dorsoventral cell migration (Llimargas and Casanova, 1997; Vincent et al., 1997) whereas EGF and Wg signalling pathways are involved in determination of dorsal trunk identity (Wappner et al.,

1997; Chihara and Hayashi, 2000; Llimargas, 2000; Llimargas and Lawrence, 2001). In embryos mutant for the Dpp receptor *thickveins* (*tkv*) and two Dpp dependent transcription factors *knirps* (*kni*) and *knirps like* (expressed normally in a subset of cells forming the dorsal branches and the lateral trunk) cell migration into dorsal branches, lateral trunk and ganglionic branches is abolished (Vincent et al., 1997; Chen et al., 1998). Thus the presence of clonal cells only in the dorsal trunk could result from their disability to migrate dorso-ventraly in response to Dpp signalling. So far only *tkv* is known to be located on the left arm of the second chromosome and being part of the Dpp signalling pathway. Thus complementation analysis will be required to assay for possible new alleles of *tkv*. However a more interesting scenario would be the identification of so far unknown gene acting downstream of Dpp. The presence of such genes have been implicated by the fact, that expression of *kni* in the trachea cannot rescue the *tkv* tracheal defects (Zelzer and Shilo, 2000).

The absence of clonal cells in the dorsal trunk (class D IV) could be due to interferance in the EGF or Wg signalling cascades, since in the embryos in which these pathways are inactivated, the dorsal trunk does not form and may be transformed into an other branch identities. (Wappner et al., 1997; Chihara and Hayashi, 2000; Llimargas, 2000; Llimargas and Lawrence, 2001). Two genes were identified to play a crucial role in establishment of dorsal trunk: *spalt*, required for identity determination and *ribbon,* involved in anterior-posterior cell migration. S*palt* is a target gene of EGF signalling (Kuhnlein and Schuh, 1996) while *ribbon* acts downstream of Wg and FGF pathways (Bradley and Andrew, 2001). As s*palt* is located on the left arm of the second chromosome, it is a possible candidate gene of class D IV phenotype. However, the phenotype could also be derived from mutations in unknown genes downstream of *spalt,* affecting tracheal cell fate and branch identity.

The two other dorsal trunk phenotypes found in the screen, dorsal trunk bending and reduced size of clonal cells in the dorsal trunk, might be caused by mutations affecting cell - cell contacts. The dorsal trunk is the only branch with a multicellular lumen and with cell contacts based exclusively on intercellular junctions. Thus, it is possible that a defect restricted to this type of tube might result from a disturbance of cells interactions. However, on the bases of current knowledge it is difficult to suggest any mechanism or molecule that could lead to a reduction in the size of clonal cells only in the dorsal trunk.

The dorsal trunk bendings phenotype was found in 10 lines, out of which eight are allelic to each other. This complementation group (D1) was studied in more details (section 3.4.1). The clonal cells within the dorsal trunk, causing the bend, are smaller than neighbouring wild type cells and misshapen. However, reduction in size and shape change cannot wholly explain the observed phenotype, since small clonal cells in the dorsal trunk found in other mutant lines do not cause any

bending. Also, cell adhesion defects are unlikely to be responsible for the phenotype since no difference could be observed in the distribution of the adherens junction component, β-catenin (arm) between mutant clonal cells and their neighbours. However, in order to exclude an involvement if cell-to-cell jucntions, other junctional markers should be analysed as well. Thus, the phenotype requires further investigation which together with the mapping and identification of the affected gene may give us an insight into the mechanism causing the bendings.

The terminal branching defects characteristic for group E are of most interest to us. Within this class five distinct phenotypic subgroups could be identified: terminal branches crossing or fusing (fig. 3.7) decreased and expanded branching of terminal cells (figs 3.8 and 3.9), absence or reduced number of clonal cells in terminal branches and problems with lumen formation (fig. 3.10).
Extensive cytoplasmic outgrowth and ramification of terminal cells are controlled by FGF signalling, dependent on the physiological needs of the target tissues (Jarecki et al., 1999). Although the branching pattern of terminal cells is variable, it is not unorganised. Ramification points are regularly spaced and terminal branches do not cross or fuse (Ghabrial et al., 2003). In the screen we found 24 mutant lines in which branch crossing or fusion occurred in clonal cells. 60% of them (14 lines) are homozygous lethal but only one complementation group (with two lines) was found. This would suggest that there are mutations in 13 independent genes causing homozygous lethality and terminal cell phenotype which is rather unlikely. Thus, we expect that at least some of the lines have additional lethal mutations on the chromosome and the terminal branches defect does not lead to lethality as such.

Not much is known about the mechanism which prevents neighbouring cells to cross or fuse their terminal branches. The *bnl* expression being restricted to a small target area and this gradient is enough to attract a migrating terminal cell but it is unlikely to regulate the spatial spreading of the cytoplasmic extensions on its own. Thus, there must be other cues involved in that process. The first evidence of such an involvment came from the study of Kato et al. (2004) which revealed a role for Hedgehog (Hh) and Dpp signalling in the restriction of the epidermal spreading area of the dorsal terminal branches. Hh functions as a permissive signal to allow migration of terminal cell extensions laterally and along the anterior-posterior axis while Dpp prevents dorsal migration. However, the effectors are unknown and it is unclear whether the signalling acts directly on the cell cytoskeleton or is mediated by a nuclear transduction of the signal. It is also possible that other factors and tissues are involved in spatial restriction of terminal branching since it has been reported that mesodermal cells contribute to correct migration of dorsal branches in the embryo (Franch-Marro and Casanova, 2000) and thus might be involved also in migration of terminal branches. This

is another aspect requiring further investigation. Thus some candidate mutations from the screen may turn out to affect genes expressed in signal receiving terminal cell, responding to the restrictive cue.

The next interesting phenotypes observed within class E are reduced or expanded branching of terminal cells. One speculation on the class of genes mutated in these lines is the signalling response to oxygen needs. Under high oxygen conditions, terminal branches had fewer ramifications whereas at low oxygen concentrations branching was more extensive (Jarecki et al., 1999). It is not fully understood how the *Drosophila* cells sense hypoxia and how it leads to induction of *bnl* expression which in turn regulates branch outgrowth. Thus a more detailed analyses and mapping of the identified mutants might uncover additional genes involved in hypoxia sensing and its cellular response.

4.2.1 The phenotypes of candidate lines selected for further investigation.

From the 114 lines, three complementation groups were considered most promising and chosen for further analysis and mapping. The selection criteria were based on interesting phenotype and larger number of alleles (in complementation group). The first criterion was fulfilled by two complementation groups (E2 and E3) showing defects in lumen formation in terminal branches and consist of two lines each. The second criterion was fulfilled by group D1 which consists of eight lines with dorsal trunk bendings at clonal cells position (discussed earlier).

The lumen of terminal cells is a channel formed within the cytoplasm of a single cell. How it is formed and maintained is poorly understood. Similar structures are found also in other organisms, for eg: the finest capillaries of the vertebrate vascular system. *In vitro* studies of angiogenesis showed that cytoplasmic vesicles providing apical membrane are essential for lumen formation (Folkman and Haudenschild, 1980; Davis and Camarillo, 1996). *In vivo* analyses of vascular lumen formation in zebrafish together with results from earlier *in vitro* studies led to a model in which the formation and intracellular fusion of endothelial vacuoles drives vascular lumen formation (Kamei et al., 2006). It is likely that this is a common mechanism for tube formation during development and could be applied also during formation of lumen in terminal cells of *Drosophila*.

In the two lines from group E3 (2L3443 and 2L2218), lumen formation is completely or partially abolished (section 3.4.3.1). The phenotype indicates that the formation of cytoplasmic vesicles is impaired rather than their fusion, although how it is, remains unclear. The mutation was mapped to a region of ~160kb in the 26D10-26F3 interval on the cytogenetic map. There are 21 predicted

genes in this region. None of them had a previous involvement in tracheal development. In the case of five predicted genes, no functional information is available. To map the mutation I decided to sequence these genes and compare the mutant sequence with its wild type allele. So far eight of the genes including four with unknown function were analysed but no mutation was found.

