

From the Zoologischen Institut  
(Leader of the institution: Prof. Dr. Stanislav Gorb)  
Research Group Molecular Physiology  
(Head of the research group: Prof. Dr. Thomas Roeder)  
at Kiel University

**Targeted expression of the Epidermal growth factor  
receptor in the respiratory system of *Drosophila  
melanogaster* to develop a lung cancer model**

Dissertation  
to acquire the doctoral degree (Dr. med.)  
at the Faculty of Medicine  
at Kiel University

Presented by  
Lijo Jose Vattoly  
from Pullur, India

Kiel, 2021

1st Reviewer: Prof. Dr. rer. nat. Thomas Roeder

2nd Reviewer: Prof. Dr. Heiner Schäfer

Date of oral examination: 03.05.2022

Approved for printing, Kiel, 12.01.2022

Signed: Prof. Dr. Anna Trauzold  
(Chairperson of the Examination Committee)

## Table of Contents

Table of Contents.....	I
List of abbreviations and units .....	III
Abbreviations.....	III
Units .....	VI
List of figures.....	VII
List of tables .....	VIII
1. Introduction .....	1
1.1 An overview of cancer and hallmarks of cancer cells.....	1
1.2 Process of Carcinogenesis.....	3
1.3 Lung cancer .....	4
1.3.1 Classification and mutation prediction of lung cancer .....	5
1.4 <i>Drosophila melanogaster</i> a unique model for research.....	6
1.4.1 <i>Drosophila melanogaster</i> as model organism to study cancer .....	7
1.5 Tracheal system of <i>Drosophila</i> larvae.....	7
1.6 Inka cells and its role in ecdysis .....	10
1.7 EGFR pathway .....	10
1.8 UAS-Gal4 system.....	13
1.9 Aim of the study.....	15
2. Materials and Methods .....	16
2.1 Devices .....	16
2.2 General materials .....	16
2.3 General chemicals.....	17
2.4 Fly Husbandry.....	18
2.5 <i>Drosophila</i> fly lines and crossings .....	18
2.6 Microscopy.....	19
2.6.1 Dissection of trachea, intestine and filet preparation from L3 larvae.....	20
2.6.2 Immunohistochemistry .....	21
2.6.3 Quantification of nucleus number and size .....	22
2.7 Collection of L1 and L2 larvae.....	23
2.8 Development of pupae and larvae.....	23
2.9 Statistics .....	23
3. Results.....	24
3.1 Targeted expression of Egfr <sup>CA</sup> throughout the larval tracheal system.....	24
3.1.1 Morphological changes in trachea expressing Egfr <sup>CA</sup> .....	25
3.1.2. Analysis of the trachea of Egfr <sup>CA</sup> overexpressing animals.....	27

3.1.3. Increased lethality in early larval stages .....	28
3.1.4. Female biased sex ratio in Egfr <sup>CA</sup> expressing pupae .....	30
3.2 Targeted expression of Egfr <sup>CA</sup> in tracheal endocrine cells of <i>Drosophila</i> Larvae .....	31
3.2.1. Lethality in larval and pupal stage caused by the Egfr <sup>CA</sup> expression at the Inka cells. ....	33
3.2.2. Analysis of the pupal trachea at the cellular level .....	34
4. Discussion.....	36
4.1 Impacts of upregulated Egfr <sup>CA</sup> in the whole tracheal system of <i>Drosophila</i> larvae .....	36
4.2 Reason for the increased larval lethality of expressed Egfr <sup>CA</sup> .....	38
4.3 Explanations for a female-biased survival of upregulated Egfr <sup>CA</sup> .....	39
4.4 Targeted expression of Egfr <sup>CA</sup> in Inka cells of larval tracheal system does not provoke any morphological or structural changes. ....	40
4.5 Impact of the upregulated Egfr <sup>CA</sup> in Inka cells in pupae .....	41
5. Zusammenfassung .....	43
Abstract.....	45
6. References .....	46
7. Eidesstattliche Erklärung .....	54
8. Acknowledgment.....	64

