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UNIVERSITY OF CRETE

BIOLOGY DEPARTMENT

Bachelor Thesis

Investigation of tumor growth mechanisms and the interaction with tracheal and hematopoietic system in Drosophila.

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ABSTRACT

Drosophila melanogaster is a widely used genetic model organism, with characteristics that enable scientists to study complex pathways involved in biomedical research, including cancer. Notch signaling pathway is one the most commonly deregulated pathways in various diseases and cancer. Notch is a key player in Drosophila brain development and its ectopic over-activation can lead to tumorigenesis with dramatic results. Transcriptomic data from previous work in our lab has shown that after allografting Notch induced brain tumors into adult flies, various genes get up- or down- regulated as the tumor progresses to a more aggressive state.

It has been indicated that the tracheal system, the analog of the respiratory system in mammals, plays important role in tumor growth and metastasis (Grifoni et al., 2015, Tamamouna 2021). In the transcriptomic analysis one of the genes that was found upregulated in the most aggressive tumor stage was *branchless*. Branchless encodes a Drosophila FGF homolog which is important for tracheal cell migration and branching (Sutherland et al., 1996). Considering the above results, our study attempts to clarify the interplay between tumor and the tracheal system in adult Drosophila with a focus on the FGF/FGFR homologs, *branchless* and *breathless*.

As tumor progresses over time, another gene that gets upregulated is Tep4. Tep4 is a thioester-containing protein which is involved in immune responses in Drosophila and other insects by promoting recruitment of immune cells in response to infections (Shokal & Eleytherianos, 2017). In this work, we examine the expression of Tep4 in the larval and adult stages and we try to define the differential expression pattern upon tumorigenic conditions.

Finally, guided by the transcriptomic data along with immunohistochemistry, we have observed that Drosophila blood cells respond to allografted tumors. Here we wanted to investigate the response of the larval hemocytes in the primary brain hyperplasia by examining the proliferation rate of hemocytes and their differentiation into distinct subtypes.

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CONTENT TABLE

1. INTRODUCTION

1.1 Drosophila as a genetic model

Drosophila melanogaster has been used as a model organism in modern biological science for over 100 years and makes for the first major complex organism to have its genome sequenced (Charles & Nichols, 2011). The Drosophila genome is 60% homologous to that of humans and about 75% of the genes responsible for human diseases have homologs in flies (Fig.1). It also offers a powerful genetic toolkit including gene-knockout and transgenic stocks and gives the ability to generate flies with complex genotypes. Drosophila strains are highly reproductive and have a brief generation time (Yamamura et al., 2020). These features allow scientists to study complex pathways relevant in biomedical research, including cancer.

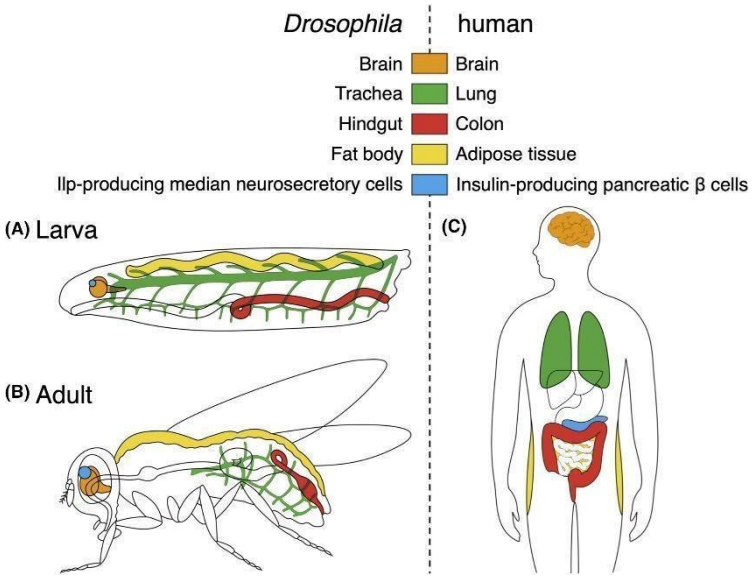


Figure 1: Tissue/organ similarity between Drosophila and human. These similarities allow construction of fly models for human diseases including cancer affecting tissue/organ functions (Yamamura et al., 2020)

D. melanogaster represents a simple system for modelling cancer. Most of the signaling pathways controlling cell growth and invasion in mammals have conserved functions in flies allowing the study of tumor biology in a simple model organism. Furthermore, the systematic study of tumorigenesis is more accessible because fewer mutations are required for tumor development. Indeed, heritable tumor causing mutations have been documented since researchers first started studying D. melanogaster genetics. Genetic screens combined with the availability of powerful recombination techniques also enabled the functional characterization of conserved oncogenes and tumor suppressor genes in a whole organism. Mammalian homologues of potential cancer-causing genes and pathways have also been discovered (Brumby & Richardson, 2005).

1.2 Notch signaling as a key player in Drosophila brain development

The Notch pathway is involved in cell proliferation, differentiation and survival. It is one of the most commonly activated signaling pathways in various diseases, such as T-cell leukemia, breast cancer, prostate cancer, colorectal cancer and lung cancer as well as central nervous system (CNS) malignancies (Yuan et al., 2015).

Notch signaling is activated when a ligand binds to a Notch receptor. It requires cell-to-cell contacts that enable Notch receptor and Delta/Serrate/Jagged ligand interactions. A receptor-ligand pulling force unfolds a protective domain within the Notch receptor to allow sequential proteolytic cleavage and release of the Notch intracellular domain (NICD). Later, NICD translocates to the nucleus where it binds a conserved transcription factor to upregulate Notch target genes (Yuan et al., 2015, Koval et al., 2017). Drosophila melanogaster has one Notch receptor and two ligands, Delta (Dl) and Serrate (Ser) showing a high level of conservation with their mammalian orthologs.

Neuroblasts (NBs) in Drosophila, also known as Neural Stem Cells (NSCs) are a common system to study self-renewal, differentiation and tumorigenesis mechanisms in vivo. During brain development, NBs arise from the neuroepithelium and start to proliferate in order to generate diverse neurons and glial cells in a spatially and temporally regulated way. The larval NBs are divided into type I and type II NBs. Type I NBs are located ventrally in the brain lobes and constitute all NBs in the Ventral Nerve Cord (VNC). They undergo repeated self-renewing asymmetric divisions and generate ganglion mother cells (GMC), which divide once to produce neurons and/or glial cells. Type II NBs are only in the dorsoposterior side of the brain, while each brain lobe contains eight type II lineages. Type II NBs divide asymmetrically to produce several transit amplifying Intermediate Neural Precursors (INPs), which later become mature INPs. Mature INPs divide asymmetrically to generate several GMCs, which then also divide to produce differentiated neurons or glia (Fig. 2). It is well known that Notch signaling events take place from the GMC/INP to the NB. During NB asymmetric cell divisions and self-renewal, the Notch signaling pathway appears to play a key role by ensuring the expression of key target genes (dpn, E (spl)…)(Zacharioudaki et al., 2012, Zacharioudaki et al., 2014, Magadi et al., 2020, Homem et al., 2012).

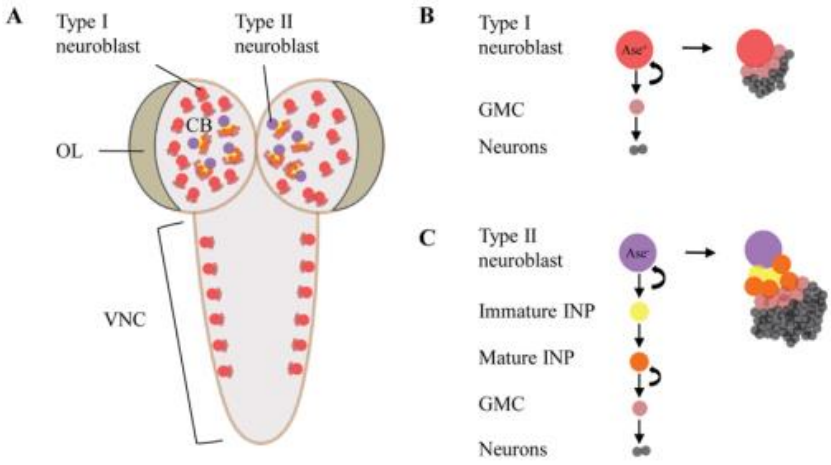


Figure 2*:* (A) Neuroblast lineages in the Drosophila larval brain (B, C) Type I and type II Neuroblasts asymmetric cell division (Li et al., 2014)

The two neuroblast lineages show important differences in their responsiveness towards Notch signaling. An over-activated Notch activity leads to hyperplasia in type II NBs, while type I NBs show better resistance in the same Notch hyperactivity lesions (Li et al., 2014). Thus, over-activation of Notch signaling leads to NB over-proliferation and tumorigenesis, with type II NBs being more sensitive in over-proliferating signals and enhanced tumor formation. (Zacharioudaki et al., 2012, 2019).

1.3 Drosophila tracheal system & FGF/FGFR signaling

The tracheal system of Drosophila melanogaster is a branched network of epithelial tubes that ramifies throughout the body and serves as a respiratory system which transports oxygen to the tissues and organs. During embryogenesis, the tracheal primordia appear as segments of tracheal placodes that invaginate into the body cavity and undergo stereotyped branching processes to form a network of tubular epithelium. During the larval period, terminal cells undergo extensive growth and branching and in the pupal stage, the diploid tracheal cells proliferate and remodel the tracheal system into the adult pattern (Hayashi & Kondo, 2018). The adult tracheal system of Drosophila is composed mainly of spiracles and air sacs connected to tracheal tubes for gas transportation (Pitsouli &Perrimon, 2010) (Fig. 3).

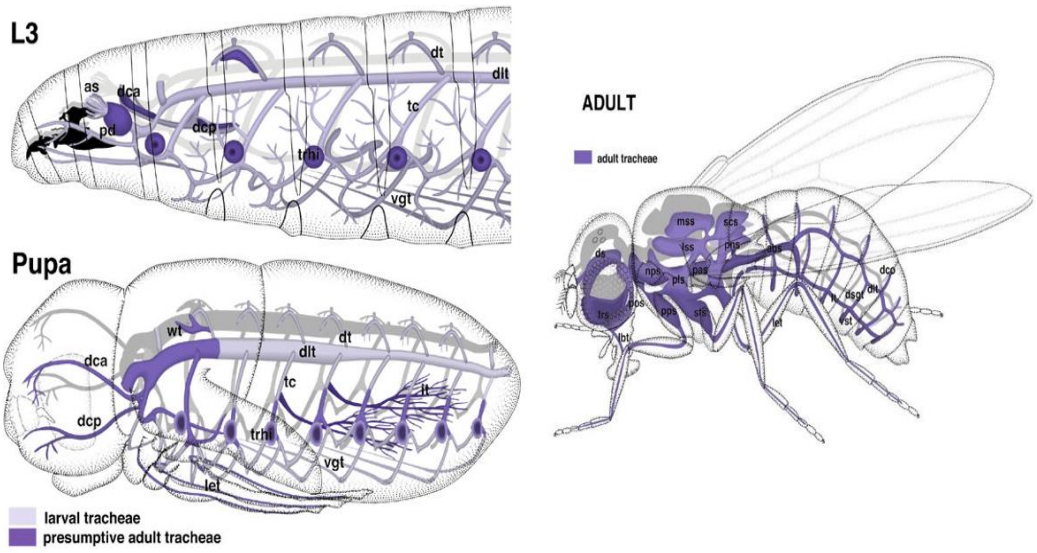


Figure 3: Larval, pupal and adult tracheal system (Hartenstein, 1993)

The transcription factor Trachealess (Trh) is expressed in all tracheal cells from the onset of tracheal placode specification through adulthood. Trh is a central regulator of tracheal cell identity, since in its absence the tracheal system does not develop. It regulates a multitude of other tracheal genes, most notably breathless (btl). Breathless (btl) encodes a homolog of FGF receptors (FGFRs), classical receptor tyrosine kinases (RTKs) and branchless (bnl) encodes a homolog of mammalian FGFs. The fibroblast growth factor signaling pathway (FGFR signaling) is an evolutionarily conserved signaling cascade that regulates several basic biologic processes. FGFs are a large family of peptide growth factors, with nine different mammalian FGF genes and four genes encoding FGF receptors (FGFRs). Drosophila melanogaster presents a simpler model system in which to study FGF signaling, with only three ligands [Pyramus (Pyr), Thisbe (Ths) and Branchless (Bnl)] and two FGF receptors [Heartless (Htl) and Breathless (Btl)] identified. Btl/FGFR is a transmembrane receptor tyrosine kinase (RTK) and consists of three extracellular immunoglobulin-like domains and one intracellular split tyrosine kinase domain. Bnl/FGF is a secreted glycoprotein that is sequestered by heparin sulfate proteoglycans (HPSG), which stabilize the FGF/FGFR interaction by protecting FGFs from degradation in the extracellular matrix and the cell surface. The binding of the ligand to the receptor leads to FGFR's dimerization and trans phosphorylation of tyrosine kinase domains. Activation of downstream signaling occurs via the intracellular FGFR substrate 2 and phospholipase Cγ (PLC-g), leading to subsequent upregulation of RAS/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT signaling pathways. (Touat et al., 2015)

Branchless (Bnl), initiates the branching process by triggering cell migration. Bnl is expressed around the invaginated tracheal sac by groups of ectodermal or mesodermal cells. The tracheal cells closest to the source of the Bnl ligand sense it through the FGF receptor (FGFR) Breathless (Btl) which induces the migration of some

cells away from the sac to generate ovoid extensions. During this process, the actively migrating cells remain fully assimilated in the tracheal epithelium (Affolter et al., 2009, Ghabrial et al., 2003).