The lines from group E2 (2L3637 and 2L4501) show also no lumen formation in the terminal branches. Additionally, clonal cells on secondary branches lack the typical gas filling (section 3.4.2.1). This indicates that the mutation is not only affecting the formation or maintenance of the subcellular lumen but also the one formed by single cell, wrapped around the lumen. The fact that in some clonal cells parts of the lumen were still present (visualised by the absence of cytoplasmic marker signal in luminal space; fig.3.19) but gas filling was missing, together with the observation of properly formed lumen of secondary branches in the mutant embryos suggest, that the observed phenotype may result not from lumen formation but lumen maintenance defect. One possible explanation of the phenotype and its occurance only in terminal cells and secondary branches could be, that secretion of cuticular lining of the branches is affected which may lead to lumen collapse. However, this hypothesis requires further investigation. If it were true, our mutants would indicate that the proper chitin metabolism and cuticle formation might not only be involved in the control of lumen size in the embryonic tracheal system (Araujo et al., 2005; Devine et al., 2005; Tonning et al., 2005; Moussian et al., 2006; Luschnig et al., 2006; Wang et al., 2006) but also in later stages of development.

5. Conclusions

The aim of this study was to perform a genetic mosaic screen in order to reveal new molecules involved in tracheal development. The identification of 114 lines showing trachea specific defects indicate that the chosen approach was a suitable tool for studying tubulogenesis. Although so far none of the mutations have beed mapped to individual genes, and no mechanism explaining the different phenotypes could be proposed, the screen provides a collection of mutants, whose detailed analyses will help our understanding of such processes like lumen formation in terminal cells, their branching control, lumen formation and stabilisation in secondary branches or cell behaviour in multicellular tubes.

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7. Appendix

Table A.1 Deficiencies stocks used for mapping

Df symbol	Genotype	Cytology	References
A) Deficiencies used for fine mapping			
Df(2L)ED292	w^{1118}; Df(2L)ED292, P{3'.RS5+3.3'}ED292/SM6a	25F5;26B2	Bloomington #9182
Df(2L)Exel7024	w1118;Df(2L)Exel7024,P+PBac{XP5.RB3}Exel7024/CyO	26A1;26A8	Exelixis, Inc./ Bloomington #7799
Df(2L)ED347	w^{1118}; Df(2L)ED347, P{3'.RS5+3.3'}ED347/SM6a	25F5;26B5	Bloomington #9272
Df(2L)ED354	w^{1118}; Df(2L)ED354, P{3'.RS5+3.3'}ED354/SM6a	26B1;26B5	Bloomington #9187
Df(2L)ED385	w^{1118}; Df(2L)ED385, P{3'.RS5+3.3'}ED385/SM6a	26B1;26D7	Bloomington #9341
Df(2L)BSC6	Df(2L)BSC6, dp^{ov1} cn^{1}/SM6a	26D3-E1; 26F4-7	Bloomington #6338
Df(2L)Exel7027	w^{1118}; Df(2L)Exel7027, P+PBac{XP5.RB3}Exel7027/CyO	26F5;27B1	Exelixis, Inc./ Bloomington #7801
Df(2L)BSC7	w^{1118}; Df(2L)BSC7/CyO	26D10-E1; 27C1	Bloomington #6374
Df(2L)ED6569	w^{1118}; Df(2L)ED6569, P{3'.RS5+3.3'}ED6569/SM6a	27A1;27C4	Bloomington #8940
Df(2L)Liprin-α^{R60}	w^{*}; Df(2L)Liprin-α^{R60}, Liprin-α^{R60}/CyO, P{ActGFP}JMR1	27A1;27A1	Bloomington #8561
B) Bloomington Deficiency kit for 2L			
Df(2L)net-PMF	Df(2L)net-PMF/SM6a	21A1; 21B7-8	Bloomington #3638
Df(2L)BSC16	Df(2L)BSC16, net^{1} cn^{1}/SM6a	21C3-4; 21C6-8	Bloomington #6608
Df(2L)ast2	Df(2L)ast2/SM1	21D1-2; 22B2-3	Bloomington #3084
Df(2L)BSC37	Df(2L)BSC37/CyO	22D2-3; 22F1-2	Bloomington #7144
Df(2L)JS17	Df(2L)JS17, $dpp^{d\text{-}ho}$/CyO, P{en1}wg^{en11}	23C1-2; 23E1-2	Bloomington #1567
Df(2L)BSC28	Df(2L)BSC28/SM6a, bw^{k1}	23C5-D1; 23E2	Bloomington #6875
Df(2L)BSC31	Df(2L)BSC31, net^{1} cn^{1}/CyO, b^{81f2} rk^{81f2}	23E5; 23F4-5	Bloomington #6965
Df(2L)drm-P2	y^{1} w^{*}; Df(2L)drm-P2, P{lacW}$Pdsw^{k10101}$/SM6b	23F3-4; 24A1-2	Bloomington #6507
Df(2L)ed1	Df(2L)ed1/CyO; P{ftz/lacC}1	24A2;24D4	Bloomington #5330
Df(2L)ED250	w^{1118}; Df(2L)ED250, P{3'.RS5+3.3'}ED250/SM6a	24F4;25A7	Bloomington #9270
Df(2L)BSC110	w^{1118}; Df(2L)BSC110, P+PBac{XP5.RB3}BSC110/CyO	25C1;25C3	Bloomington #8835
Df(2L)BSC109	w^{1118}; Df(2L)BSC109, P+PBac{XP5.RB3}BSC109/CyO	25C4;25C8	Bloomington #8674
Df(2L)Exel6011	w^{1118}; Df(2L)Exel6011, P{XP-U}Exel6011/CyO	25C8;25D5	Exelixis, Inc./ Bloomington #7497
Df(2L)cl-h3	Df(2L)cl-h3/SM6b	25D2-4; 26B2-5	Bloomington #781
Df(2L)BSC5	Df(2L)BSC5, w^{+mC}/SM6a	26B1-2; 26D1-2	Bloomington #6299
Df(2L)BSC6	Df(2L)BSC6, dp^{ov1} cn^{1}/SM6a	26D3-E1; 26F4-7	Bloomington #6338
Df(2L)BSC7	w^{1118}; Df(2L)BSC7/CyO	26D10-E1; 27C1	Bloomington #6374
Df(2L)spd^{j2}	w^{*}; Df(2L)spd^{j2}, $wg^{spd\text{-}j2}$/CyO, P{ftz/lacB}E3	27C1-2; 28A	Bloomington #2414
Df(2L)Dwee1-W05	Df(2L)Dwee1-W05/CyO; P{ftz/lacC}1	27C2-3; 27C4-5	Bloomington #5420
Df(2L)Trf-C6R31	y^{1} w^{67c23}; Df(2L)Trf-C6R31/CyO	28DE	Bloomington #140
Df(2L)TE29Aa-11	In(1)w[m4h],y^{1}; Df(2L)TE29Aa-11, dp^{*}/CyO	28E4-7; 29B2-C1	Bloomington #179
Df(2L)BSC111	w^{1118};Df(2L)BSC111, P+PBac{XP5.WH5}BSC111/CyO	28F5;29B1	Bloomington #8836