## List of abbreviations and units

### Abbreviations

Akt	AKR mouse thymoma kinase
APC	Adenomatous Polyposis Coli
arg	Argos
ATM	Ataxia Telangiectasia Mutated
Avg	Average
Bak	bcl-2 antagonist killer 1
Bax	bcl-2 associated X protein
Bcl	B-cell lymphoma unspecified
Bcl-XL	B-cell lymphoma-Extra Large
bFGF	basic Fibroblast Growth Factor
Bid	BH3-interacting domain death agonist
Bim	Bcl-2-interacting mediator of cell death
BMI	Body Mass Index
Bnl	Branchless
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
Btl	Breathless
CAMs	Cell Adhesion Molecules
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
CM	Concentrated Medium
CNS	Central Nervous System
Cora	Coracle
D <sub>l</sub>	dorsolateral
D <sub>m</sub>	dorsomedial
DAPI	4',6-diamino-2-phenylindole
DIC	Differential interference contrast
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DSOR1	Downstream of raf1
DRK	Downstream of Receptor Kinase
EGFR	Epidermal Growth Factor Receptor
Egfr <sup>CA</sup>	Constitutive active isoform of Egfr
EH	Eclosion Hormone
EMT	Epithelial -Mesenchymal Transition
ER	Endoplasmic Reticulum
ERBB4	erb-b2 receptor tyrosine kinase 4
ERK	Extracellular Receptor Kinase

ETH	Ecdysis Triggering Hormone
et al.	et alia
FDA	Food and Drug Administration
FGFR4	Fibroblast Growth Factor Receptor 4
flb	faint little ball
GDP	Guanosine diphosphate
GFP	Green Fluorescence Protein
Grb2	Growth factor receptor-bound protein 2
<i>grk</i>	Gurken
GTP	Guanosine triphosphate
HER	Human epidermal growth factor receptor
HL3	Hemolymph-like solution
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papillomavirus Infection
IARC	International Agency for Research on Cancer
IGF-1	Insulin-like Growth Factor-1
IGF1R	Insulin-like Growth Factor type 1 Receptor
IGFBP-2	IGF binding proteins-2
IIS	Insulin/IGF-1 signalling
KEAP1	Kelch-like ECH-associated protein 1
KEK	Kekkon
KDR	Kinase insert Domain Receptor
KRAS	Kirsten Rat Sarcoma viral oncogene homolog
<i>kren</i>	Keren
LCIN	Lung Cancer In Never smokers
LUAD	Lung Adenocarcinoma
LUSC	Lung Squamous Cell Carcinoma
MAPK	Mitogen-Activated Protein Kinase
MEK	MAPK /ERK kinase
mTOR	mechanistic target of rapamycin
NF1	Neurofibromatosis type 1
NFE2L2	Nuclear Factor, Erythroid 2 like 2
NM	Normal Medium
No.	number
n. s	not significant
NSCLC	Non-Small Cell Lung Cancer
NSCLC NOS	Non-Small Cell Lung Cancer Not Otherwise Specified
NTRK	Neurotrophic Tyrosine Receptor Kinase
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline

PBST	Phosphate Buffered Saline with Tween
PCBs	Polychlorinated biphenyl
PCR	Polymerase Chain Reaction
PFA	paraformaldehyde
pH3	phospho-Histone H3
PI <sub>3</sub> K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
Pnt	Pointed
ppk	pickpocket
pRb	Retinoblastoma protein
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RB	Retinoblastoma
RI	Rolled
RHO	Rhomboid
RKI	Robert Koch Institute
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Room Temperature
RTK	Receptor tyrosine kinase
S	Star
SCLC	Small Cell Lung Cancer
SH2	Src homology 2 domain
SJ	Septate Junctions
SOS	Son of sevenless
spi	Spitz
SQCC	Squamous Cell Carcinoma
STAT	Signal Transducer and Activator of Transcription
STK11	serine/threonine kinase 11
sty	Sprouty
TARGET	Temporal and Regional Gene Expression Targeting
TGF	Tumor Growth Factor
TP53	Tumor Protein 53
TSP-1	Thrombospondin-1
UAS	Upstream Activation Sequence
Vm	Ventromedial
VEGF	Vascular Endothelial Growth Factor
vn	Vein

**Units**

°C	degree Celsius
μg	microgram
μl	microliter
μm <sup>2</sup>	micrometer square
g	gram
Mb	Mega base
min	Minute
mM	millimolar
ml	milliliter
v/v	volume per volume
w/v	weight per volume