Recent studies have shown that tracheal cells can take up positions within the developing branches in a rather dynamic pattern. Both in the embryo and in the larva, only cells with an intact Fgf signaling pathway can take up the leading position in outgrowing tracheal structures (Ghabrial & Krasnow, 2006). Other studies exhibited that an ectopic expression of a dominant negative form of btl leads to significantly reduced migration of tracheoblasts (Sato & Kornberg, 2002). Tracheal cells close to Bnl-secreting cells in the wing imaginal disc start to proliferate and migrate towards Bnl-expressing cells (Cabernard & Affolter, 2005). Moreover, tracheae increase their terminal branching in response to infection or tumor development, while increased tracheal terminal cell branching is necessary for tumor growth. (Tamammouna et al, 2021). In Bnl-expressing cancer tissues, migration of cancer cells towards nearby tracheal tubes occurs alongside tracheal imitation, namely differentiation of cancer cells themselves into tracheal cells, ensuring oxygen supply to the tumour (Griffoni et al., 2015).

In summary, tracheogenesis is a novel cancer hallmark in Drosophila and its study might reveal new molecular alterations that resemble the phenomenon of angiogenesis in human cancers.

1.4 Drosophila immune response

The immune system in Drosophila is characterized by the absence of adaptive immunity and a robust innate immune system, with the last sharing many functional and molecular similarities to that of vertebrates. The immune cells in flies are collectively described as hemocytes and exhibit functionally and developmentally conserved features with that of vertebrate blood cells. Other tissues also play a role in immunity, eg. the epithelia of the epidermis, gut and trachea (first to encounter pathogens), as well as the fat body, an adipose-like tissue that is responsible for the humoral immune response upon activation of the Toll or Imd pathways.

The Drosophila body cavity is filled with hemolymph that contains circulating and sessile hemocytes. Hemocytes are divided into three subtypes with distinctive functions and characteristics: plasmatocytes that account for 90% of total hemocytes, lamellocytes and crystal cells. Similar to vertebrate macrophages, Drosophila plasmatocytes have prominent roles in immunity, development, and wound healing. Plasmatocytes are professional phagocytes and readily engulf invasive pathogens and clear apoptotic remains. The recognition and uptake of phagocytic targets is mediated by the hemocyte-specific scavenger receptors Crq, Eater and the phagocytic receptors Nimrod C1 (NimC1) and Draper, which belong to a broad family of receptors with conserved roles in phagocytosis. Crystal cells are particularly large cells that mediate melanization reactions in innate immunity and wound healing and are named after the large crystalline inclusions of prophenoloxidase they contain. Lamellocytes have roles in the encapsulation of large immune targets and melanization, but emerge only

in the larva and mainly upon immune challenge by parasitic organisms (Gold & Bruckner, 2014, Parsons & Foley, 2016, Yu et al., 2018) (Fig.4).

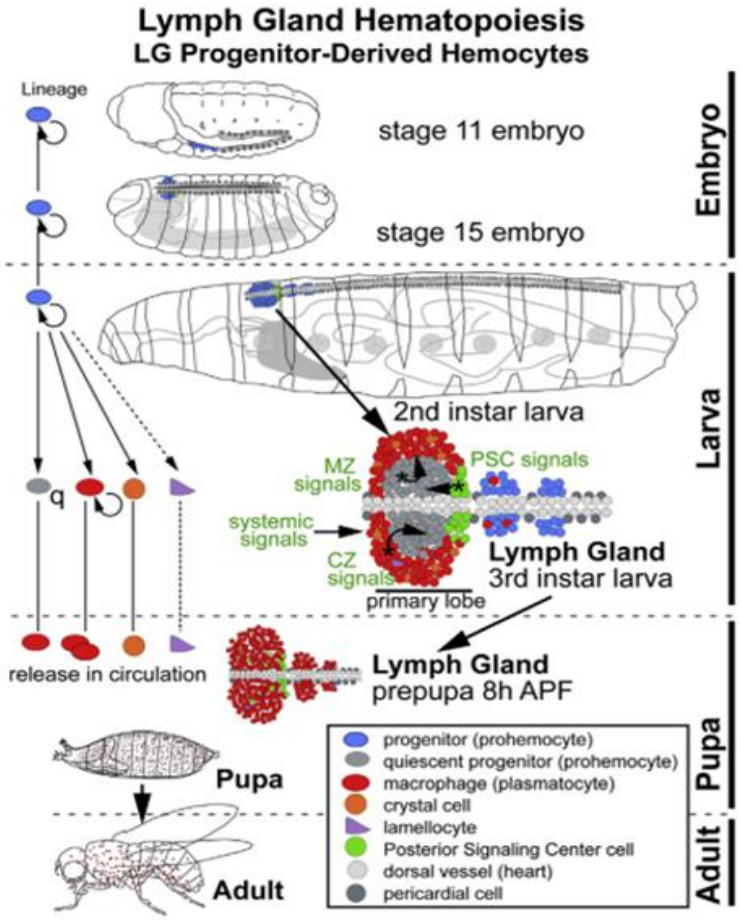


Figure 4: The hematopoietic system of Drosophila (Gold & Bruckner, 2014)

In human cancers, tumor interactions with the immune system have been extensively studied as it has been shown that these interactions with the microenvironment might account for tumor growth and metastasis (Mantovani et al 2017). In Drosophila, tumor cells activate pathways to trigger the systemic immune response leading to hemocyte proliferation and recruitment (Hauling et al., 2014). Studies have shown that, depending on the tumor model, the interaction between the Drosophila immune system and cancer cells, might promote or suppress tumor development. (Wang et al., 2014)

Studies for immune response in Drosophila have mainly focused on mechanisms for recognition of pathogens and strategies against attacks by bacteria, fungi, parasites, and viruses. One of the least well studied branches of pathogen recognition relies on TEP's. Thioester-containing proteins (TEPs) serve a major role in the host innate immune response of organisms by recognizing and promoting the elimination of the invading microbes. These proteins contain an unstable thioester bond between a Cys and a Gln residue in a conserved 4-amino-acid sequence (CGEQ) that allows covalent bond formation with microbial surfaces and promotes opsonization. TEPs emerged

early in evolution and are present in a wide variety of organisms. They have been found to participate in the mosquito immune response to certain bacteria and protozoa parasites. In vertebrates, certain TEPs act in the innate immune response by promoting recruitment of immune cells, phagocytosis, and direct lysis of microbial invaders. In Drosophila there are 6 Tep genes (Tep1-Tep6). Specifically, the Tep4 gene is expressed in larval stages as well as in adult flies. Upon bacterial challenge, Tep1, Tep2, and Tep4 are upregulated in larvae, whereas only Tep1, Tep2, Tep4, and Tep6 are upregulated in adults in response to certain bacterial, fungal, or parasitoid infection. (Shokal & Eleftherianos, 2016, Shokal & Eleftherianos, 2017).

1.5 Purpose of this study

Transcriptomic data from previous RNA-seq experiments performed in our lab have shown that after allografting larval brain tumors to adult flies, various genes get up- or down- regulated between allograft stages T0 to T3. More specifically, wild type (wt) adult host flies were injected with Notch- overexpressing brain tumors (T0 transplantation stage) and then three serial re-transplantations were performed till the T3 transplantation stage (Fig.5). An RNA-seq experiment was performed and transcriptomes of primary tumors (FACS-sorted from the larval CNS) were compared to the first and last allograft stage (T0 and T3). This experiment revealed several genes that get up- or down- regulated during the tumor progression.

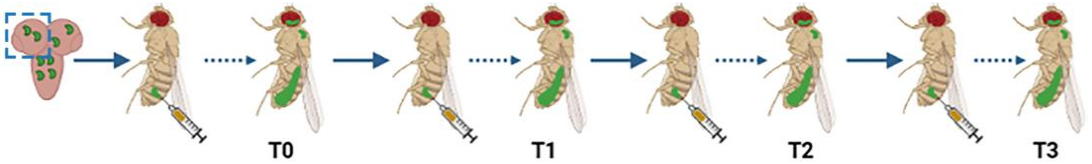


Figure 5: Serial transplantation of Notch tumor (T0-T3 stages) (Voutyraki et al., under review)

One of the genes that was observed to be upregulated in the T0 and T3 stage was *branchless* (Fig.6).We did not observe the other two FGF homologs, *pyramus* and *thisbe*, to be upregulated. It has been shown that the trachea system plays a serious role in tumor growth and metastasis (Grifoni et al., 2015, Tamamouna et al.2021). We therefore attempted to clarify the role of the tracheal system under tumorigenic allograft conditions in adult Drosophila. More specifically, we focused on FGF/FGFR signaling homologs, branchless and breathless, and their contribution in the interplay of tumor and trachea.

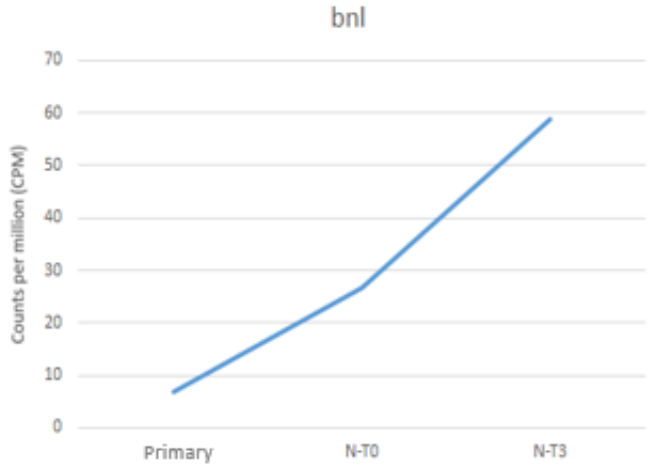


Figure 6: Branchless (bnl) is upregulated upon allografting.

The Thioester-containing protein 4 (Tep4) was also upregulated between the primary tumor and the allografts T0 and T3 (Fig.7). In fact, from our data it seems that Tep4 is possibly not expressed in the tumor cells due to really low reads in the primary tumor (FACS). We speculated that the upregulation we observe derives from the attached hemocytes (which are known to express Tep4 upon bacterial challenge) or other host cells (trachea, fat body) that may still adhere on the tumor material we analyzed. For that reason, our first attempt was to define the Tep4 expression pattern in different developmental stages and tissues as well as upon tumorigenic conditions.

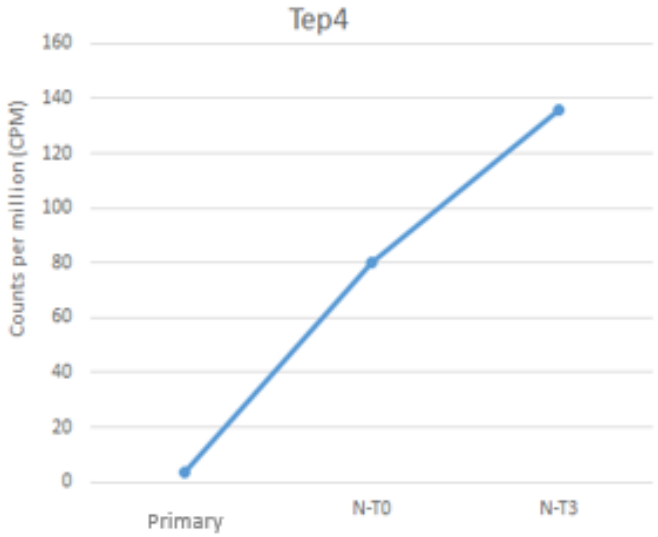


Figure 7: Thioester-containing protein 4 (Tep4) is upregulated from T0 to T3 allograft stage

2. MATERIALS & METHODS

2.1. Genetic methods and fly stocks

2.1.1 Genetics

Transgenic techniques in Drosophila melanogaster have been developed giving the opportunity to control the spatial and temporal gene expression in the living organism.

One method of achieving spatial control gene expression is the use of specifically designed enhancer sequences to drive the expression of a gene of interest. One of the most commonly used gene overexpression systems in Drosophila is the GAL4/UAS system. This system consists of two parts: the Gal4 gene, encoding the yeast (Saccharomyces cerevisiae) transcription activator protein Gal4 , and the UAS (Upstream Activation Sequence), an enhancer to which GAL4 specifically binds and activates gene transcription. The target transgene is cloned downstream of a UAS sequence and is then expressed in the same tissue-specific pattern as the GAL4 activator. Thus, a Drosophila transgenic line expressing GAL4 in a given spatiotemporal pattern can be crossed to any UAS-target line and the gene of interest will be expressed in that same pattern in the fly progeny (McGuire et al., 2004) (Fig.8).