Df(2L)ED611	w^{1118}**;** Df(2L)ED611**,** P{3'.RS5+3.3'}ED611/SM6a	29B4;29C3	Bloomington #9298
Df(2L)N22-14	Df(2L)N22-14/CyO	29C1-2; 30C8-9	Bloomington #2892
Df(2L)BSC17	Df(2L)BSC17/SM6a	30C3-5; 30F1	Bloomington #6478
Df(2L)BSC50	Df(2L)BSC50/SM6a	30F4-5; 31B1-4	Bloomington #8469
Df(2L)J2	y^{*}; Df(2L)J2/SM1	31B;32A	Bloomington #3366
Df(2L)BSC36	Df(2L)BSC36/SM6a, bw^{k1}	32D1; 32D4-E1	Bloomington #7143
Df(2L)FCK-20	Df(2L)FCK-20, dp^{ov1} bw^{1}/CyO, P{sevRas1.V12}FK1	32D1; 32F1-3	Bloomington #5869
Df(2L)Prl	Df(2L)Prl, Prl^{1} nub^{Prl}/CyO	32F1-3; 33F1-2	Bloomington #3079
Df(2L)BSC30	Df(2L)BSC30/SM6a, bw^{k1}	34A3; 34B7-9	Bloomington #6999
Df(2L)TE35BC-24	Df(2L)TE35BC-24, b^{1} pr^{1} pk^{1} cn^{1} sp^{1}/CyO	35B4-6; 35F1-7	Bloomington #3588
Df(2L)r10	Df(2L)r10, cn^{1}/CyO	35D1; 36A6-7	Bloomington #1491
Df(2L)cact-255rv64	Df(2L)cact-255rv64, $cact^{chif64}$/CyO; ry^{506}	35F-36A; 36D	Bloomington #2583
Df(2L)TW137	Df(2L)TW137, cn^{1} bw^{1}/CyO, $Dp(2;2)M(2)m^{+}$	36C2-4; 37B9-C1	Bloomington #420
Df(2L)Exel6049	w^{1118}; Df(2L)Exel6049, P{XP-U}Exel6049/CyO	40A5;40D3	Exelixis,Inc./ Bloomington #7531
Df(2L)C'	Df(2L)C'/CyO		Bloomington #4959
C) Exelixis Deficiency Kit for 2L			
Df(2L)Exel6001	w^{1118}; Df(2L)Exel6001, P{XP-U}Exel6001/CyO	21B1;21B1	Exelixis,Inc./ Bloomington #7488
Df(2L)Exel7002	w^{1118}; Df(2L)Exel7002, P+PBac{XP5.RB3}Exel7002/CyO	21B4;21B7	Exelixis,Inc./ Bloomington #7772
Df(2L)Exel8003	w^{1118}; Df(2L)Exel8003, P+PBac{XP5.RB3}Exel8003/CyO	21D1;21D2	Exelixis,Inc./ Bloomington #7774
Df(2L)Exel6002	w^{1118}; Df(2L)Exel6002, P{XP-U}Exel6002/CyO	21D2;21D3	Exelixis,Inc./ Bloomington #7489
Df(2L)Exel7005	w^{1118};Df(2L)Exel7005, P+PBac{XP5.WH5}Exel7005/CyO	21D2;21D4	Exelixis,Inc./ Bloomington #7775
Df(2L)Exel6003	w^{1118}; Df(2L)Exel6003, P{XP-U}Exel6003/CyO	21D3;21E3	Exelixis,Inc./ Bloomington #7490
Df(2L)Exel6004	w^{1118}; Df(2L)Exel6004, P{XP-U}Exel6004/CyO	21E3;21F2	Exelixis,Inc./ Bloomington #7491
Df(2L)Exel7006	w^{1118}; Df(2L)Exel7006, P+PBac{XP5.WH5}Exel7006/CyO	21F2;21F4	Exelixis,Inc./ Bloomington #7776
Df(2L)Exel6005	w^{1118}**;** Df(2L)Exel6005, P{XP-U}Exel6005/CyO	22A3;22B1	Exelixis,Inc./ Bloomington #7492
Df(2L)Exel7007	w^{1118}; Df(2L)Exel7007, P+PBac{XP5.RB3}Exel7007/CyO	22B1;22B5	Exelixis,Inc./ Bloomington #7778
Df(2L)Exel8005	w^{1118}; Df(2L)Exel8005, P+PBac{XP5.WH5}Exel8005/CyO	22B2;22B8	Exelixis,Inc./ Bloomington #7779
Df(2L)Exel6006	w^{1118}; Df(2L)Exel6006, P{XP-U}Exel6006/CyO	22B5;22D1	Exelixis,Inc./ Bloomington #8000
Df(2L)Exel7008	w^{1118}; Df(2L)Exel7008, P+PBac{XP5.RB3}Exel7008/CyO	22B8;22D1	Exelixis,Inc./ Bloomington #7780
Df(2L)Exel6007	w^{1118}; Df(2L)Exel6007, P{XP-U}Exel6007/CyO	22D1;22E1	Exelixis,Inc./ Bloomington #7493
Df(2L)Exel7010	w^{1118}; Df(2L)Exel7010, P+PBac{XP5.WH5}Exel7010/CyO	22D4;22E1	Exelixis,Inc./ Bloomington #7782
Df(2L)Exel7011	w^{1118}; Df(2L)Exel7011, P+PBac{XP5.WH5}Exel7011/CyO	22E1;22F3	Exelixis,Inc./ Bloomington #7783
Df(2L)Exel6008	w^{1118}; Df(2L)Exel6008, P{XP-U}Exel6008/CyO	22F3;23A3	Exelixis,Inc./ Bloomington #7494

Df(2L)Exel6277	w^{1118}; Df(2L)Exel6277, P{XP-U}Exel6277/CyO	23A2;23B1	Exelixis,Inc./ Bloomington #7744
Df(2L)Exel7014	w^{1118}; Df(2L)Exel7014, P+PBac{XP5.RB3}Exel7014/CyO	23C4;23D1	Exelixis,Inc./ Bloomington #7784
Df(2L)Exel7015	w^{1118}; Df(2L)Exel7015, P+PBac{XP5.WH5}Exel7015/CyO	23D1;23E3	Exelixis,Inc./ Bloomington #7785
Df(2L)Exel8008	w^{1118}; Df(2L)Exel8008, P+PBac{XP5.RB3}Exel8008/CyO	23E3;23E5	Exelixis,Inc./ Bloomington #7786
Df(2L)Exel7016	w^{1118}; Df(2L)Exel7016, P+PBac{XP5.WH5}Exel7016/CyO	23F3;23F3	Exelixis,Inc./ Bloomington #7787
Df(2L)Exel7018	w^{1118}; Df(2L)Exel7018, P+PBac{XP5.WH5}Exel7018/CyO	24A1;24C2	Exelixis,Inc./ Bloomington #7789
Df(2L)Exel6009	w^{1118}; Df(2L)Exel6009, P{XP-U}Exel6009/CyO	24C3;24C8	Exelixis,Inc./ Bloomington #7495
Df(2L)Exel8010	w^{1118}; Df(2L)Exel8010, P+PBac{XP5.RB3}Exel8010/CyO	24C8;24D4	Exelixis,Inc./ Bloomington #7790
Df(2L)Exel6010	w^{1118}; Df(2L)Exel6010, P{XP-U}Exel6010/CyO	25A6;25B1	Exelixis,Inc./ Bloomington #7496
Df(2L)Exel9062	w^{1118}; Df(2L)Exel9062, P+PBac{XP5.RB3}Exel9062/CyO	25B1;25B1	Exelixis,Inc./ Bloomington #7792
Df(2L)Exel8012	w^{1118}; Df(2L)Exel8012, P+PBac{XP5.RB3}Exel8012/ CyO	25B1;25B5	Exelixis,Inc./ Bloomington #7793
Df(2L)Exel7021	w^{1118}; Df(2L)Exel7021, P+PBac{XP5.RB3}Exel7021/ CyO	25B3;25B9	Exelixis,Inc./ Bloomington #7795
Df(2L)Exel8013	w^{1118}; Df(2L)Exel8013, P+PBac{XP5.RB3}Exel8013/ CyO	25B8;25B10	Exelixis,Inc./ Bloomington #7796
Df(2L)Exel7022	w^{1118}; Df(2L)Exel7022, P+PBac{XP5.WH5}Exel7022/ CyO	25B10;25C3	Exelixis,Inc./ Bloomington #7794
Df(2L)Exel6011	w^{1118};Df(2L)Exel6011, P{XP-U}Exel6011/ CyO	25C8;25D5	Exelixis,Inc./ Bloomington #7497
Df(2L)Exel6012	w^{1118}; Df(2L)Exel6012, P{XP-U}Exel6012/ CyO	25D5;25E6	Exelixis,Inc./ Bloomington #7498
Df(2L)Exel7023	w^{1118}; Df(2L)Exel7023, P+PBac{XP5.WH5}Exel7023/ CyO	25E5;25F1	Exelixis,Inc./ Bloomington #7797
Df(2L)Exel6256	w^{1118}; Df(2L)Exel6256, P{XP-U}Exel6256/ CyO	25E6;25F2	Exelixis,Inc./ Bloomington #7724
Df(2L)Exel8016	w^{1118}; Df(2L)Exel8016, P+PBac{XP5.WH5}Exel8016/ CyO	25E6;25F2	Exelixis,Inc./ Bloomington #7798
Df(2L)Exel6013	w^{1118}; Df(2L)Exel6013, P{XP-U}Exel6013/ CyO	25F2;25F4	Exelixis,Inc./ Bloomington #7499
Df(2L)Exel6014	w^{1118}; Df(2L)Exel6014, P{XP-U}Exel6014/ CyO	25F5;26A3	Exelixis,Inc./ Bloomington #7500
Df(2L)Exel7024	w^{1118}; Df(2L)Exel7024, P+PBac{XP5.RB3}Exel7024/ CyO	26A1;26A8	Exelixis,Inc./ Bloomington #7799
Df(2L)Exel6015	w^{1118}; Df(2L)Exel6015, P{XP-U}Exel6015/ CyO	26B9;26C1	Exelixis,Inc./ Bloomington #7501
Df(2L)Exel6016	w^{1118};Df(2L)Exel6016, P{XP-U}Exel6016/ CyO	26C1;26D1	Exelixis,Inc./ Bloomington #7502
Df(2L)Exel9038	w^{1118}; Df(2L)Exel9038, P{XP-U}Exel9038/CyO	26C2;26C3	Exelixis,Inc./ Bloomington #7800
Df(2L)Exel7027	w^{1118}; Df(2L)Exel7027, P+PBac{XP5.RB3}Exel7027/CyO	26F5;27B1	Exelixis,Inc./ Bloomington #7801
Df(2L)Exel7029	w^{1118};Df(2L)Exel7029,P+PBac{XP5.WH5}Exel7029/CyO	27C4;27D4	Exelixis,Inc./ Bloomington #7802
Df(2L)Exel8019	w^{1118}; Df(2L)Exel8019, P+PBac{XP5.WH5}Exel8019 /CyO	27E2;27E4	Exelixis,Inc./ Bloomington #7803
Df(2L)Exel6017	w^{1118}; Df(2L)Exel6017, P{XP-U}Exel6017/CyO	27E4;27F5	Exelixis,Inc./ Bloomington #7503
Df(2L)Exel7031	w^{1118}; Df(2L)Exel7031, P+PBac{XP5.WH5}Exel7031 /CyO	27F3;28A1	Exelixis,Inc./ Bloomington #7804
Df(2L)Exel6018	w^{1118}; Df(2L)Exel6018, P{XP-U}Exel6018 /CyO	28B1;28C1	Exelixis,Inc./ Bloomington #7504