## List of figures

<b>Figure 1:</b> Hallmarks of cancer.....	2
<b>Figure 2:</b> Process of Carcinogenesis.....	3
<b>Figure 3:</b> Lifecycle of <i>Drosophila melanogaster</i> .....	6
<b>Figure 4:</b> Tracheal system in <i>Drosophila</i> larvae..	9
<b>Figure 5:</b> Illustration of Inka cells in <i>Drosophila</i> .....	10
<b>Figure 6:</b> Epidermal growth factor receptor (EGFR) pathway in <i>D. melanogaster</i> .....	13
<b>Figure 7:</b> The binary UAS-Gal4 system of <i>Drosophila melanogaster</i> .....	14
<b>Figure 8:</b> Tracheal phenotype caused by $Egfr^{CA}$ expression induced by <i>ppk4</i> -Gal4 and <i>btl</i> -Gal4, respectively. ....	24
<b>Figure 9:</b> Morphological and structural changes caused by ectopic expression of $Egfr^{CA}$ in the trachea.....	25
<b>Figure 10:</b> $Egfr^{CA}$ upregulation leads to changes in number and size of the nucleus of the tracheal cells.....	26
<b>Figure 11:</b> Comparison of number and size of nucleus in control and upregulated $Egfr^{CA}$ .....	26
<b>Figure 12:</b> Cellular level analysis of upregulated $Egfr^{CA}$ .....	28
<b>Figure 13:</b> Effect of deregulated $Egfr^{CA}$ expression on larval lethality in L1 and L2 stages.....	29
<b>Figure 14:</b> Incidence of death during the life stages of $Egfr^{CA}$ upregulated larvae..	29
<b>Figure 15:</b> Effect of overexpressed $Egfr^{CA}$ on larval lethality in L1 and L2 stages.....	30
<b>Figure 16:</b> Disproportional distribution of the sexes in upregulated $Egfr^{CA}$ survivors.....	31
<b>Figure 17:</b> The airway epithelium of <i>Drosophila</i> larvae with Inka cells..	31
<b>Figure 18:</b> Illustration of the c929 cells (Inka cells) in the trachea expressing $Egfr^{CA}$ with DIC and DAPI. ....	32
<b>Figure 19:</b> Cellular level analysis of upregulated $Egfr^{CA}$ at the Inka cells of the trachea.....	32
<b>Figure 20:</b> Larval development of $Egfr^{CA}$ expressing animals..	33
<b>Figure 21:</b> Larval development and lethality of the pupae in $Egfr^{CA}$ expression in Inka cells of the trachea..	34
<b>Figure 22:</b> Cellular level analysis of pupal trachea of upregulated $Egfr^{CA}$ .....	34
<b>Figure 23:</b> Cancer like phenotype in the pupal trachea of upregulated $Egfr^{CA}$ .....	35

## List of tables

<b>Table 1:</b> Drosophila fly lines .....	19
<b>Table 2:</b> Antibodies and dilutions .....	22