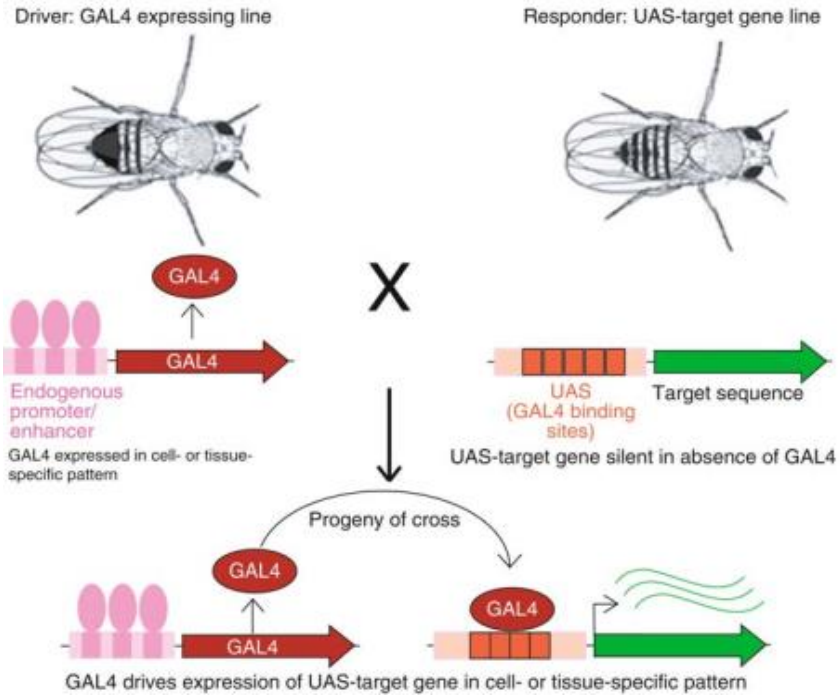


Figure 8: The GAL4/UAS system (Elliot & Brand, 2018)

A system that allowed researchers to take advantage of transgenic GAL4 and UAS lines, while adding the important feature of the temporal control of target gene expression was developed on the basis of a temperature sensitive GAL80 protein. In

yeast, the transcriptional activity of GAL4 is suppressed by the GAL80 repressor in the absence of galactose. In the presence of galactose, this suppression is absent, allowing GAL4 to activate the genes required for its metabolism. Based on that, a temperature-sensitive variant of GAL80 (GAL80ts) was selected and used in Drosophila. When flies grow at 18°C Gal80ts suppresses Gal4 activity, while at permissive temperatures (29°C or above) Gal80ts does not bind to GAL4 allowing the UAS driven gene expression (Fig.9). This system is called the TARGET system (Rodríguez et al., 2012).

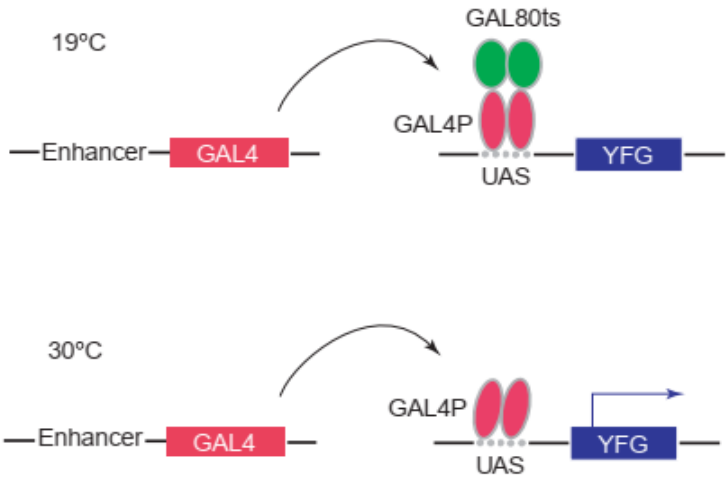


Figure 9: The TARGET system (McGuire et al., 2004). YFG = your favourite gene

Another tool for the spatial and temporal regulation of transgene expression relies on the use of the yeast site-specific recombinase, flipase (Flp) and its recognition target sequence (FRT). In this approach, FLP recombinase is cloned downstream of the heat shock promoter and allows temporal control of FLP induced recombination. More specifically, a transcription-termination (polyadenylation) cassette is inserted between a specific promoter and GAL4 or between the UAS sequence and the gene of interest. The inserted transcription-termination cassette is removed and allows the transgene to be expressed in the tissue of interest. “Flp-out”-out can be combined with the GAL4-UAS system. Transgene expression is induced in a random mosaic fashion upon the induction of FLP expression from heat shock (Rodriguez et al., 2012) (Fig.10).

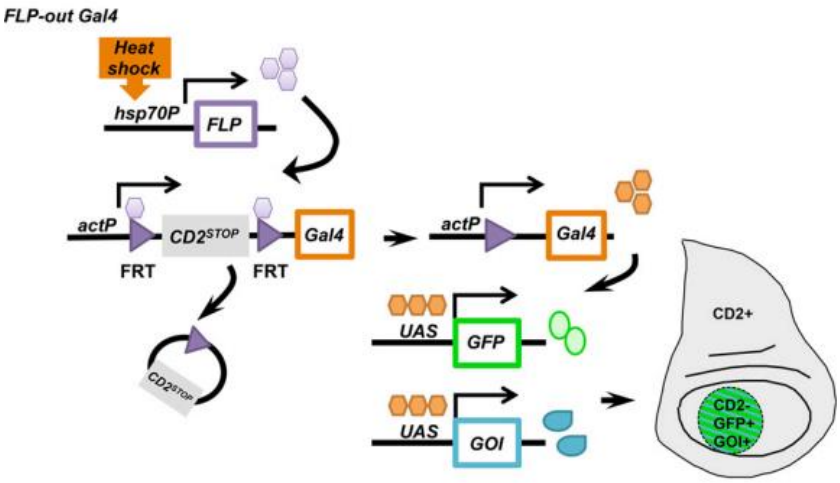


Figure 10: The FLP-OUT system (Germani et al., 2018) GOI = gene of interest

On the other hand, the GAL4-UAS system can be combined with the RNA interference (RNAi) method for specific gene knock down. RNAi is an endogenous cellular mechanism which was first discovered in Caenorhabditis elegans and plants. It is triggered by double-stranded RNA (dsRNA), which leads to the degradation of homologous RNAs and results in gene silencing. Exploiting that mechanism, Drosophila lines have been generated to express a unique transgene encoding the dsRNA hairpin with complementarity to endogenous genes, thus leading to their silencing. The hairpin RNA expression can be under control of the Gal4-UAS system leading to tissue-specific gene silencing. Thus, a fly line expressing Gal4 in specific temporal or spatial patterns can be crossed with UAS–RNAi transgenes resulting in progeny with tissue specific gene knockdown (Heigwer et al., 2018).

2.1.3 Fly Stocks

For this particular experimental procedure Drosophila fly stocks are obtained from the Bloomington Drosophila Stock Center (BDSC) and maintained at 18◦C or 25◦C on standard fly food.

For Flip-out clones, male flies with genotype: hs-FLP; act-FRT>STOP>FRT-Gal4, UAS-GFP, were crossed with females carrying the gene of interest and the appropriate UAS combinations. The crosses were maintained at 25◦C, were transferred every 2 days and the 3rd day after egg laying underwent a 45 minutes heat shock at 37◦C. Phenotypes were analyzed in late 3rd instar larvae 3 days later.

Using the TARGET system, crosses were maintained at 18 ◦C for 9 days and moved to 29◦C 2days before brain dissection. For the btl knockdown, crosses were maintained at 18 ◦C and adult flies moved to 29◦C right after their eclosion. Flies were transferred every 4 days.

Fly strains used:

Flip out system:	hsFLP; act-FRT>STOP>FRT-Gal4,UAS-GFP
TARGET system:	tubGal80ts,UAS-stingerRFP/Cyotb; grhGal4/TM6B
Trachea-tumor interaction experiments:	w; btl-Gal4,UAS-GFP,tub-Gal80ts /CyO
	yw;UAS-LacZ
	UAS-BtlDN (III)
	UAS-NΔecd/ Cyotb; UAS-*white*Ri
	UAS-NΔecd/Cyotb; bnl-LacZ/TM6B
	bnl-LacZ/TM3
	UAS-NΔecd/Cyotb
	UAS-bnl-Ri/TM6B
Tep4 experiments:	yw;UAS-mCD8GFP
	yw; tep4Gal4/SM6A
	y,ver,sc/ UAS-tep4-Ri
Hemocyte experiments:	Cyo/sp; srp(hemo)moesin 3xmCherrry / TM3ser

Balancer chromosomes with dominant markers in fly strains (such as CyO, TM6B, TM3) enable us to follow our transgenes and prevent the recovery of crossovers.

2.2 Immunohistochemistry

2.2.1 Immunofluorescence

Fluorescence immunohistochemistry was performed to determine gene expression in tissues.

Primary antibodies used in this study were: guinea pig anti-Deadpan 1:1000 (Magadi et al., 2020), mouse anti-Prospero 1:100 (DSHB), mouse anti-B-gal 1:20 (DSHB), mouse anti-GFP 1:10 (DSHB), mouse anti-NimC1 1:30 (obtained from I.Ando, Hungary), rat anti-Elav 1:300 (DSHB), rabbit anti-GFP 1:10000 (Minotech), rabbit anti-PH3 1:1000 (Abcam) and also DAPI and Hoechst (1:100) (staining of the nuclei).

Secondary antibodies used: mouse Alexa 488 (1:2000), rabbit Alexa 488 (1:2000), mouse Alexa 555 (1:2000), guinea pig Alexa 647 (1:2000), mouse Alexa 633 (1:1000) and rat Cy3Fab (1:4000).

Samples were imaged on a Leica TCS SP8 confocal microscope (FORTH-IMBB).

2.2.2 Larval Brain Immunohistochemistry

For brain staining procedure inverted larvae were fixed for 20 minutes in 4% formaldehyde in 1XPBS (4% FA solution). After 3 rinses with 1XPBS, the samples incubated in blocking PBT solution (1x PBS, 0.5% BSA, 0.2% Triton X-100) at room temperature for 1-3 hours. Primary antibody diluted in PBT solution and overnight incubation at 4◦C was performed. Samples washed 3 times in PT solution (PBS supplemented with 0.2% Triton X-100) for 10 minutes/wash. Secondary antibody incubation was performed the same way as described for the first. Samples washed in PT solution and the brains transferred onto a glass slide in 25-30µl of NPG medium (80% glycerol in PBS with 0.5% *N*-propyl-gallate), covered with 18X18mm coverslip and sealed with nail polish.

2.2.3 Allograft Tumor Immunohistochemistry

Initially, the abdominal area was separated from the thorax of 2-3 adult flies for each sample. After an incision was made in the skin cuticle of the abdomen, the sample placed in a well of a 24-well plate and fixed for 30 minutes in 4% FA solution. Samples rinsed 3X with 1XPBS and incubated in blocking PBT solution (1x PBS, 0.5% BSA, 0.2% Triton X-100) at 4◦C overnight. The abdomen transferred into a well of 96-well plate and additional overnight incubation at 4◦C in primary antibody diluted in PBT was performed. Three 10-minute washes in PT solution followed the next day and secondary antibody incubation was performed the same way as the first. Samples washed 3X in PT solution and were transferred onto a glass slide in NPG medium. There the abdominal tissue was dissected in smaller parts, covered with a coverslip and sealed with nail polish.

2.2.4 Larval & Adult Hemocyte Immunohistochemistry

For larval hemocyte staining, the skin of the larvae was torn in sterile PBS solution, so that hemocytes could be isolated. For adult hemocyte staining, an incision was made in the skin cuticle of the abdomen in adult flies and sterile PBS injected in the thorax to perfuse through the small cut. After hemocytes from 10 adult flies or larvae were isolated using sterile PBS, they transferred in poly-lysine treated round coverslips in a well of a 24-well plate and fixed for 10 minutes in 4% FA solution. After 3 rinses with 1XPBS, the samples were incubated in PBT at room temperature for at least 20 minutes. The plate was sealed with parafilm and overnight incubation at 4◦C in primary antibody diluted in PBT solution was performed. Three rinses with PT solution followed the next day and secondary antibody incubation performed the same way as the first. Samples were washed in PT solution and the coverslip was transferred onto a glass slide with 10µl NPG medium and sealed with nail polish.

2.2.5 Cryosections

For each sample, proboscis and wings of 2-3 flies were removed. The flies were fixed for 2, 5 hours in 4% FA solution. After 3 washes with PBS, the samples were incubated overnight in 25% sucrose solution (diluted in H2O) at 4◦C. Next day, after the flies were dried out, they were embedded into OCT in cryoboxes. Samples were instantly frozen on dry ice and stored at -80◦C until the section procedure.

For sectioning, Leica CM1850 Cryostat was equilibrated at -25◦C. The frozen sample was placed onto the cryostat chuck, trimmed for excessive OCT and sliced into 40-50

µm thick sections. Frozen sections were transferred on Superfrost Plus Microscope Slides (25x75x1.0 mm, Thermo Scientific) and placed at -20◦C overnight. Next day, the slices were fixed for 12 minutes with 200µl 4% FA solution. After 3 gentle rinses, the samples were mounted with DAPI-containing mounting medium (Drop-n-Stain EverBrite™ Mounting Medium, BIOTIUM, cat.23009), were covered with 24X50mm coverslips and sealed with nail polish.

2.3 Tumor Transplantations

Transplant injections were performed with the help of Chrysanthi Voutyraki using a nanoinjector (Nanoject II Auto-Nanoliter Injector, Drummond Scientific Company, 3-000-205A). Donor larval hyperplastic combinations were generated as described in the results section. They were sliced into single brain lobes and implanted into the abdomen of 3-5 days old female host flies, following the protocol for tissue allograft method (Rossi & Gonzalez, 2015b). Malignant GFP or RFP-positive tumour pieces (T0) were dissected out of the abdomen of host flies and were re-transplanted into new host flies (T1). After the injections, host flies were kept either in 25◦C or 29◦C incubators and treated according to survival assay (2.4 Survival & Viability assay).