Df(2L)Exel9031	w^{1118}; Df(2L)Exel9031, PBac{RB3.WH3}Exel9031 /CyO	28B4;28C1	Exelixis,Inc./ Bloomington #7805
Df(2L)Exel7034	w^{1118}; Df(2L)Exel7034, P+PBac{XP5.RB3}Exel7034/CyO	28E1;28F1	Exelixis,Inc./ Bloomington #7807
Df(2L)Exel8021	w^{1118}; Df(2L)Exel8021, P+PBac{XP5.WH5}Exel8021/CyO	29C1;29D1	Exelixis,Inc./ Bloomington #7808
Df(2L)Exel7038	w^{1118}; Df(2L)Exel7038, P+PBac{XP5.RB3}Exel7038/CyO	29C4;29D4	Exelixis,Inc./ Bloomington #7809
Df(2L)Exel7039	w^{1118}; Df(2L)Exel7039, P+PBac{XP5.WH5}Exel7039/CyO	29D5;29F1	Exelixis,Inc./ Bloomington #7810
Df(2L)Exel7040	w^{1118}; Df(2L)Exel7040, P+PBac{XP5.WH5}Exel7040/CyO	29F1;29F6	Exelixis,Inc./ Bloomington #7811
Df(2L)Exel6021	w^{1118}; Df(2L)Exel6021, P{XP-U}Exel6021/CyO	29F7;29B1	Exelixis,Inc./ Bloomington #7505
Df(2L)Exel8022	w^{1118}; Df(2L)Exel8022, P+PBac{XP5.RB3}Exel8022/CyO	30B3;30B5	Exelixis,Inc./ Bloomington #7813
Df(2L)Exel9064	w^{1118}; Df(2L)Exel9064, PBac{RBr}Exel9064/CyO	30B4;30B5	Exelixis,Inc./ Bloomington #7814
Df(2L)Exel6022	w^{1118}; Df(2L)Exel6022, P{XP-U}Exel6022/CyO	30B5;30B11	Exelixis,Inc./ Bloomington #7506
Df(2L)Exel7042	w^{1118}; Df(2L)Exel7042, P+PBac{XP5.WH5}Exel7042/CyO	30B10;30C1	Exelixis,Inc./ Bloomington #7812
Df(2L)Exel9040	w^{1118}; Df(2L)Exel9040, P+PBac{XP5.WH5}Exel9040/CyO	30C1;30C1	Exelixis,Inc./ Bloomington #7815
Df(2L)Exel6024	w^{1118}; Df(2L)Exel6024, P{XP-U}Exel6024/CyO	30C1;30C9	Exelixis,Inc./ Bloomington #7507
Df(2L)Exel6025	w^{1118}; Df(2L)Exel6025, P{XP-U}Exel6025/CyO	30C9;30E1	Exelixis,Inc./ Bloomington #7508
Df(2L)Exel7043	w^{1118}; Df(2L)Exel7043, P+PBac{XP5.RB3}Exel7043/CyO	30D1;30F1	Exelixis,Inc./ Bloomington #7816
Df(2L)Exel8024	w^{1118}; Df(2L)Exel8024, P+PBac{XP5.WH5}Exel8024/CyO?	31A2;31B1	Exelixis,Inc./ Bloomington #7817
Df(2L)Exel9032	w^{1118}; Df(2L)Exel9032, PBac{RB5.WH5}Exel9032/CyO	31A3;31B1	Exelixis,Inc./ Bloomington #7818
Df(2L)Exel7046	w^{1118}; Df(2L)Exel7046, P+PBac{XP5.WH5}Exel7046/CyO	31C3;31D9	Exelixis,Inc./ Bloomington #7819
Df(2L)Exel7048	w^{1118}; Df(2L)Exel7048, P+PBac{XP5.RB3}Exel7048/CyO	31E3;31F5	Exelixis,Inc./ Bloomington #7999
Df(2L)Exel8026	w^{1118}; Df(2L)Exel8026, P+PBac{XP5.RB3}Exel8026/CyO	31F5;32B1	Exelixis,Inc./ Bloomington #7820
Df(2L)Exel7049	w^{1118}; Df(2L)Exel7049, P+PBac{XP5.RB3}Exel7049/CyO	32B1;32C1	Exelixis,Inc./ Bloomington #7821
Df(2L)Exel6027	w^{1118}; Df(2L)Exel6027, P{XP-U}Exel6027/CyO	32D1;32D5	Exelixis,Inc./ Bloomington #7510
Df(2L)Exel6028	w^{1118}; Df(2L)Exel6028, P{XP-U}Exel6028/CyO	32D5;32E4	Exelixis,Inc./ Bloomington #7511
Df(2L)Exel6029	w^{1118}; Df(2L)Exel6029, P{XP-U}Exel6029/CyO	32E4;32F2	Exelixis,Inc./ Bloomington #7512
Df(2L)Exel6030	w^{1118}; Df(2L)Exel6030, P{XP-U}Exel6030/CyO	33A2;33B3	Exelixis,Inc./ Bloomington #7513
Df(2L)Exel6031	w^{1118}; Df(2L)Exel6031, P{XP-U}Exel6031/CyO	33B3;33C2	Exelixis,Inc./ Bloomington #7514
Df(2L)Exel6032	w^{1118}; Df(2L)Exel6032, P{XP-U}Exel6032/CyO	33C2;33D4	Exelixis,Inc./ Bloomington #7515
Df(2L)Exel6033	w^{1118}; Df(2L)Exel6033, P{XP-U}Exel6033/CyO	33E4;33F2	Exelixis,Inc./ Bloomington #7516
Df(2L)Exel6034	w^{1118}; Df(2L)Exel6034, P{XP-U}Exel6034/CyO	33F2;34A1	Exelixis,Inc./ Bloomington #7517
Df(2L)Exel 8028	w^{1118}; Df(2L)Exel 8028, P+PBac{XP5.WH5}Exel8028/CyO	34A1;34A2	Exelixis,Inc./ Bloomington #7822
Df(2L)Exel7055	w^{1118}; Df(2L)Exel7055, P+PBac{XP5.WH5}Exel7055/CyO	34A2;34A7	Exelixis,Inc./ Bloomington #7823