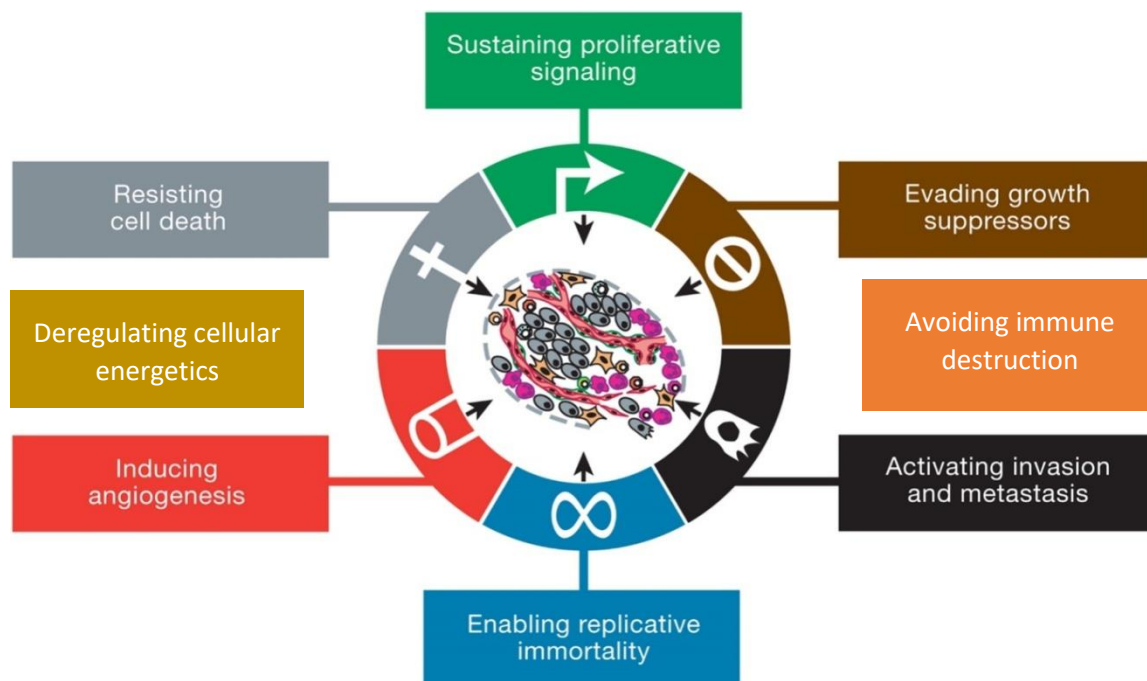
## 1. Introduction

### 1.1 An overview of cancer and hallmarks of cancer cells

Cancer comprises a group of different diseases that can affect different parts of the body. It is an uncontrolled growth of abnormal cells in the body and they have the ability to invade nearby tissues. They can spread through the blood and lymph systems to other parts of the body (NIH, 2019; Organization, 2017). Cancer is one of the leading causes of mortality and morbidity worldwide. In Germany and the US, cancer was ranked as the 2<sup>nd</sup> leading cause of death after heart disease (RKI, 2015; Siegel et al., 2021). The International Agency for Research on Cancer (IARC) reported that in 2020 about 19.3 million new cancer cases and 10 million cancers associated deaths occurred worldwide. Globally, it was determined that 1 in 5 individuals acquire cancer during their lifetime, and 1 in 8 men and 1 in 11 women die from cancer. Breast cancer followed by colorectal and lung cancer was found frequently in women, whereas lung cancer followed by prostate cancer was frequent among men. Increased occurrence of cancer can be ascribed to growth and aging of the population and westernization of lifestyles such as tobacco and alcohol use, unhealthy diet, physical inactivity leading to obesity and increased BMI (Body Mass Index) (Sung et al., 2021). Carcinogenesis, the process which transforms normal cells into tumor cells are triggered by the interaction between an individual's genetic structure and foreign factors such as physical carcinogens – ultraviolet and ionizing radiation; chemical carcinogens – asbestos, tobacco smoke, aflatoxin (a food contaminant), and arsenic (a drinking water contaminant); and biological carcinogens – infections from certain viruses, bacteria, or parasites (Organization, 2017).

Homeostasis is strictly maintained in differentiated cells; where else this is completely deregulated in cancer cells. Two major classes of genes are particularly relevant for carcinogenesis: oncogenes and tumor suppressor genes. Cancer cells have various hallmarks distinguishing them from normal cells which are acquired through a variety of mechanisms, including activation of oncogenes and mutations in tumor suppressor genes (Fig. 1). Cancer cells can generate many of their own growth signals which in turn disrupt the homeostasis. The antiproliferative property, which prevents the unwanted growth of the cells, conveyed through retinoblastoma protein (pRb) and its two relatives, p107 and p130, is lost by any interruption in the pRb pathway thus leading to cell proliferation and imbalance. The tumor