When the tumor tissue had populated the entire body cavity of the host (strong fluorescent signal detected under a fluorescent microscope), the tumor was either dissected for immunohistochemistry or the fly was prepared for cryosectioning.

2.4 Survival & Viability assay

Injected flies were mainly kept at 29°C and were examined daily for viability. They were transferred every 2 days and injected hosts were kept in a horizontal position (Survival assay).

Fly strains of w; btl-Gal4,UAS-GFP,tub-Gal80ts/CyO were crossed with yw;UAS-LacZ (control line) or UAS-BtlDN and kept at 18°C (3.1 Trachea-tumor interactions) .The adult flies (F1 generation) collected within 1-2 days after eclosing at 18^0C and were transferred at 29^0C. They were examined daily for viability (Viability assay).

For the quantification of the above assays, GraphPad Prism was used for graphs and statistical analysis.

3. RESULTS

3.1 Trachea-tumor interactions

It is well known that the FGF Receptor homolog Breathless (Btl) affects the branching properties of the tracheal system (Lee et al., 1996). In this set of experiments, we wanted to validate that our genetic manipulations will effectively interfere with the branching capacity of the larval tracheal system. For this reason, we overexpressed a dominant negative form of Btl (UAS-btl^{DN}) using the trachea specific Gal4 driver btl-Gal4 combined with UAS-GFP and tubGal80ts for temporal control (btl-Gal4, UAS-GFP,tub-Gal80ts/CyO). Fly strains of w; btl-Gal4,UAS-GFP,tub-Gal80ts/CyO were crossed with yw;UAS-LacZ (control line) or UAS-Btl^{DN}. The crosses were treated as described in section 2.1.1. Staged L3 larvae were observed under a fluorescent stereoscope and as shown in Fig.11, UAS-btlDN larval tracheal branches are morphologically different compared to the control (UAS-LacZ). More specifically, btl knockdown leads to larvae with missing tracheal branches (as white arrow shows) while others have thinner branches (white *).

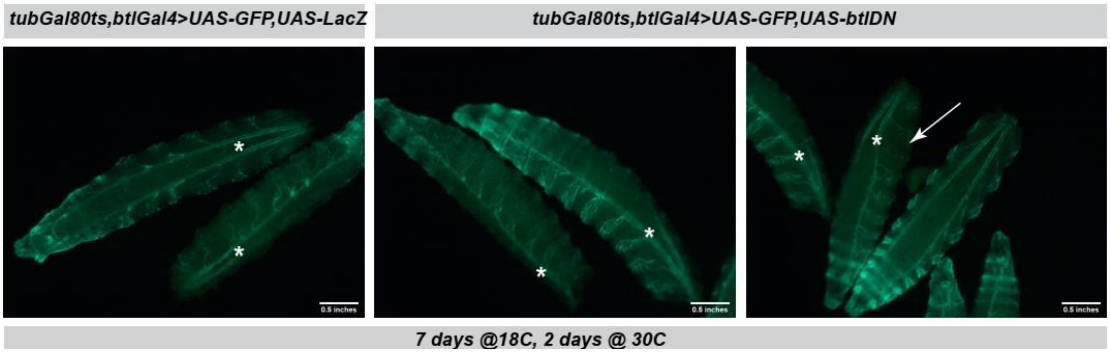


Figure 11: Btl knockdown affects larval branches: Mesoscopic observation under GFP stereoscope of control (left) and btl knocked-down (right) larvae.

To assess whether btl knockdown affects the viability of adult flies, a survival assay was performed. The adult flies collected within 1-2 days after eclosing at 18^{0}C and were transferred at 29^{0}C, where we daily observed their survival. The following graph indicates in the viability between control and >btl^{DN} flies in two replicates. A student t-test was performed showing difference between the two conditions in both replicates. First replicate shows that Btl^{DN} flies (mean: 41,90) die earlier comparing to control (mean: 44,84). Second replicate indicates that control flies die earlier (mean: 27,88) in contrast to btl depleted flies (mean: 35,34). The inconsistency of results lead us to repeat a replicate with a bigger number of flies (Fig.12).

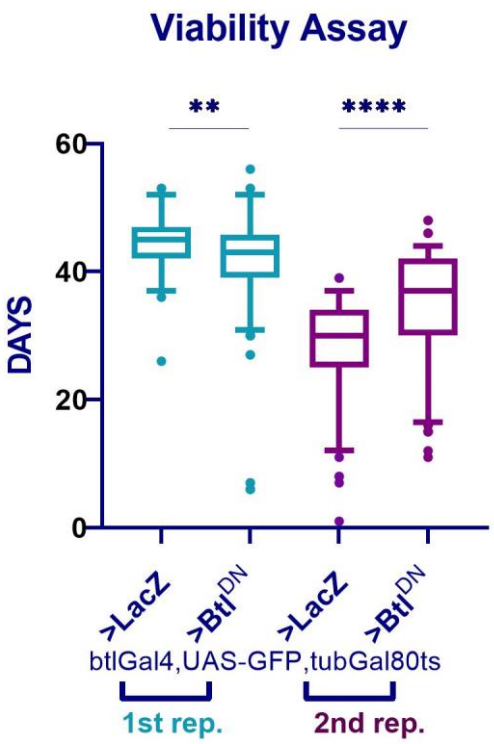


Figure 12: Viability assay between control (>LacZ) and >BtlDN: For Box plots: median values (middle bars) and first to third interquartile ranges (boxes); whiskers are 5% and 95%; dots indicate outliers. ** P < 0.01, ****P<0.0001 (unpaired t-test).

Studies have shown that trachea remodeling happens in response to tumorigenic conditions and branchless (FGF)-breathless (FGFR) pathway plays an important role in tumor properties (Tamamouna et al., 2021, Grifoni et al., 2015). Transcriptomic analysis of Notch tumors in our lab has shown that Branchless (Bnl) is upregulated at the T0 and T3 transplantation stage (described in 1.5). The following experiments were performed to examine the expression of branchless (Bnl) in hyperplastic lineages, in the larval brain we overexpressed Notch and simultaneously LacZ was expressed under the control of bnl regulatory elements (bnl-LacZ). Fly strains of UAS-NΔecd/Cyotb; bnl-LacZ/TM6B were crossed with hsFLP; act-FRT>STOP>FRT-Gal4, UAS-GFP (flipout) or tubGal80ts, UAS-stingerRFP/Cyotb; grhGal4/TM6B (TARGET system). As a control we used the fly line bnl-LacZ/TM3. Larval brains were dissected, fixed and stained with specific antibodies to determine the expression of bnl-lacZ on Notch overexpressing tumors. The result suggests that Branchless is mainly expressed superficially (glial expression) but not in deeper layers of the brain, both in Notch overexpressing (N-bnlLacz) and control (bnlLacZ) brains. Bnl expression is also rarely found in specific neuroblasts. Figures 13 show bnl expression in control and Notch hyperplastic brains in a superficial section. This is consistent with the low number of RNA reads in the transcriptome of primary tumours.

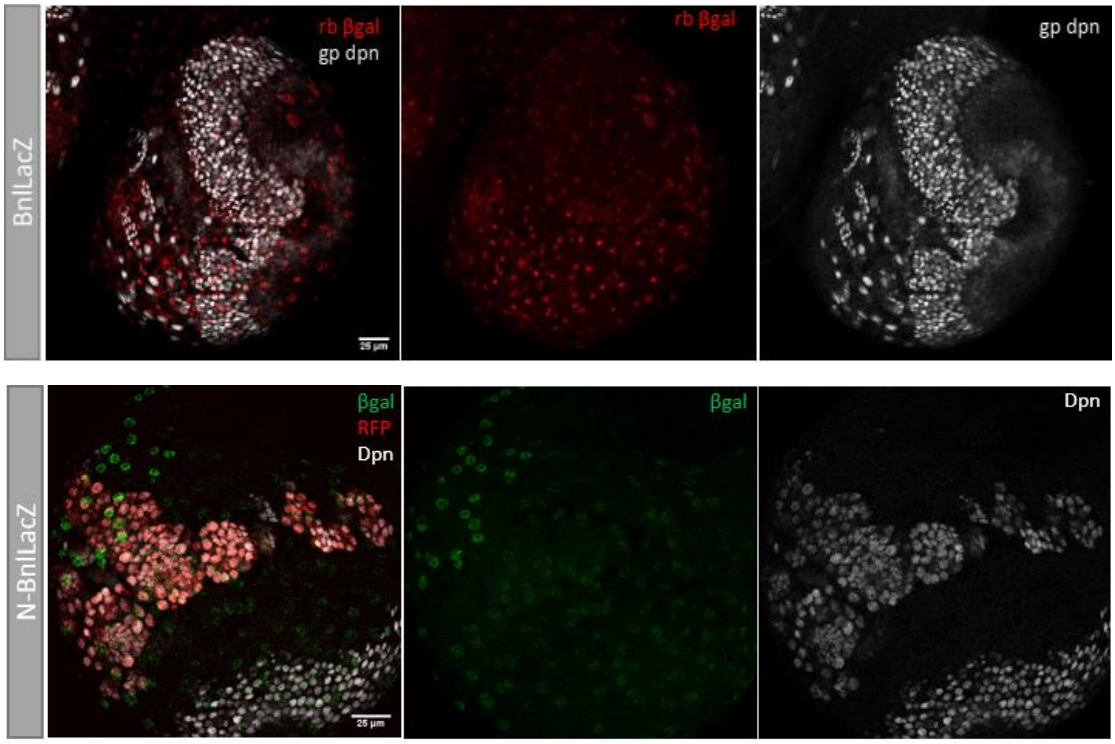


Figure 13: Branchless expression in control and Notch overexpressing brains: Staining in fixed larval brain samples, in bnlLacZ/TM3 and UAS-NΔecd/Cyotb; bnl-LacZ/TM6B combined with the TARGET system. Bgal (red or green) is marker for lacZ expression and Dpn (grey) is a neuroblast marker (Scale bar: 25µm).

In order to better characterize the impact of Bnl on tumors, we overexpressed Notch to induce hyperplasia, while at the same time expression of Bnl was knocked down via RNAi. For this set of experiments, we used the TARGET system as described in 2.1.1. As a control we combined UAS-NΔecd with a control UAS-*white*Ri line (UAS-NΔecd/ Cyotb; UAS-wRi). Larval brains were dissected, fixed and stained with specific antibodies to determine the effect of branchless on Notch overexpressing tumors. We observed that qualitatively there is no significant difference between control (N-wRi) and Bnl knockdown (N-bnlRi) tumorigenic brains (Fig.14). We should further quantify the differences on the clone size between the two conditions in future experiments.

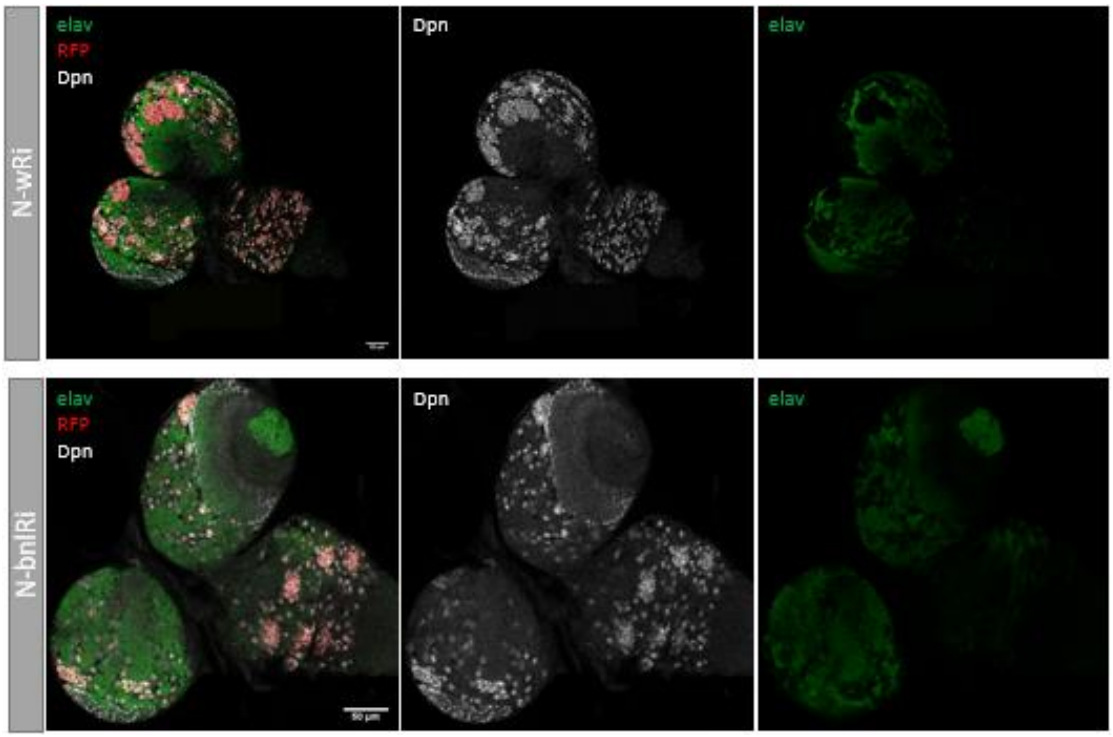


Figure 14: Branchless knockdown in Notch overexpressing tumors (N-bnlRi) and control (N-wRi): Staining in fixed larval brain samples, in UAS-NΔecd; UAS-bnlRi or UAS-wRi (control) combined with the TARGET system. Elav is an antibody for neurons and Dpn (grey) is a neuroblast marker (Scale bar: 50μm).