Df(2L)Exel7059	w^{1118}; Df(2L)Exel7059, P+PBac{XP5.RB3}Exel7059/CyO	34D3;34E1	Exelixis,Inc./ Bloomington #7826
Df(2L)Exel6035	w^{1118}; Df(2L)Exel6035, P{XP-U}Exel6035/CyO	35A3;35B2	Exelixis,Inc./ Bloomington #7518
Df(2L)Exel6036	w^{1118}; Df(2L)Exel6036, P{XP-U}Exel6036/CyO	35B1;35B2	Exelixis,Inc./ Bloomington #7519
Df(2L)Exel8033	w^{1118}; Df(2L)Exel8033, P+PBac{XP5.WH5}Exel8033/CyO	35B1;35B8	Exelixis,Inc./ Bloomington #7828
Df(2L)Exel8034	w^{1118}; Df(2L)Exel8034, P+PBac{XP5.WH5}Exel8034/CyO	35C5;35D2	Exelixis,Inc./ Bloomington #7830
Df(2L)Exel7063	w^{1118}; Df(2L)Exel7063, P+PBac{XP5.RB3}Exel7063/CyO	35D2;35D4	Exelixis,Inc./ Bloomington #7831
Df(2L)Exel6038	w^{1118}; Df(2L)Exel6038, P{XP-U}Exel6038/CyO	35D6;35E2	Exelixis,Inc./ Bloomington #7521
Df(2L)Exel7066	w^{1118}; Df(2L)Exel7066, P+PBac{XP5.WH5}Exel7066/CyO	36A1;36A12	Exelixis,Inc./ Bloomington #7833
Df(2L)Exel6039	w^{1118}; Df(2L)Exel6039, P{XP-U}Exel6039/CyO	36A10;36B3	Exelixis,Inc./ Bloomington #7522
Df(2L)Exel7067	w^{1118}; Df(2L)Exel7067, P+PBac{XP5.RB3}Exel7067/CyO	36A12;36B2	Exelixis,Inc./ Bloomington #7834
Df(2L)Exel8036	w^{1118}; Df(2L)Exel8036, P+PBac{XP5.RB3}Exel8036/CyO	36B1;36C9	Exelixis,Inc./ Bloomington #7835
Df(2L)Exel7068	w^{1118}; Df(2L)Exel7068, P+PBac{XP5.RB3}Exel7068/CyO	36C7;36C10	Exelixis,Inc./ Bloomington #7838
Df(2L)Exel9044	w^{1118}; Df(2L)Exel9044, PBac{WHr}Exel9044/CyO	36C10;36C11	Exelixis,Inc./ Bloomington #7836
Df(2L)Exel7069	w^{1118}; Df(2L)Exel7069, P+PBac{XP5.RB3}Exel7069/CyO	36C10;36D1	Exelixis,Inc./ Bloomington #7837
Df(2L)Exel7070	w^{1118}; Df(2L)Exel7070, P+PBac{XP5.WH5}Exel7070/CyO	36D2;36E1	Exelixis,Inc./ Bloomington #7839
Df(2L)Exel8038	w^{1118}; Df(2L)Exel8038, P+PBac{XP5.RB3}Exel8038/CyO	36D3;36E3	Exelixis,Inc./ Bloomington #7840
Df(2L)Exel9033	w^{1118}; Df(2L)Exel9033, PBac{WHr}Exel9033	36F2;36F2	Exelixis,Inc./ Bloomington #7841
Df(2L)Exel6041	w^{1118}; Df(2L)Exel6041, P{XP-U}Exel6041/CyO	36F5;37A2	Exelixis,Inc./ Bloomington #7523
Df(2L)Exel7071	w^{1118}; Df(2L)Exel7071, P+PBac{XP5.RB3}Exel7071/CyO	37A1;37A4	Exelixis,Inc./ Bloomington #7843
Df(2L)Exel7072	w^{1118}; Df(2L)Exel7072, P+PBac{XP5.WH5}Exel7072/CyO	37A2;37B6	Exelixis,Inc./ Bloomington #7844
Df(2L)Exel7073	w^{1118}; Df(2L)Exel7073, P+PBac{XP5.WH5}Exel7073/CyO	37B1;37B9	Exelixis,Inc./ Bloomington #7845
Df(2L)Exel8039	w^{1118}; Df(2L)Exel8039, P+PBac{XP5.WH5}Exel8039/CyO	37B8;37B11	Exelixis,Inc./ Bloomington #7846
Df(2L)Exel6042	w^{1118}; Df(2L)Exel6042, P{XP-U}Exel6042/CyO	37B8;37C5	Exelixis,Inc./ Bloomington #7524
Df(2L)Exel8040	w^{1118}; Df(2L)Exel8040, P+PBac{XP5.WH5}Exel8040/CyO	37C1;37C5	Exelixis,Inc./ Bloomington #7847
Df(2L)Exel6043	w^{1118}; Df(2L)Exel6043, P{XP-U}Exel6043/CyO	37C5;37D7	Exelixis,Inc./ Bloomington #7525
Df(2L)Exel7075	w^{1118}; Df(2L)Exel7075, P+PBac{XP5.RB3}Exel7075/CyO	37D2;37E1	Exelixis,Inc./ Bloomington #7848
Df(2L)Exel 8041	w^{1118}; Df(2L)Exel 8041, P+PBac{XP5.RB3}Exel8041/CyO	37D7;37F2	Exelixis,Inc./ Bloomington #7849
Df(2L)Exel9043	w^{1118}; Df(2L)Exel9043, P+PBac{XP5.RB3}Exel9043/CyO	37E1;37E1	Exelixis,Inc./ Bloomington #7913
Df(2L)Exel6044	w^{1118}; Df(2L)Exel6044, P{XP-U}Exel6044/CyO	37F2;38A4	Exelixis,Inc./ Bloomington #7526
Df(2L)Exel6045	w^{1118}; Df(2L)Exel6045, P{XP-U}Exel6045/CyO	38A4;38A7	Exelixis,Inc./ Bloomington #7527
Df(2L)Exel7077	w^{1118}; Df(2L)Exel7077, P+PBac{XP5.RB3}Exel7077/CyO	38A7;38B2	Exelixis,Inc./ Bloomington #7850

Df(2L)Exel6046	w^{1118}; Df(2L)Exel6046, P{XP-U}Exel6046/CyO	38C2;38C7	Exelixis,Inc./ Bloomington #7528
Df(2L)Exel7078	w^{1118}; Df(2L)Exel7078, P+PBac{XP5.WH5}Exel7078/CyO	38C7;38D4	Exelixis,Inc./ Bloomington #7851
Df(2L)Exel7079	w^{1118}; Df(2L)Exel7079, P+PBac{XP5.WH5}Exel7079/CyO	38E6;38F3	Exelixis,Inc./ Bloomington #7852
Df(2L)Exel7080	w^{1118}; Df(2L)Exel7080, P+PBac{XP5.RB3}Exel7080/CyO	38F3;39A2	Exelixis,Inc./ Bloomington #7853
Df(2L)Exel6047	w^{1118}; Df(2L)Exel6047, P{XP-U}Exel6047/CyO	39A2;39B4	Exelixis,Inc./ Bloomington #7529
Df(2L)Exel6048	w^{1118}; Df(2L)Exel6048, P{XP-U}Exel6048/CyO	39B4;39D1	Exelixis,Inc./ Bloomington #7530
Df(2L)Exel7081	w^{1118}; Df(2L)Exel7081, P+PBac{XP5.WH5}Exel7081/CyO	39D1;39E6	Exelixis,Inc./ Bloomington #7855
Df(2L)Exel6049	w^{1118}; Df(2L)Exel6049, P{XP-U}Exel6049/CyO	39E7;40D3	Exelixis,Inc./ Bloomington #7531

Table A.2 Oligos used for sequencing of candidate genes.