Moreover, we desired to examine the way Drosophila strains with limited tracheal branching respond under tumorigenic conditions. For this purpose, we generated fly hosts expressing a dominant negative form of Btl in the trachea (UAS-btlDN; btlGal4,UAS-GFP,tubGal80ts) and control flies (yw; UAS-LacZ; btlGal4,UAS-GFP,tubGal80ts) as described before. The flies were injected at 3-5 days after eclosion at 29^0C. For the injections, we used control Notch-overexpressing hyperplastic lobes combined with either a control line (UAS-NΔecd; UAS-wRi), or a transgene for bnl knockdown (UAS-NΔecd; UAS-bnl-Ri) and the TARGET system (T0 transplantations). After the tumor grew for several days, it was dissected out and re-transplanted into new hosts (allograft T1). Survival and RFP detection assays were performed alongside abdomen dissections in order to understand the tumor properties and the tracheal response. The survival assay did not indicate a significant difference between the different hosts or the tumor background (Fig. 15).

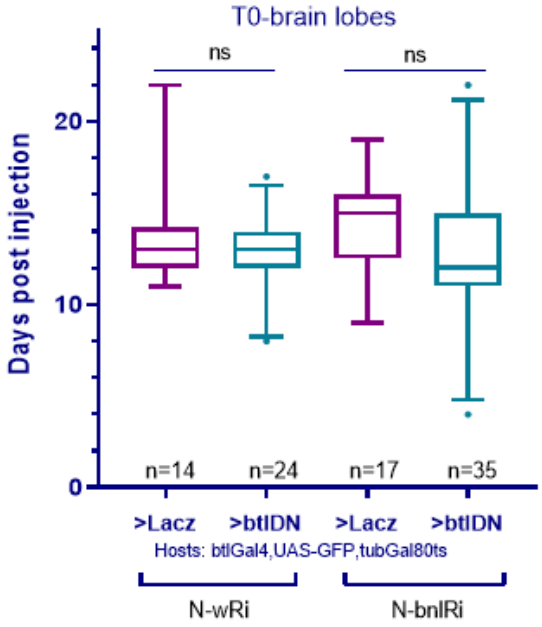


Figure 15: Survival assay in Btl tracheal knock-down (btlDN) and control (LacZ) hosts injected in T0 stage with N-wRi and N-bnlRi lobes. There is no significant difference between the different conditions. (ns=not statistically significant change.)

In T1 transplantation stage we also performed a survival assay which agrees with the T0 transplantation, where no significant differences between the genotypes were found. We also performed injections with sterile PBS as control to clarify whether the survival of the flies is affected by the injury of the injections per se (Fig. 16). BtlDN did not affect the flies' lifespan after injury.

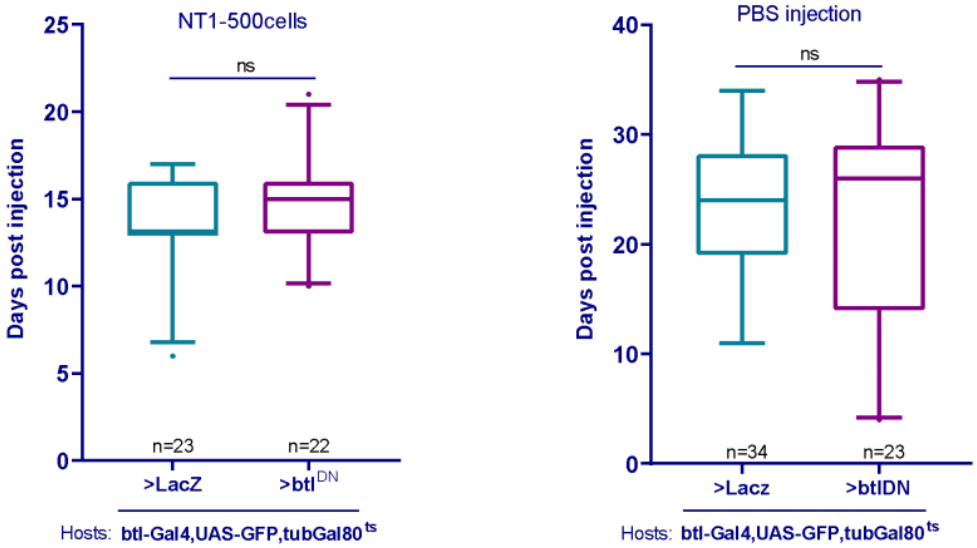


Figure 16: Survival assay in Btl tracheal knock-down (btlDN) and control (LacZ) hosts injected in T1 stage with 500cells of control tumor (left graph) and with PBS as a control (right graph). (ns=not statistically significant change.)

The above graphs show survival assays performed in triplicates of hosts injected in T0 and T1 allograft stage alongside to PBS injection for control. Collectively, we conclude that under this tumorigenic condition the flies die faster (Fig.12, 16 and 17) but the Bnl/Btl signaling does not seem to have an impact on tumor progression.

In addition, we desired to determine the division rates of tumor cells in proximity to trachea. Thus, abdomen immunohistochemistry of our injected hosts followed. Our samples stained with the mitotic marker Ph3 which is a specific biomarker that stains cells in late G2 and mitosis (pH3 stains the condensed chromatin just before chromosomal segregation). Tumour explant immunohistochemistry did not give us clear results to understand better interactions between trachea and tumor (Fig.17, 18). For that reason, we decided to perform cryosection assay in hosts 6-7 days after injection when the RFP signal of the tumor was sufficient for such an analysis.

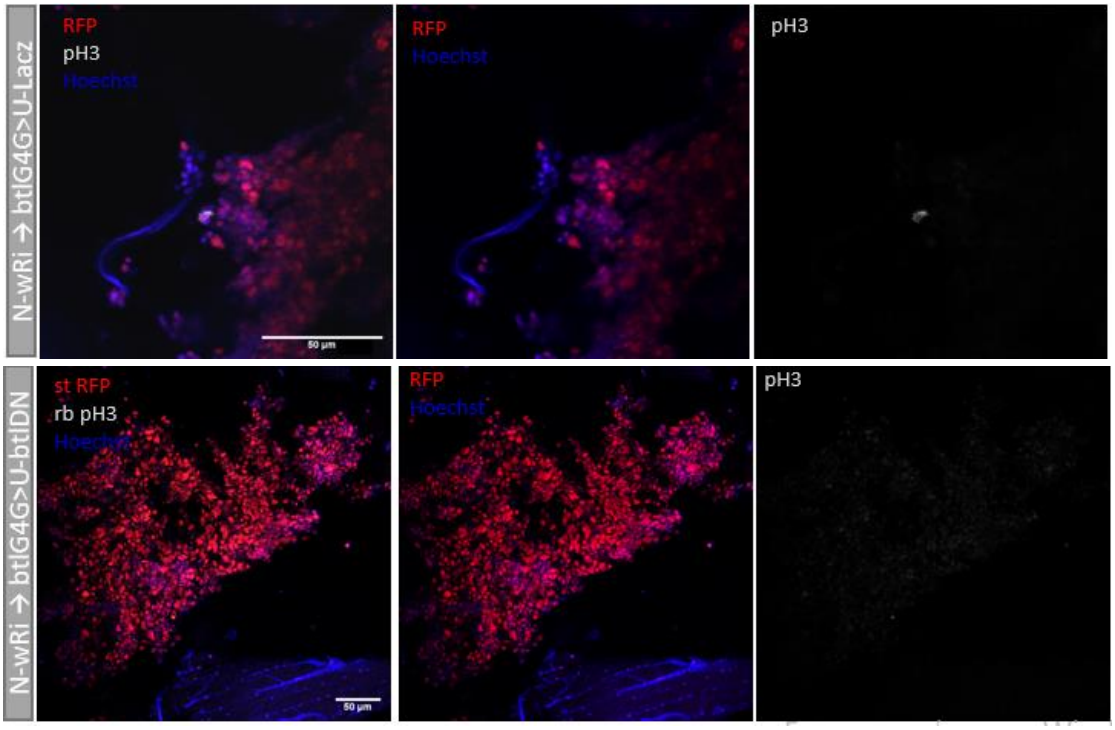


Figure 17: Abdomen immunohistochemistry of hosts injected with control: The abdomen stained with pH3 (gray) and Hoechst (blue). RFP (red) is endogenous RFP expressed in tumor cells (Scale bar: 50μm).

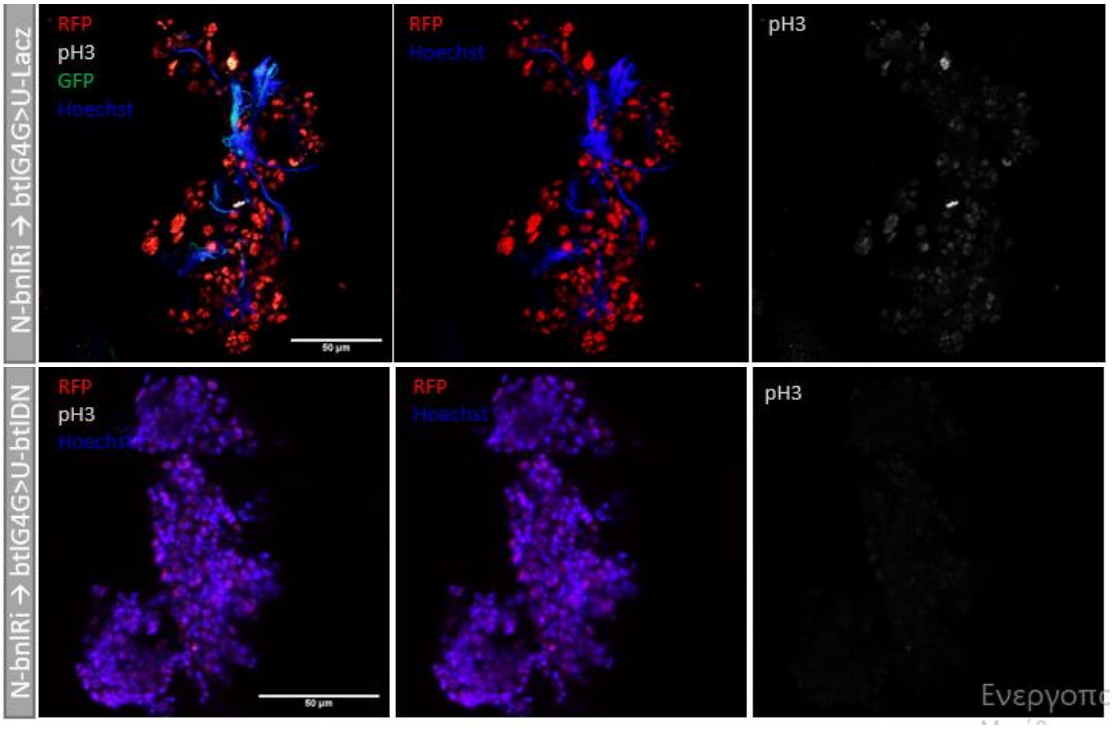
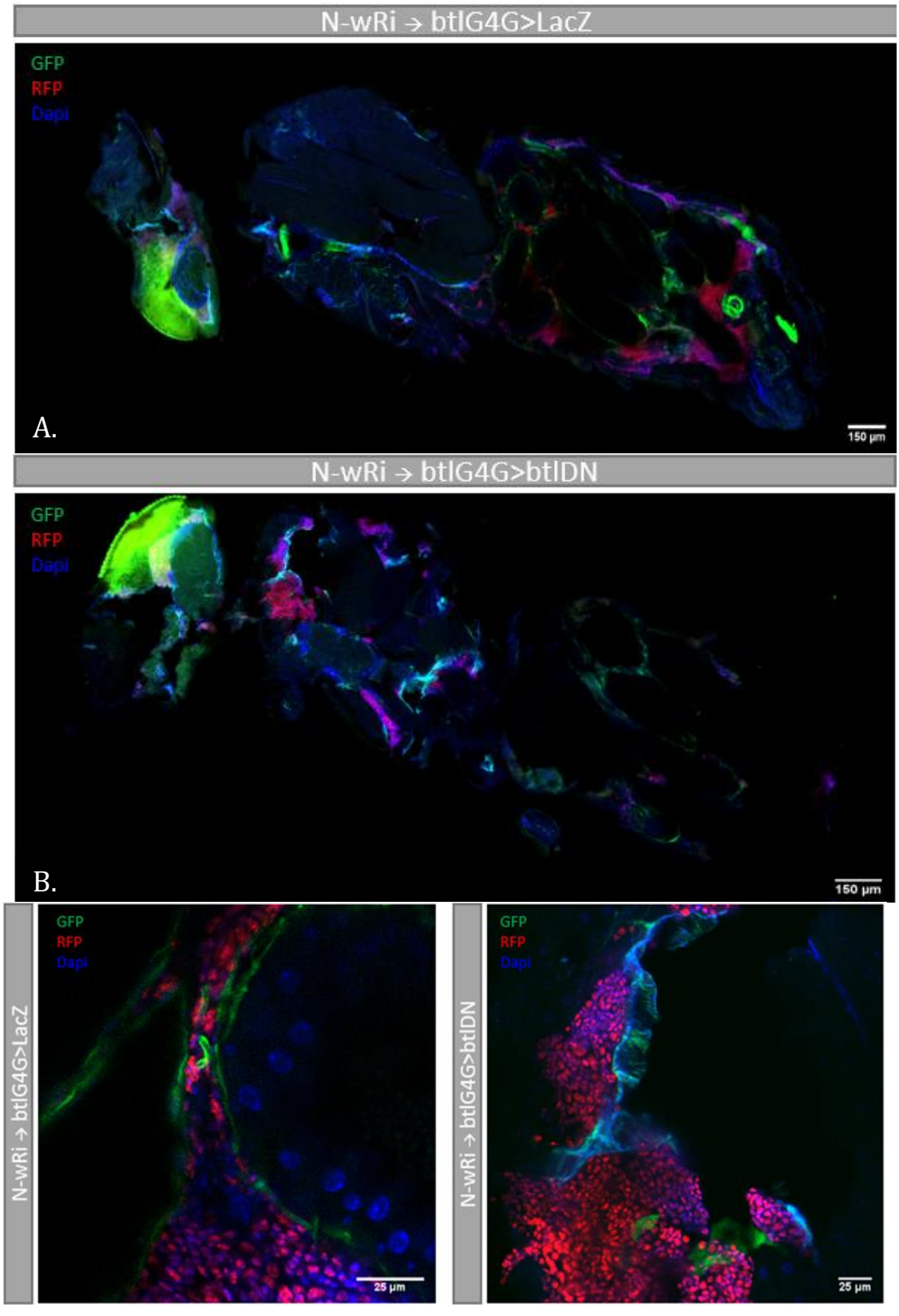


Figure 18: Abdomen immunohistochemistry of hosts injected with Bnl depleted tumors: The abdomen fixed, dissected and stained with pH3 (gray) and Hoechst (blue). RFP (red) is endogenous RFP expressed in tumor cells and endogenous GFP (green) expressed in trachea was difficult to detect without GFP antibody in almost all our samples (Scale bar: 50μm).

We performed cryosections to our fly hosts with RFP labelled tumor and GFP lebelled trachea in order to define the interplay between tracheal structures and tumor in the different Bnl/Btl manipulations. Our cryosection scans do not indicate any difference along tracheal structures and their interactions with tumors, although the Bnl/Btl knock down in both tumor and host (Fig.19).

Given that no significant differences were observed in the different conditions with all three experimental approaches (viability assays, explant immunohistochemistry, and cryosections), we conclude that FGF/FGFR signaling does not affect the interactions between the trachea and the tumor. However, this cannot be fully stated as need to examine more fly hosts for the aforementioned experiments.


N-wRi → btlG4G>LacZ
GFP
RFP
Dapi
A.
150 µm
N-wRi → btlG4G>btlDN
GFP
RFP
Dapi
B.
150 µm
N-wRi → btlG4G>LacZ
GFP
RFP
Dapi
25 µm
N-wRi → btlG4G>btlDN
GFP
RFP
Dapi
25 µm

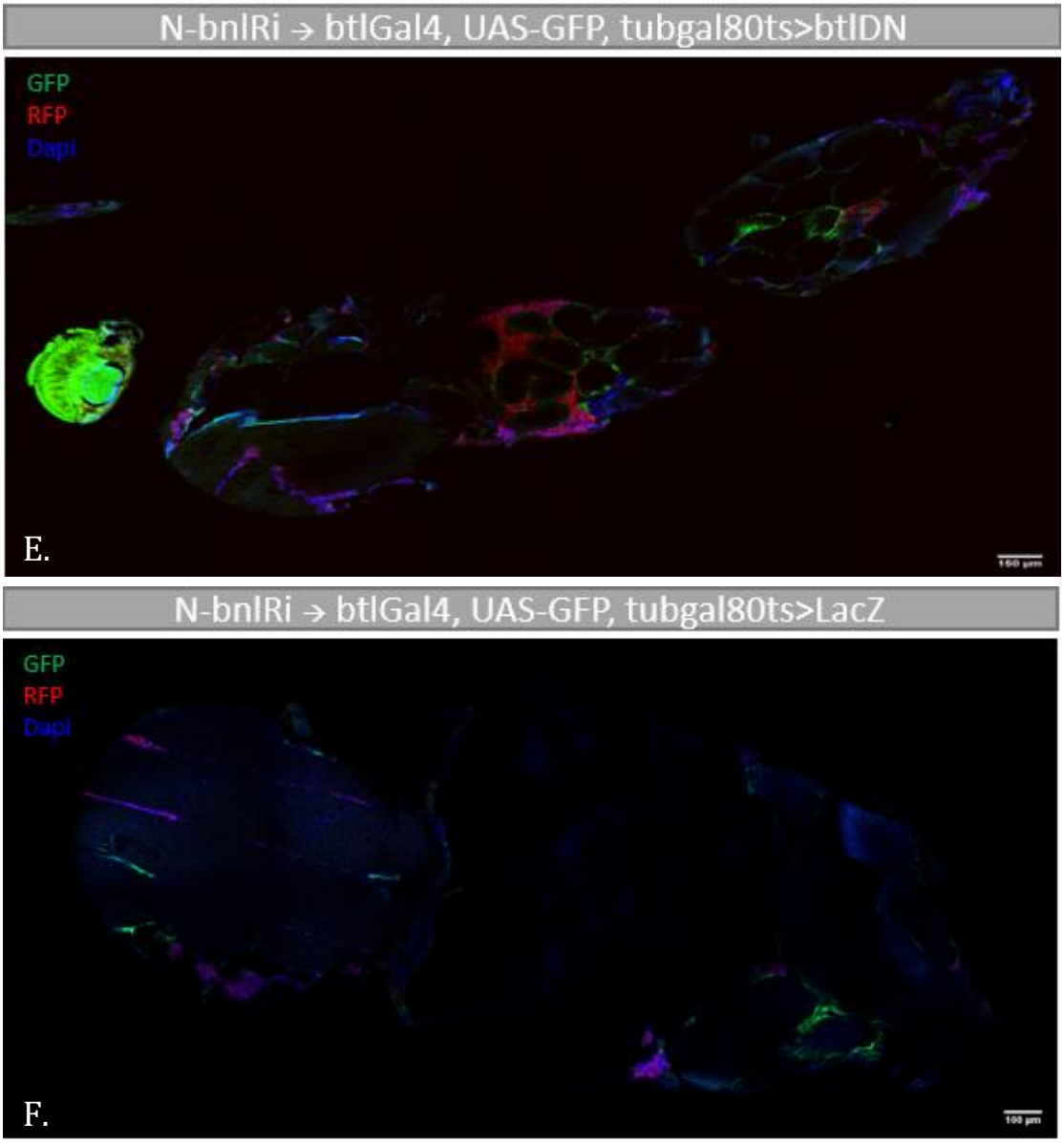


Figure 19: Cryosections of injected flies. (A, B) Red tumors of control in LacZ and Btl^{DN} hosts (Scale bar: 150µm) and close ups (C, D) showing that tumor cells embrace tracheal structures (Scale bar: 25µm). (E, F) Red Bnl depleted tumors in LacZ and Btl^{DN} hosts (Scale bar: 150µm, 100µm). GFP (green) is endogenous GFP expressed in tracheal cells. It is observed that Btl knockdown does not grossly alter trachea network shape in the adult. Moreover, N-BnlRi tumours interact with the tracheal structures despite the Bnl/Btl pathway silencing.

3.2 Tep4 expression pattern

In order to investigate the expression pattern of Tep4, we combined tep4-Gal4 with UAS-GFP by crossing yw; UAS-mCD8GFP with yw; tep4Gal4/SM6A. We bled L3 larvae and performed larval hemocyte staining with a hemocyte specific antibody, NimC1. We conclude that tep4 is expressed in many but not all larval hemocytes (Fig. 20)

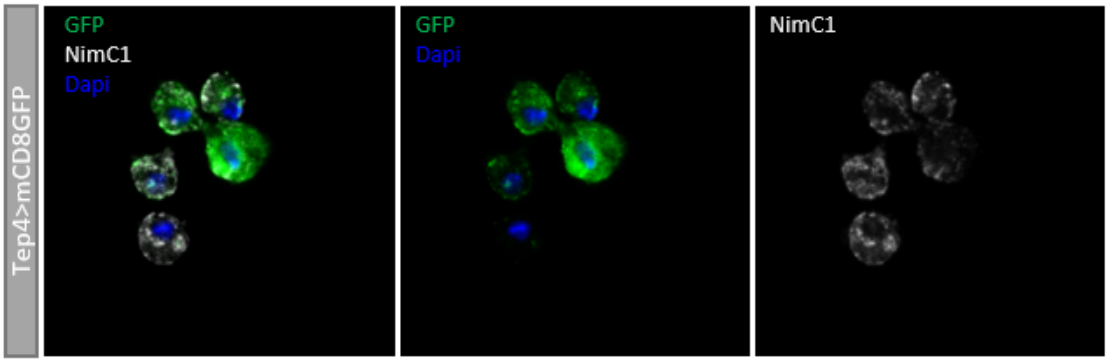


Figure 20: Tep4 is expressed in larval hemocytes: Staining in fixed larval hemocyte samples. GFP (green) is endogenous GFP overexpressed with Tep4-Gal4, NimC1 (gray) is a hemocyte marker and DAPI (blue) is for nuclear staining.

In addition, we generated a stock tep4-Gal4/Cyotb, UAS-stingerGFP/TM6B to investigate its expression in larval tissues. Data from immunohistochemistry on larval hemocytes (Fig. 20), larval brains (Fig. 21) and mesoscopic observation under GFP stereoscope (Fig.22) led us to the conclusion that tep4 is expressed on epidermal cells, fat body, midline glia, and hemocytes.

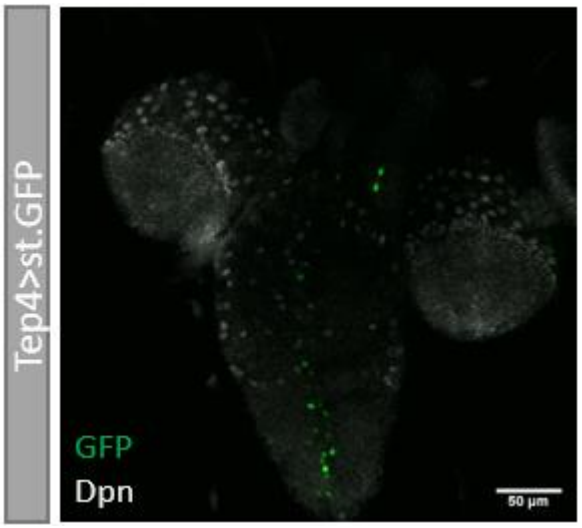


Figure 21: Tep4 expression in midline glia: Staining in fixed larval brain samples, in Tep4GAL4/UAS-st.GFP. GFP (green) marks Tep4 expression and Dpn (grey) is NB marker (Scale bar: 50µm.)

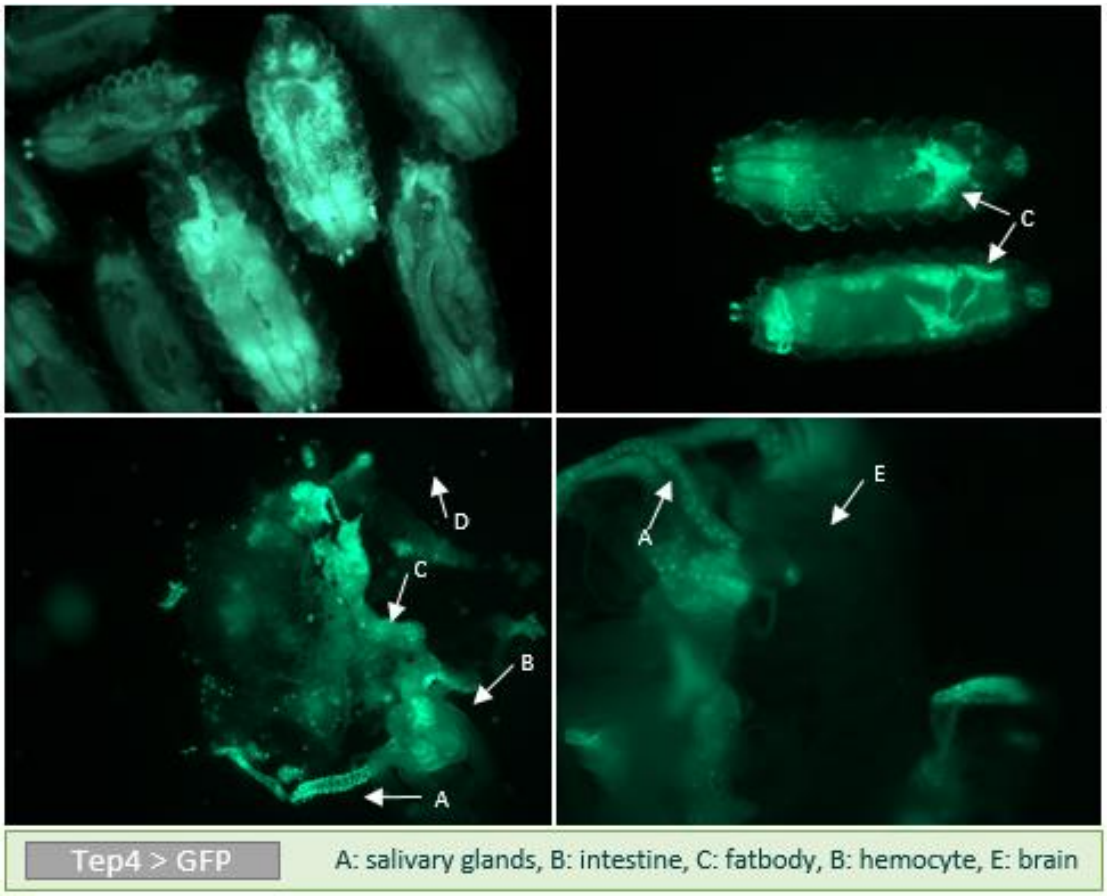


Figure 22: Mesoscopic observation of larvae expressing GFP driven by Tep4. It is indicated that tep4 is expresses in salivary glands (A), intestine (B) and fatbody (C), haemocytes (D), but not in the CNS at this level of resolution (E)

The fly stock tep4-Gal4/Cyotb, UAS-stingerGFP/TM6B was then crossed with a strain that labels all fly hemocytes (Cyo/sp; srp(hemo)moesin 3xmCherrry/TM3ser) to simultaneously label all Tep4 expressing cells and all hemocytes. We performed cryosections in adult flies on different days (Day 6 and Day15) to assess whether tep4 expression changes in adult life. However, the same as larval expression pattern is followed in the adult fly with tep4 expressed mainly in fatbody, some hemocytes and intestine (Fig. 23).