Gene	Oligo	Sequence
CG13766	CG13766F1	GAGGCGTAAACCATTCCAAA
	CG13766R1	GGAGGGAAGCAAGCGTCTA
	CG13766F2	GGCCACTTCCACATCCACTA
	CG13766R2	TTTGGAACGTGCACAAAAAG
	CG13766F3	TACGCAGTTGGTGACAGCTC
	CG13766R3	GCTACCTGGTCCCACTGTTT
CG31633	CG31633F1	GCGGTGAATGATATCCAAGAA
	CG31633R1	CGAACGAAGCCTTTGAAAAT
	CG31633F2	CCAATTCAATACATTCGTCAGG
	CG31633R2	TGGAAGCATAAGGGGTCAAG
	CG31633F3	CAAGAGCGGAAAGCAATAGG
	CG31633R3	TTGATCTACTTGGGCGATGA
	CG31633F4	AGCACAGCTGAAGGTCCAGT
	CG31633R4	GGAACCCAACTGTGGAATGT
CG11320	CG11320F1	GAAGTTTGATGCGAGTGCTG
	CG11320R1	GTCAGGATGTGGTTCGTTCC
	CG11320F2	CAGTATCGGAGTCGCTTCCT
	CG11320R2	ATGTTTGGCGTCTGAGTTCC
CG11043	CG11043F1	CAGCCGAGGAAAATTGCTTA
	CG11043R1	CTTGGCCAGCAGCATTTC
	CG11043F2	GCGTCCCATATCCACAACTC
	CG11043R2	CTACCAGATCCTGCCCAATC
CG11053	CG11053F1	AAGAGGCCCTAATGGGAATG
	CG11053R1	CCCAGAATCATCTCCTCTCG
	CG11053F2	AGTTTCCACCAGGACCTCAA
	CG11053R2	TCGATCACCTTCCTCAGTCA
	CG11053F3	CTGACTGAGGAAGGTGATCG
	CG11053R3	GGCTCATCATAAGCTGGCTA
osm6	osm6F1	TTTGAATTCGAGTCGCCAAC
	osm6R1	GAGCGACAACAAGCACTTCA
	osm6F2	GTCCTTAAGCAGGCGGTTG

	osm6R2	GAGCCGGAGTTCAATACCAA
	osm6F3	CACATGGACTCGCAAACAAC
	osm6R3	GCATGAAAAATGTCAAGCAAGC
CG34009	CG34009F1	TTGAACAGCGGGATAGTTATGT
	CG34009R1a	AATTTAGGCGTTTGCTGGAA
	CG34009F2	GGCGAAAGGAATCACAATCA
	CG34009R2	GAAAATGTGCGCCGAATAAA
	CG34009F3	GCGGGTTACAAAAGTTCAGC
	CG34009R3	ATTGCACAGCACCGTCTTTC
	CG34009F4	GCGGAGAATAAAGGCCAACT
	CG34009R4	AGGCCAGAATGAAGAAACGA
	CG34009F5	GTTTTCGCAGCAGAATGTTG
	CG34009R5	CCCTTGAAATTGAGAAAACG
	CG34009F6	TGTCCAATCCCCTAAATTCC
	CG34009R6	TCGAGCTCTTTTCGTTTTGG
CG11015	CG11015F1	AAGTTCGGTTGATTGTTGCAC
	CG11015R1	GTGGGTCCAAATCTGATGCT
	CG11015F2	GACACCACAGTGCGGAGTTA
	CG11015R2	GTTTAAACGCGGTGTGTAGC
	CG11015F3	GATTAGCTACACACCGCGTTT
	CG11015R3	CCCACTTTGAGTGAGTTGAATC
CG11050	CG11050F1	CAGATCAAGTGCTTTCAATTCG
	CG11050R1	CCGAACTTCGCTTGACTAGAG
	CG11050F2	GGAGCCACAAAGACACTGGT
	CG11050R2	AAATCAAAACACGCAGGACA
	CG11050F3	GCATTGCACTTTGTTAGAACG
	CG11050R3	GGACATCCCTCTGCTCGTAG
	CG11050F4	TACGAGCATGGACAGACTGC
	CG11050R4	AGAATTCGATCCGCAACTTT
	CG11050F5	CCTACCTCGACACGCTCATAG
	CG11050R5	CCAACCGCTTTGGATAATGT
CG9596	CG9596F1	AGTGCGGCCACTACTTCATC
	CG9596R1	GGAACAACCTGAGGCGATAG
	CG9596F2	GGAGGTCCCTTTGCTCCTAT
	CG9596R2	CAACGACTCCAGCAAAATCA
	CG9596F3	CTTGGCGGTAGAAGATGTCC
	CG9596R3	TCACATGCAACAGGTGACTAGA

Table A.3 List of lines identified in the screen

Line	Class	Lethal	Phenotype
2L0036	A	yes	no clones
2L0044	A	yes	no clones
2L0058	A	yes	no clones, convoluted DT in homozygous embroys
2L0067	A	yes	no clones, no negative clones in wing imaginal disc
2L0218	A	yes	no clones
2L0243	A	yes	no clones

2L0358	A	yes	no clones, no clones in wing imaginal disc, mutated FRT site
2L0364	A	yes	no clones
2L0437	A	yes	no clones
2L0446	A	yes	no clones
2L455	A	yes	no clones
2L0476	A	yes	no clones
2L0483	A	yes	no clones
2L0567	A	yes	no clones
2L0695	A	yes	no clones
2L0702	A	yes	no clones
2L0719	A	yes	no clones
2L0728	A	yes	no clones
2L0882	A	yes	no clones
2L1066	A	yes	no clones
2L1083	A	yes	no clones
2L1145	A	yes	no clones
2L1159	A	yes	no clones
2L1219	A	yes	no clones
2L1290	A	yes	no clones
2L1313	A	yes	no clones
2L1498	A	yes	no clones
2L1573	A	yes	no clones
2L1623	A	yes	no clones, does not complement varicose
2L1625	A	yes	no clones
2L1673	A	yes	no clones
2L1681	A	yes	no clones
2L1688	A	yes	no clones
2L1713	A	yes	no clones
2L1810	A	yes	no clones
2L1846	A	yes	no clones
2L1916	A	yes	no clones
2L1930	A	yes	no clones
2L1936	A	yes	no clones
2L1961	A	yes	no clones
2L1979	A	yes	no clones
2L1990	A	yes	no clones
2L2001	A	yes	no clones
2L2020	A	yes	no clones
2L2069	A	yes	no clones
2L2071	A	yes	no clones
2L2077	A	yes	no clones
2L2081	A	yes	no clones
2L2087	A	yes	no clones
2L2115	A	yes	no clones
2L2236	A	yes	no clones
2L2238	A	yes	no clones
2L2246	A	yes	no clones
2L2268	A	yes	no clones
2L2279	A	yes	no clones
2L2283	A	yes	no clones
2L2299	A	yes	no clones

2L2314	A	yes	no clones
2L2338	A	yes	no clones
2L2402	A	yes	no clones
2L2438	A	yes	no clones
2L2448	A	yes	no clones
2L2450	A	yes	no clones
2L2460	A	yes	no clones
2L2491	A	yes	no clones
2L2502	A	yes	no clones
2L2508	A	yes	no clones
2L2525	A	yes	no clones
2L2556	A	yes	no clones
2L2608	A	yes	no clones
2L2613	A	yes	no clones
2L2624	A	yes	no clones
2L2626	A	yes	no clones
2L2640	A	yes	no clones
2L2714	A	yes	no clones
2L2739	A	yes	no clones
2L2765	A	yes	no clones
2L2787	A	yes	no clones
2L2850	A	yes	no clones
2L2855	A	yes	no clones
2L2964	A	yes	no clones
2L2979	A	yes	no clones
2L3002	A	yes	no clones
2L3025	A	yes	no clones
2L3025	A	yes	no clones
2L3039	A	yes	no clones
2L3065	A	yes	no clones
2L3104	A	yes	no clones
2L3114	A	yes	no clones
2L3147	A	yes	no clones
2L3185	A	yes	no clones
2L3196	A	yes	no clones
2L3198	A	yes	no clones
2L3201	A	yes	no clones
2L3209	A	yes	no clones
2L3214	A	yes	no clones
2L3215	A	yes	no clones, does not complement varicose
2L3221	A	yes	no clones
2L3233	A	yes	no clones
2L3247	A	yes	no clones
2L3253	A	yes	no clones
2L3254	A	yes	no clones
2L3259	A	yes	no clones
2L32361	A	yes	no clones
2L3266	A	yes	no clones
2L3314	A	yes	no clones
2L3319	A	yes	no clones
2L3342	A	yes	no clones

2L3357	A	yes	no clones
2L3413	A	yes	no clones
2L3450	A	yes	no clones
2L3452	A	yes	no clones
2L3453	A	yes	no clones
2L3511	A	yes	no clones
2L3527	A	yes	no clones
2L3524	A	yes	no clones
2L3551	A	yes	no clones
2L3606	A	yes	no clones
2L3614	A	yes	no clones
2L3619	A	yes	no clones
2L3643	A	yes	no clones
2L3671	A	yes	no clones
2L3687	A	yes	no clones
2L3698	A	yes	no clones
2L3730	A	yes	no clones
2L3768	A	yes	no clones
2L3792	A	yes	no clones
2L3794	A	yes	no clones
2L3814	A	yes	no clones
2L3838	A	yes	no clones
2L3854	A	yes	no clones
2L3894	A	yes	no clones
2L3905	A	yes	no clones
2L3907	A	yes	no clones
2L3920	A	yes	no clones
2L3944	A	yes	no clones
2L3949	A	yes	no clones
2L3988	A	yes	no clones
2L3997	A	yes	no clones
2L4048	A	yes	no clones
2L4064	A	yes	no clones
2L4103	A	yes	no clones
2L4108	A	yes	no clones
2L4178	A	yes	no clones
2L4211	A	yes	no clones
2L4222	A	yes	no clones
2L4223	A	yes	no clones
2L4229	A	yes	no clones
2L4252	A	yes	no clones
2L4257	A	yes	no clones
2L4267	A	yes	no clones
2L4304	A	yes	no clones
2L4306	A	yes	no clones
2L4315	A	yes	no clones
2L4337	A	yes	no clones
2L4366	A	yes	no clones
2L4411	A	yes	no clones
2L4450	A	yes	no clones
2L4481	A	yes	no clones

2L4578	A	yes	no clones
2L4713	A	yes	no clones
2L4741	A	yes	no clones
2L4745	A	yes	no clones
2L0141	B	no	low number of clones, no positive clones in wing imaginal disc
2L0224	B	yes	low number of clones, wt clones in wing imaginal disc
2L0557	B	yes	low number of clonal cells
2L0611	B	yes	low number of clones
2L0872	B	no	low number of clonal cells
2L0948	B	yes	low number of clonal cells
2L0951	B	no	low number of clonal cells, especially in DT
2L4739	B	yes	low number of clonal cells
2L2629	B	yes	low number of clonal cells
2L4152	B	no	low number of clonal cells
2L1160	B I	no	extremly low number of clonal cells
2L1261	B I	yes	very low number of clones
2L1901	B I	yes	very low number of clones
2L2058	B I	yes	very low number of clonal cells
2L2622	B I	no	very low number of clonal cells
2L3089	B I	no	low number of clonal cells
2L3390	B I	yes	extremly low number of clonal cells
2L4332	B I	yes	no clones/very few clonal cells
2L4405	B I	yes	no clones/very few clonal cells
2L4449	B I	yes	very low numebr of clonal cells
2L1399	C	yes	small clonal cells
2L1832	C	yes	small clones
2L1913	C	yes	small clonal cells
2L2101	C	yes	small clonal cells
2L2269	C	yes	small clonal cells
2L2282	C	no	small clonal cells
2L2553	C	yes	small clonal cells
2L2572	C	yes	small clonal cells
2L2816	C	yes	small clonal cells
2L2976	C	yes	small clonal cells
2L3275	C	yes	small clonal cells
2L3430	C	yes	small clonal cells
2L3547	C	yes	small clonal cells
2L3569	C	yes	small clonal cells
2L3611	C	yes	small clonal cells
2L3651	C	yes	small clonal cells
2L3948	C	yes	small clonal cells
2L4034	C	yes	small clonal cells
2L4744	C	yes	small clonal cells
2L0944	C	yes	small clonal cells
2L1714	C I	no	very small clonal cells
2L1749	C I	yes	very small clonal cells
2L2416	C I	yes	very small clonal cells
2L2871	C I	yes	very small clonal cells
2L3937	C I	yes	extremly small clonal cells
2L4067	C I	yes	very small clonal cells
2L1554	C I	yes	extremly small clonal cells

2L0689	C II	?	low number of clones, small clonal cells
2L1360	C II	yes	low number of clonal cells, small
2L1663	C II	yes	very small clonal cells, low number
2L1805	C II	yes	small clonal cells, low number
2L2073	C II	no	small clonal cells, low number
2L2526	C II	yes	very small clonal cells, low number
2L2648	C II	yes	very small clonal cells, low number
2L2953	C II	yes	small clonal cells, low number
2L3278	C II	yes	low number, small clonal cells
2L3293	C II	no	extremly small clonal cells
2L3301	C II	yes	extremly small clonal cells, low number
2L3311	C II	yes	extremly small clonal cells, low number
2L3456	C II	yes	very low number,small clonal cells
2L3500	C II	yes	small clonal cells, low number
2L3642	C II	yes	extremly small clonal cells, low number
2L3908	C II	yes	small clonal cells, very low number
2L3940	C II	yes	very small clonal cells, low number
2L4081	C II	yes	very small clonal cells, low number
2L4508	C II	yes	very small clonal cells, low number
2L4509	C II	no	very small clonal cells, low number
2L0128	D	no	cells in DT larger than in wt
2L0196	D	no	DT narrowed within clonal cells (not fully penetrant)
2L0372	D	no	smaller lumen and irregular shape of clonal cells in DT
2L3090	D	yes	rounded up clonal cells in DT and secondary branches
2L3191	D	yes	irregular lumen in DT at clonal position
2L4771	D	yes	bendings of fusion cells in DT, no clonal cells in TB and other fusion cells
2L1506	D I	yes	small clonal cells, DT bendings
2L1687	D I	yes	bendings of DT at clonal position, smaller clonal cells
2L2181	D I	yes	DT bendings
2L3179	D I	yes	slight DT bendings
2L3696	D I	yes	DT bendings
2L3910	D I	yes	DT bendings
2L4021	D I	yes	slight DT bendings, very low number of clonal cells in TB
2L4333	D I	yes	DT bendings, TB shorter branches
2L0439	D I	yes	bendings of DT at clonal position
2L2676	D I/EV	yes	almost no TB, TB with reduced number of branches, slight DT bendings
2L3146	D II	yes	small clonal cells, almost only in DT, low number
2L3680	D II	no	low number of clonal cells, only in DT
2L3707	D II	yes	low number of clonal cells, only in DT
2L3038	D II	yes	small clonal cells, only in DT
2L0028	D III	yes	small clones in DT, low number of clones, no positive clones in wing imaginal disc
2L1693	D III	yes	small clonal cells in DT, low number
2L3418	D III	yes	small clonal cells in DT
2L3477	D III	yes	small clones in DT, reduced number of clonal cells in TB
2L3585	D III	yes	small clonal cells in DT
2L2769	D IV	yes	low number of clonal cells in DT
2L2875	D IV	yes	no clonal cells in DT
2L3602	D IV	yes	no clonal cells in DT