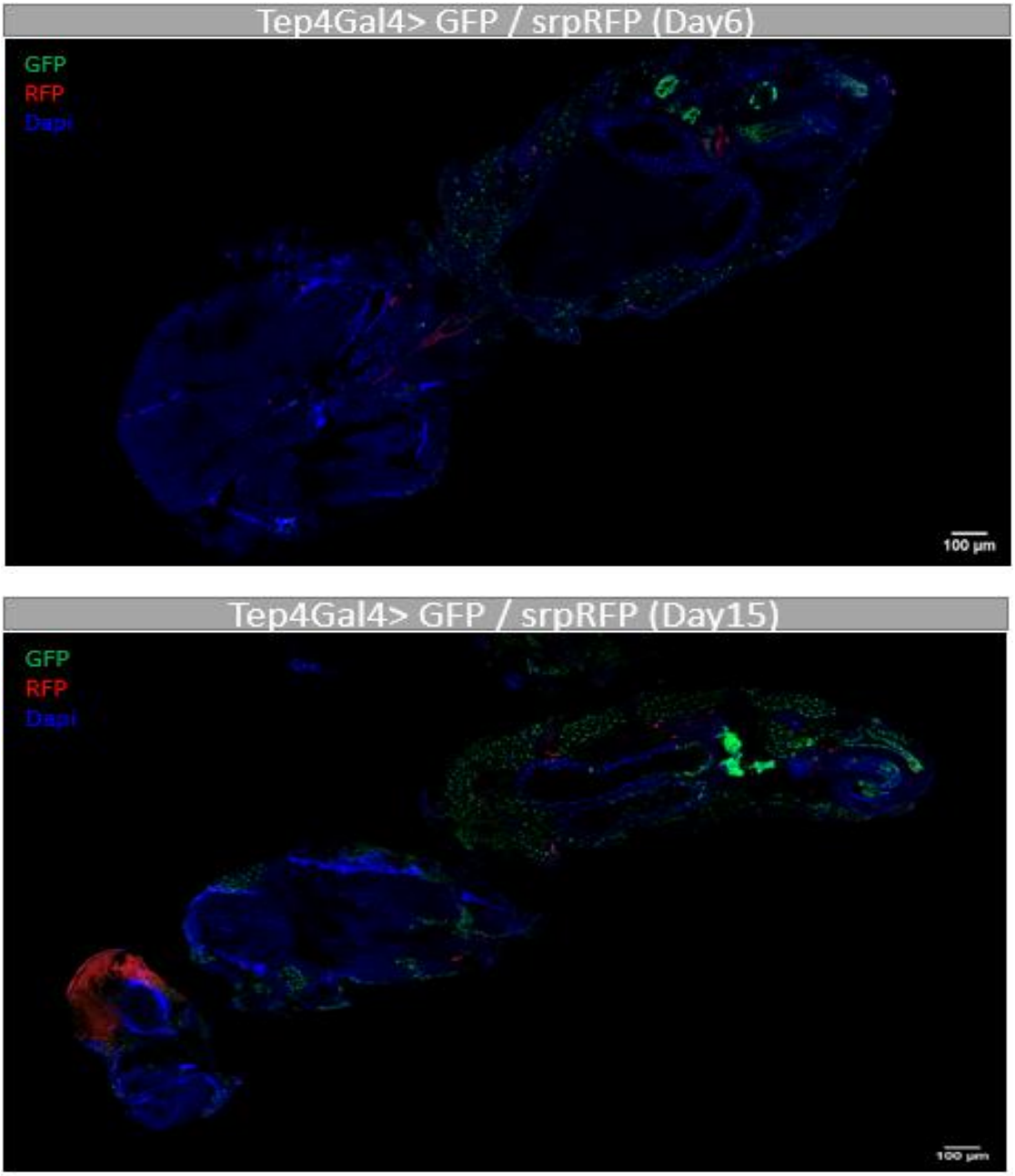


Figure 23: Tep4 expression in Day 6 and Day 15 adult fly: GFP (green) marks tep4 expression, RFP (red) is expresses in hemocytes and Dapi (blue) is for nuclear staining along with tracheal network autofluorescence. Both cryosections indicate that tep4 is mainly expressed in fat body, intestine and hemocytes (Scale bar: 100µm).

Furthermore, to investigate the induction of Tep4 in tumor bearing hosts, we performed allograft experiments. Using tep4Gal4/ Cyotb, UAS-stingerGFP/TM6B as fly hosts, we performed N-T1 transplantation. Cryosections were performed at 7 days post injection in order to determine the Tep4 expression pattern. Figure 25 indicate that tep4 expression shows a difference in comparison to not injected adults. Interestingly we found that tep4 is induced in tracheal cells as the GFP signal is co-localized with the trachea auto fluorescence (Fig.24).

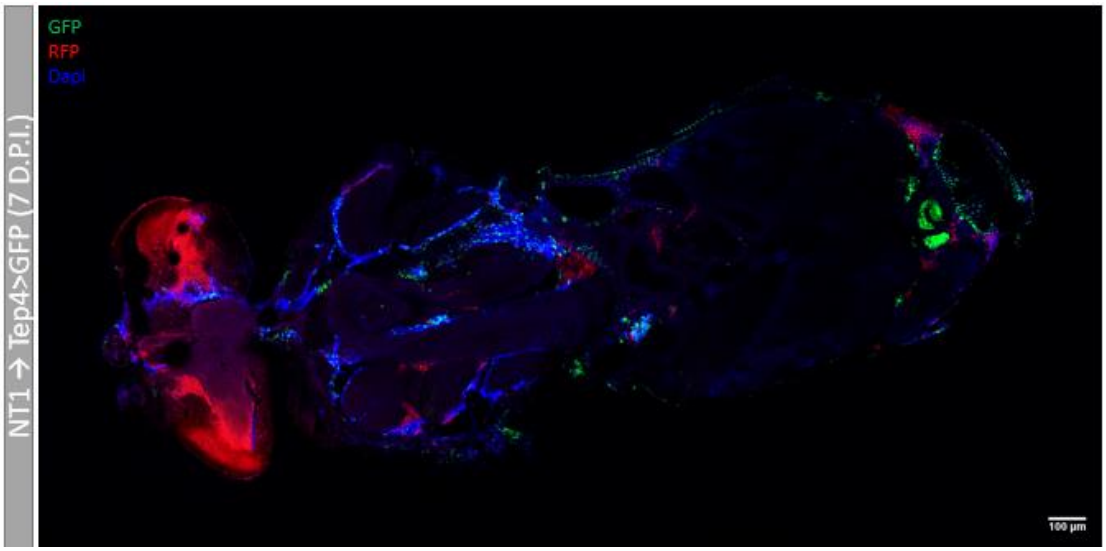


Fig.24: Tep4 expression changes upon tumorigenic condition: Cryosection of adult host expressing GFP (green) with tep4 driver injected with red tumor (RFP) in T1 transplantation stage (7 days post injection). Dapi (blue) is for nuclear staining along with tracheal network autofluorescence. (Scale bar: 100µm). Tep4 seems to be expressed in tracheal structures which was not observed in control cryosections in fig.23.

It is obvious that Tep4 expression pattern changes upon tumorigenic condition which combined to the role of Tep4 in the immune system may indicate a fly systemic response to the tumor.

Consequently, we desired to define more the role of Tep4 in tumor bearing host. For that reason, we attempted to knock down *tep4* via RNAi in our hosts. We crossed tep4Gal4/ Cyotb, UAS-stingerGFP/TM6B flies with UAS-tep4Ri (II) strains and a control (UAS-LacZ). We were able to recognize F1 generation carrying *tep4Ri* (or *Lacz*) using the balancer CyOtb. Non tubby pupae and adults with non-curly wings carry the gene of our interest. In control cross, we collected adult flies with curly and non-curly wings. The Tep4 knock-down cross, in contrast, produced only adults with curly wings although there were non-tubby pupae in the vial. Interestingly, we conclude that flies with *tep4* depletion die in a late pupal stage before eclosing as an adult. The above findings give new perspectives about Tep4 protein and its role in Drosophila.

3.3 Hemocyte response

Tumour growth generates signals that have been linked to hemocyte proliferation and recruitment (Shaukat et al., 2015). The following experiments aimed to investigate whether hemocytes are able to sense primary brain tumors and proliferate. For that purpose, we used a recombinant fly strain: UAS-NΔecd, srp(hemo)H2A3xmCherry/ Cyotb; UAS-stingerGFP/TM6B (and control line: UAS-LacZ, srp(hemo) H2A3xmCherry /Cyotb ; UAS-stingerGFP/TM6B). In this way we were able to mark hemocytes (endogenous nuclear RFP) and tumor cells (endogenous GFP) after crossing these flies with tubGal80ts/CyoYFP; grhGal4/TM6B. First, we performed larval brain immunohistochemistry to make sure that hyperplasia was induced (Fig.25).

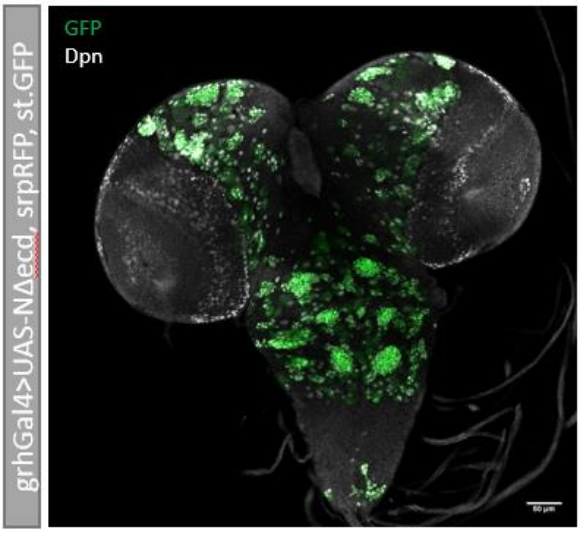


Figure 25: Notch overexpressing tumor: Staining in fixed larval brain samples, in UAS-NΔecd, srp (hemo) moesinH2A3xmCherry / Cyotb; UAS-stingerGFP/TM6B combined with the TARGET system. Dpn (grey) is NB marker. Red hemocytes (RFP) were not detected (Scale bar: 50µm).

Immunohistochemistry on larval hemocytes performed to elucidate hemocyte proliferation using a pH3 antibody and hemocyte differentiation using subtype specific antibodies (L1, L2). We did not observe any difference between control and tumorigenic condition in proliferation qualitatively. In both conditions, hemocytes seem to proliferate and NimC1 marker shows that hemocytes are mainly plasmatocytes (Fig. 26). Thus, we are not sure whether hemocytes respond to Notch primary hyperplastic brains.

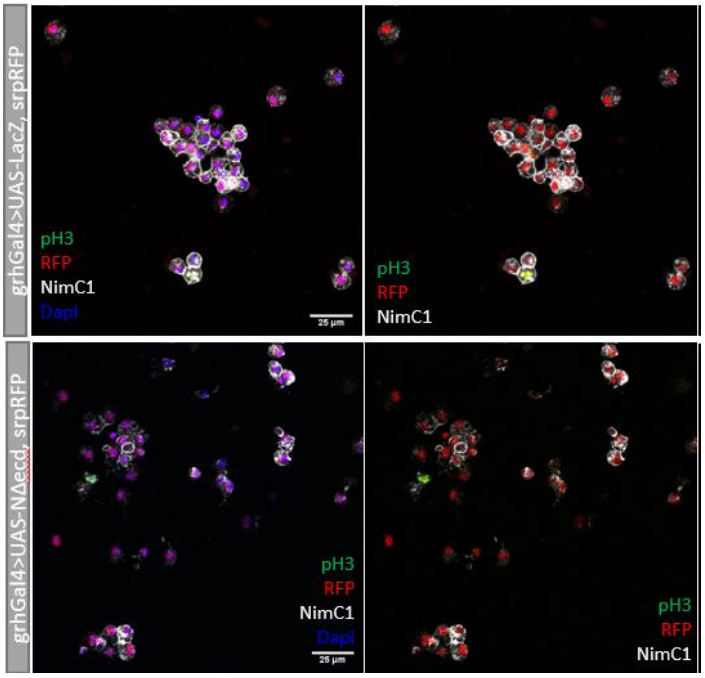


Figure 26: Larval hemocyte staining upon primary hyperplasia and control: RFP (red) expressed endogenously in hemocytes, NimC1 (grey) is a marker for plasmatocytes and pH3 (green) give details for proliferation (scale bar: 25µm)

4. DISCUSSION

Notch ectopic overexpression in Drosophila larval brain can lead to neuroblasts over-proliferation and results to dramatic effects. When this hyperplastic brain is injected into adults, it becomes an aggressive cancer and spreads through the whole host body. In our lab, we have confirmed with an RNA sequencing experiment that in area tumour explant some genes show upregulation in comparison to different allograft stages.

FGF homolog Branchless (bnl) is upregulated in allograft stage T0. Thus, we tried to elucidate branchless impact in our Notch induced tumors. We indicate that Bnl expressed in larval brains both in control and hyperplasia. Moreover, branchless silencing did not affect qualitatively our tumors. One future perspective is to clarify whether the bnl upregulation is related with the tumor or the host. For that reason, we need to perform extra experiments with N-BnlLacZ hyperplastic lobe injection to track bnl expression in the allograft stages. Furthermore, tumor cell seem to be aligned on the tracheal structure, indicating the air supply to tumor as a possible role for trachea. We suspected that FGF/FGFR signaling may be a key player in trachea and tumor interaction. It is obvious that cancer cells aligned between tracheal structures whether Bnl/Btl signaling is depleted or not. Tumor continued to associate with trachea despite the btl knock down. This result leads us to conclude that FGF/FGFR signaling may not have any impact in the tumor's progression. However, we need to perform extra experiments to ensure this. For that reason, we continue to focus on tracheal role in tumorigenic condition. Tumor injection in adults with tracheal ablation via apoptotic factors (e.g. hid) could be performed and tumor progress in this type of hosts may be interesting to define.

In addition, we observed that Tep4 was upregulated between the allograft stages but we did not detect any expression in our primary Notch hyperplasia. In this work, we define the expression pattern of our hosts, showing that Tep4 is expressed in many tissues and follows the same pattern both in larval and adult life. We also reveal that Tep4 changes its expression upon tumorigenic condition and observe this change mainly in the trachea. Considering that, a future perspective would be a specific tep4 knock down in trachea and its effect in tumors progression. Moreover, a complete tep4 silencing seems to be lethal at the pharate adult stage, indicating a possible non-immune role of Tep4 in the process of eclosion. These findings redirect our interest to Tep4 role, since Tep proteins are part of the innate immune system. A future goal is to combine our genetic tools with Tep4-RNAi lines and Gal80ts to control temporal the tep4 depletion. This way, we could overcome the Tep4 RNAi lethality in order to test its possible immune effect on the tumour.

Finally, we could assume that hemocyte and other opsonizing molecules may play a role in tumor's control. We attempted to define whether hemocytes respond to

primary hyperplasia in the larva. We could not clarify their response but we did observe proliferation in plasmatocytes.

REFERENCES

Affolter, M., & Caussinus, E. (2008). Tracheal branching morphogenesis in Drosophila: New insights into cell behaviour and organ architecture. In *Development* (Vol. 135, Issue 12, pp. 2055–2064). https://doi.org/10.1242/dev.014498

Affolter, M., Zeller, R., & Caussinus, E. (2009). Tissue remodelling through branching morphogenesis. In *Nature Reviews Molecular Cell Biology* (Vol. 10, Issue 12, pp. 831–842). https://doi.org/10.1038/nrm2797

Brumby, A. M., & Richardson, H. E. (2005). Using Drosophila melanogaster to map human cancer pathways. In *Nature Reviews Cancer* (Vol. 5, Issue 8, pp. 626–639). https://doi.org/10.1038/nrc1671

Caussinus, E., Gonzalez, C. Induction of tumor growth by altered stem-cell asymmetric division in Drosophila melanogaster. Nat Genet 37, 1125–1129 (2005). https://doi.org/10.1038/ng1632

del Valle Rodríguez, A., Didiano, D., & Desplan, C. (2012). Power tools for gene expression and clonal analysis in Drosophila. In *Nature Methods* (Vol. 9, Issue 1, pp. 47–55). https://doi.org/10.1038/nmeth.1800

Elliott, D. A., & Brand, A. H. (n.d.). *The GAL4 System A Versatile System for the Expression of Genes*.

Gervais, L., & Bardin, A. J. (2021). Tracheal remodelling supports stem cells. In *Nature Cell Biology* (Vol. 23, Issue 6, pp. 580–582). Nature Research. https://doi.org/10.1038/s41556-021-00695-w

Ghabrial, A. S., & Krasnow, M. A. (2006). Social interactions among epithelial cells during tracheal branching morphogenesis. *Nature*, *441*(7094), 746–749. https://doi.org/10.1038/nature04829

Ghabrial, A., Luschnig, S., Metzstein, M. M., & Krasnow, M. A. (2003). Branching Morphogenesis of the Drosophila Tracheal System. *Annual Review of Cell and Developmental Biology*, *19*, 623–647. https://doi.org/10.1146/annurev.cellbio.19.031403.160043

Grifoni, D., Sollazzo, M., Fontana, E., Froldi, F., & Pession, A. (2015). Multiple strategies of oxygen supply in Drosophila malignancies identify tracheogenesis as a novel cancer hallmark. *Scientific Reports*, *5*. https://doi.org/10.1038/srep09061

Hauling, T., Krautz, R., Markus, R., Volkenhoff, A., Kucerova, L., & Theopold, U. (2014). A Drosophila immune response against Ras-induced overgrowth. *Biology Open*, *3*(4), 250–260. https://doi.org/10.1242/bio.20146494

Hayashi, S., & Kondo, T. (2018). Development and function of the drosophila tracheal system. *Genetics*, *209*(2), 367–380. https://doi.org/10.1534/genetics.117.300167
Heigwer, F., Port, F., & Boutros, M. (2018). Rna interference (RNAi) screening in Drosophila. *Genetics*, *208*(3), 853–874. https://doi.org/10.1534/genetics.117.300077

Heigwer, F., Port, F., & Boutros, M. (2018). Rna interference (RNAi) screening in Drosophila. *Genetics*, *208*(3), 853–874. https://doi.org/10.1534/genetics.117.300077

Homem, C. C. F., & Knoblich, J. A. (2012). Drosophila neuroblasts: A model for stem cell biology. *Development (Cambridge)*, *139*(23), 4297–4310. https://doi.org/10.1242/dev.080515

Kovall, R. A., Gebelein, B., Sprinzak, D., & Kopan, R. (2017). The Canonical Notch Signaling Pathway: Structural and Biochemical Insights into Shape, Sugar, and Force. In *Developmental Cell* (Vol. 41, Issue 3, pp. 228–241). Cell Press. https://doi.org/10.1016/j.devcel.2017.04.001

Lee, T., Hacohen, N., Krasnow, M., & Ls, D. J. M. (n.d.). *Regulated Breathless receptor tyrosine kinase.activity required to pattern cell migration andbranchmg m the Drosophila tracheal system*.

Li, S., Wang, H., & Groth, C. (2014). Drosophila neuroblasts as a new model for the study of stem cell self-renewal and tumour formation. In *Bioscience Reports* (Vol. 34, Issue 4, pp. 401–414). Portland Press Ltd. https://doi.org/10.1042/BSR20140008

Magadi, S. S., Voutyraki, C., Anagnostopoulos, G., Zacharioudaki, E., Poutakidou, I. K., Efraimoglou, C., Stapountzi, M., Theodorou, V., Nikolaou, C., Koumbanakis, K. A., Fullard, J. F., & Delidakis, C. (2020). Dissecting Hes-centred transcriptional networks in neural stem cell maintenance and tumorigenesis in Drosophila. *Development (Cambridge)*, *147*(22). https://doi.org/10.1242/dev.191544

McGuire, S. E., Roman, G., & Davis, R. L. (2004). Gene expression systems in Drosophila: A synthesis of time and space. In *Trends in Genetics* (Vol. 20, Issue 8, pp. 384–391). https://doi.org/10.1016/j.tig.2004.06.012

Mirzoyan, Z., Sollazzo, M., Allocca, M., Valenza, A. M., Grifoni, D., & Bellosta, P. (2019). Drosophila melanogaster: A model organism to study cancer. In *Frontiers in Genetics* (Vol. 10). Frontiers Media S.A. https://doi.org/10.3389/fgene.2019.00051

Pandey, U. B., & Nichols, C. D. (2011). Human disease models in drosophila melanogaster and the role of the fly in therapeutic drug discovery. *Pharmacological Reviews*, *63*(2), 411–436. https://doi.org/10.1124/pr.110.003293

Parsons, B., & Foley, E. (2016). Cellular immune defenses of Drosophila melanogaster. *Developmental and Comparative Immunology*, *58*, 95–101. https://doi.org/10.1016/j.dci.2015.12.019

Pitsouli, C., & Perrimon, N. (2010). Embryonic multipotent progenitors remodel the Drosophila airways during metamorphosis. *Development*, *137*(21), 3615–3624. https://doi.org/10.1242/dev.056408

Sato, M., & Kornberg, T. B. (2002). Wilk et al., 1996) and Tango (Ohshiro and Saigo. In *Developmental Cell* (Vol. 3).

Shaukat, Z., Liu, D., & Gregory, S. (2015). Sterile inflammation in drosophila. In *Mediators of Inflammation* (Vol. 2015). Hindawi Limited. https://doi.org/10.1155/2015/369286

Shokal, U., & Eleftherianos, I. (2017). Thioester-containing Protein-4 regulates the drosophila immune signaling and function against the pathogen photorhabdus. *Journal of Innate Immunity*, *9*(1), 83–93. https://doi.org/10.1159/000450610

Shokal, U., & Eleftherianos, I. (2017). Evolution and function of thioester-containing proteins and the complement system in the innate immune response. In *Frontiers in Immunology* (Vol. 8, Issue JUN). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2017.00759

Suchting, S., Freitas, C., le Noble, F., Benedito, R., Bré ant, C., Duarte, A., & Eichmann, A. (2007). *The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching*. www.pnas.org/cgi/content/full/

Tamamouna, V., Rahman, M. M., Petersson, M., Charalambous, I., Kux, K., Mainor, H., Bolender, V., 2†, B. A. E., & Pitsouli, C. (n.d.). *Remodeling of oxygen-transporting tracheoles drives intestinal regeneration and*. https://doi.org/10.1101/2021.01.23.427889

Touat, M., Ileana, E., Postel-Vinay, S., André, F., & Soria, J. C. (2015). Targeting FGFR signaling in cancer. *Clinical Cancer Research*, *21*(12), 2684–2694. https://doi.org/10.1158/1078-0432.CCR-14-2329

Voutyraki C, Choromidis A, Theodorou V, Efraimoglou C, Anagnostopoulos G, Ss, M., Zacharioudaki E, & Delidakis C. (n.d.). *Repression of differentiation genes by the Hes transcription factors fuels neural tumour growth in Drosophila*.

Wang, L., Kounatidis, I., & Ligoxygakis, P. (2014). Drosophila as a model to study the role of blood cells in inflammation, innate immunity and cancer. In *Frontiers in Cellular and Infection Microbiology* (Vol. 3, Issue JAN). Frontiers Media S.A. https://doi.org/10.3389/fcimb.2013.00113

Weaver, M., & Krasnow, M. A. (2008). Dual origin of tissue-specific progenitor cells in Drosophila tracheal remodeling. *Science*, *321*(5895), 1496–1499. https://doi.org/10.1126/science.1158712

Yamamura, R., Ooshio, T., & Sonoshita, M. (2021). Tiny Drosophila makes giant strides in cancer research. In *Cancer Science* (Vol. 112, Issue 2, pp. 505–514). Blackwell Publishing Ltd. https://doi.org/10.1111/cas.14747

Yu, S., Luo, F., & Jin, L. H. (2018). The Drosophila lymph gland is an ideal model for studying hematopoiesis. *Developmental and Comparative Immunology*, *83*, 60–69. https://doi.org/10.1016/j.dci.2017.11.017

Yu, S., Luo, F., & Jin, L. H. (2018). The Drosophila lymph gland is an ideal model for studying hematopoiesis. *Developmental and Comparative Immunology*, *83*, 60–69. https://doi.org/10.1016/j.dci.2017.11.017

Yuan, X., Wu, H., Xu, H., Xiong, H., Chu, Q., Yu, S., Wu, G. S., & Wu, K. (2015). Notch signaling: An emerging therapeutic target for cancer treatment. In *Cancer Letters* (Vol. 369, Issue 1, pp. 20–27). Elsevier Ireland Ltd. https://doi.org/10.1016/j.canlet.2015.07.048

Zacharioudaki, E., & Bray, S. J. (2014). Tools and methods for studying Notch signaling in Drosophila melanogaster. *Methods*, *68*(1), 173–182. https://doi.org/10.1016/j.ymeth.2014.03.029

Zacharioudaki, E., Magadi, S. S., & Delidakis, C. (2012). bHLH-O proteins are crucial for Drosophila neuroblast self-renewal and mediate notch-induced overproliferation. *Development*, *139*(7), 1258–1269. https://doi.org/10.1242/dev.071779