2L1668	E	yes	abnormally formed TB
2L1923	E	yes	wrong formation of TB
2L2084	E	yes	thicker lumen in dorsla branches, additional branching points
2L2806	E	no	untypical TB branching
2L2944	E	no	untypical TB, thicker, with reduced branching
2L3193	E	no	abnormally formed TB
2L3393	E	yes	abnormally formed TB
2L3807	E	yes	abnormal ratio of clonal cells in TB and other types of branches
2L4105	E	yes	smaller diameter of TB
2L4515	E	yes	smaller diameter of TB
2L0525	E I	yes	TB cross/fusion
2L1030	E I	yes	intra/inter fusion and branch crossing in TB
2L1210	E I	no	TB cross/fusion, low number of TB
2L1472	E I	no	TB crossing, spacing problem in lateral branches
2L1629	E I	no	expanded TB, crossed, maybe more branches
2L1816	E I	yes	TB crossing
2L2365	E I	yes	TB crossing
2L2408	E I	yes	TB crossing
2L2442	E I	no	TB crossing/fusion
2L2445	E I	no	TB crossing/fusion
2L2646	E I	yes	TB crossing
2L2993	E I	yes	TB crossing
2L3063	E I	yes	TB crossing/fusion
2L3075	E I	yes	TB crossing
2L3200	E I	no	TB crossing, irregular branching
2L3244	E I	yes	TB intra - crossing
2L3294	E I	yes	TB crossing
2L3300	E I	no	TB crossing, maybe expanded
2L3316	E I	no	TB crossing, weak, enlarged cell body
2L3327	E I	yes	TB crossing/fusion
2L3469	E I	yes	TB crossing
2L4753	E I	no	TB crossing/fusion
2L1878	E I	no	TB crossing
2L2022	E I	no	TB crossing
2L0253	E II	yes	low number of clones, no typical terminal outgrowth, smaller cells, only negative clones in wing imaginal dic
2L2129	E II	yes	less or no branches in TB
2L3120	E II	yes	reduced branching in TB
2L3333	E II	yes	low number of clones, TB reduced number of branches
2L3458	E II	no	reduced number of branches in TB
2L3935	E II	yes	reduced number of branches in TB
2L4099	E II	yes	reduced number of branches in TB
2L4409	E II	no	very small clonal cells, lumen formation problems in TB, fewer branches, fewer TB
2L4485	E II	yes	small clonal cells, TB formed irregulary, reduced number of branches
2L4567	E II	yes	extremly small clonal cells, reduced number of TB, reduced number of branches
2L4613	E II	yes	reduced number of branches, small clonal cells and low number
2L0876	E III	no	partila formation of lumen in TB, not fully penetrant
2L2218	E III	yes	no lumen in TB , almos no branches, very low number of clonal cells
2L2741	E III	yes	lumen formation problem in TB, TB fusion/cross

2L3152	E III	yes	almost no clonal cells in TB, if they are no lumen
2L3260	E III	yes	no lumen in TB, low number of clonal cells in TB
2L3340	E III	yes	lumen formation problem in TB,
2L3443	E III	yes	low number of clonal cells, lumen formation problem in TB
2L3463	E III	yes	lumen in TB placed on the side, sometimes two lumen in part of branches
2L3637	E III	yes	lumen formation problem
2L3664	E III	yes	reduced number of branches in TB, small clones, lumen formation problem
2L3686	E III	yes	misplaced lumne in TB
2L3789	E III	yes	sligth TB lumen formation problem - lumne stops at fine branches
2L3881	E III	yes	thicker TB without lumen
2L4010	E III	yes	lumen in TB stops at beginning if the branch
2L4117	E III	yes	no lumen in TB
2L4158	E III	yes	lumen formation problem in TB, change in diameter in transverse connective
2L4501	E III	yes	lumen formation problem in TB and secondary branches
2L4658	E III	no	no lumen in TB
2L4765	E III	yes	very small clonal cells, lumen formation problem in TB, abnormal branching pattern
2L1475	E IV	yes	elongated TB
2L2853	E IV	yes	expanded TB longer branches
2L3522	E IV	yes	expanded TB
2L4098	E IV	no	more branches in TB
2L0507	E V	no	small clonal cells, few in TB
2L0508	E V	no	small clonal cells, few in TB
2L0957	E V	no	no clonal cells in TB
2L1514	E V	yes	very low number, clonal cells very small, no in TB
2L1691	E V	yes	very low number of clones, no in TB
2L1806	E V	yes	very low number of clones no in TB
2L3369	E V	yes	reduced number of TB
2L3874	E V	yes	no clonal cells in TBs
2L4127	E V	yes	low number of clonal cells, no clonal cells in TB, small
2L4324	E V	yes	no clonal cells in TB
2L4506	E V	yes	low number of clonal cells, no clonal cells in TB
2L4700	E V	no	no/fewer clonal cells in TB
2L4737	E V	yes	almost no clonal cells in TB
2L0419		yes	tracheal histoblasts are smaller if consisting of clonal cells
2L0445		no	wrongly shaped and too small clonal cells at DT and secondary branches
2L1281		yes	no clones in epidermal cells, tracheal clones wt
2L1296		no	no clonal cells in epidermis
2L3574		yes	clonal cells only in epidermis

Abstract

Many animal organs are built of ramified tubular epithelial structures. How they form and what controls branching events, direction of growth and tube size are important questions for understanding branch morphogenesis. The *Drosophila* tracheal (respiratory) system is an excellent model to investigate this process. Extensive studies on tracheal development in the last years resulted in revealing mechanisms involved in tube formation. However there are still many open questions, especially concerning late events of tracheal morphogenesis, such as branching of terminal cells during the larval life. Thus we performed a genetic mosaic screen to identified new genes involved in tracheal development. As a screening tool the MARCM system was applied and mutant lines were examined in third instar larvae.

Out of 4779 analysed lines 344 showed phenotypes different from the wild type. These lines were classified into five general phenotypic classes: A - no mutant clones, B - low number of mutant clonal cells, C – small mutant clonal cells, D - dorsal trunk defects, E – terminal branching defects. Three phenotypic groups were characterised in more detail – one from class D, showing dorsal trunk bendings and consisting of eight alleles (D1), and two from class E, showing defects in formation of lumen in terminal cells (E2 and E3), both consist of two alleles. The phenotypes in groups D1 and E3 were mapped to the genomic region of ~190kb and ~160kb respectively.

Although none of the mutations have been mapped to individual genes and no mechanisms explaining different phenotypes could be proposed, the screen provided collection of mutants, whose analysis will help in better understanding of cellular processes of tracheal development.

9. Zusammenfassung

Viele tierische Organe sind aus Netzwerken verzweigter epithelialer Röhren (Tubuli) aufgebaut. Wie diese entstehen und welcher Kontrolle die Bildung von Verzweigungen, deren gerichtetes Wachstum sowie die Größe der entstehenden Röhren unterliegen, sind wichtige Fragen zum Verständnis der Morphogenese dieser Strukturen. Das Atmungssystem (Tracheensystem) von *Drosophila* bietet ein gutes Modellsystem, um diese Prozesse zu untersuchen. Umfangreiche Studien zur Entwicklung des Tracheensystems in den letzten Jahren haben viel zu unserem Verständnis der Mechanismen, die der Tubusformation zugrunde liegen, beigetragen. Allerdings sind noch immer viele Fragen offen, insbesondere bezüglich der späteren Prozesse der Tracheenentwicklung, wie zum Beispiel die Verzweigung der terminalen Zellen während des Larvenstadiums. Wir führten einen genetischen Mosaik-Screen durch, um neue Gene zu identifizieren, die an der Tracheenentwicklung beteiligt sind. Das MARCM System wurde für den Screen verwendet; die mutanten Linien im dritten Larvenstadium untersucht.

344 von insgesamt 4779 untersuchten Linien zeigten Phänotypen, die sich vom Wildtyp unterschieden. Diese Linien wurden in 5 generelle phänotypische Klassen unterteilt: A – keine mutanten Klone, B – geringe Anzahl mutanter klonaler Zellen, C – mutante klonale Zellen mit kleiner Zellgröße, D – Defekte im Dorsalstamm (dorsal trunk), E – Defekte in der Verzweigung der terminalen Zellen. Drei phänotypische Gruppen wurden detaillierter analysiert - eine der Klasse D, die Krümmungen des Dorsalstamms aufwies und aus 8 Allelen bestand (D1), sowie zwei der Klasse E, die Defekte in der Lumenbildung in den terminalen Zellen aufwiesen (E2 und E3), beide mit jeweils zwei Allelen. Die Phänotypen der Gruppen D1 und E3 wurden der genomischen Region von ~190 kb resp. ~160 kb zugeordnet.

Obwohl keine der Mutationen individuellen Genen zugewiesen werden konnte und keine Modelle aufgestellt werden konnten, um die unterschiedlichen Phänotypen zu erklären, stellt der Screen dennoch eine Sammlung von Mutanten zur Verfügung, deren Analyse dazu beitragen wird, die der Tracheenentwicklung zugrunde liegenden zellulären Prozesse besser zu verstehen.

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit − einschließlich Tabellen, Karten und Abbildungen −, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie − abgesehen von unten angegebenen Teilpublikationen − noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Maria Leptin betreut worden.

Keine Teilpublikationen

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