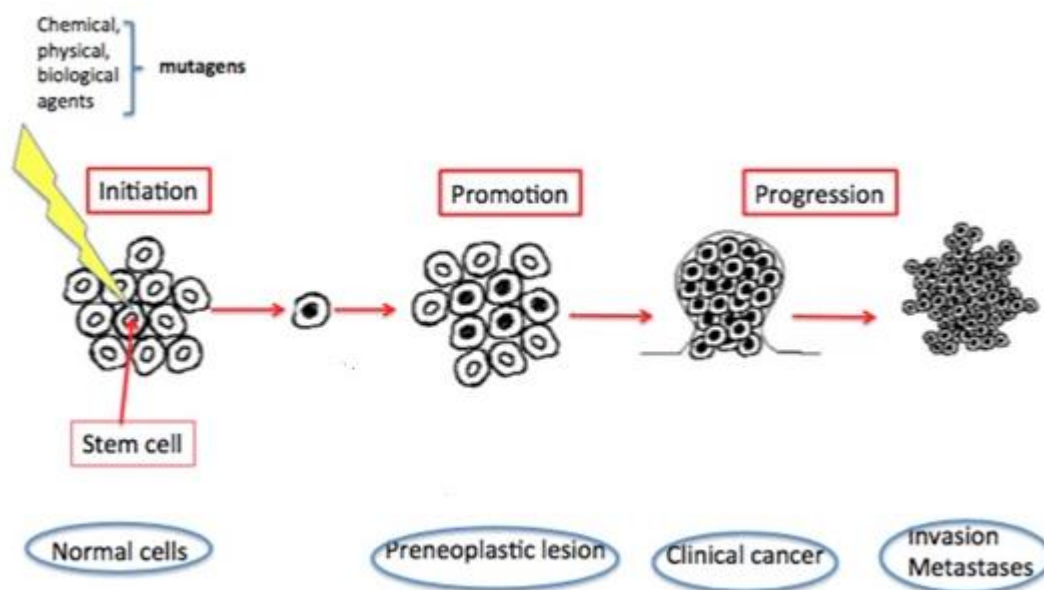
cell population is not only maintained by increasing its cell proliferation property, but also by affecting the normal apoptosis property of the cell. This apoptotic pathway is inhibited by overexpression of anti-apoptotic proteins (Bcl-2, Bcl-XL, and Bcl-W) and under-expression of proapoptotic proteins (Bax, Bak, Bid, and Bim). Cancer cells acquire a limitless replicative potential and are also able to escape immune recognition and destruction by T and B lymphocytes, macrophages, and natural killer cells thus leading to tumor progression. They can reprogram their glucose metabolism by limiting their energy production largely to glycolysis. Tumor vascularisation is maintained by changing the balance of angiogenesis inducers (VEGF and bFGF) and countervailing inhibitors (TSP-1). Activation of Epithelial - Mesenchymal Transition (EMT) and change in expression of cadherins, cell adhesion molecules (CAMs), and integrins contribute to tissue invasion and metastasis. The main characteristics of cancer cells, genomic instability and mutations, as well as inflammation developed by tumors are main factors that enable these capabilities (Fouad and Aanei, 2017; Hanahan and Weinberg, 2017).



**Figure 1: Hallmarks of cancer.** Cancer cells have the capability to flourish themselves by producing their own growth factors and interrupting various protein pathways, replicate unlimitedly. They are able to metastasize and invade, withstand programmed cell death, setup tumor associated neovasculature. They evade immune destruction is by delivering signals and are also capable of reprogramming their energy metabolism (according to Hanahan and Weinberg, 2011).

## 1.2 Process of Carcinogenesis

Carcinogenesis, otherwise known as oncogenesis or tumorigenesis happens as a result of interaction between an external factor (chemical, biologic, physical, or genetic) and normal cells leading to genetic transformation or mutation. This complicated process of cancer formation can broadly be classified into 3 stages; namely initiation, promotion, and progression (Fig. 2). The foremost irreversible stage, initiation, involves genetic alteration (mutations, transversions, transitions, small deletions in DNA), which distracts the biochemical signalling pathways related to cellular proliferation, survival, and differentiation. This initial stage can happen spontaneously and be induced promptly. Promotion, a reversible and lengthier stage of carcinogenesis in which the initiated cell gets transformed into preneoplastic cells by interacting with tumor promoters (alcohol, high estrogen, dietary fat, ultraviolet radiations, chronic irritation, and chemicals like saccharin, dioxin, tryptophan, polychlorinated biphenyl (PCBs)), causes a change in expression profile of these cells (Pitot, 1993). Chemopreventive agents (resveratrol, antioxidants, polyphenol) can play a significant role in this stage by affecting the growth rate (Siddiqui et al., 2015).



**Figure 2: Process of Carcinogenesis.** In the initiation stage, by the exposure of mutagens, the normal stem cells get genetically altered. These cells undergo tumor promotion into preneoplastic cells, which progress to clinical cancer and further into invasive metastatic cancer (modified from Burgio and Migliore, 2015).

Tumor progression is an irreversible phase characterized by the appearance of malignant neoplasms with genetic and phenotypic alterations which further grow in size thus invading nearby cells. Metastasis is the primary reason for cancer - related morbidity and mortality.

This metastatic stage is accomplished when the cancer cells disentangle from the primary tumor and travel through the circulatory and lymphatic system escaping the immune attack and invading and proliferating in a distant site (Seyfried and Huysentruyt, 2013). Metastasis involves the spread of cancer cells from the primary site to other parts of the body through the bloodstream or the lymph system. Chemopreventive agents are known to inhibit angiogenesis and invasion of primary tumors, and thus could be utilized to inhibit the metastasis of cancer.

### **1.3 Lung cancer**

Based on the report from GLOBOCAN 2020, lung cancer contributes 2.2 million new cases, which is 11.4% of total cancer cases and 1.8 million deaths, which is 18% of total cancer deaths in both sexes. It is the second most frequently occurring cancer worldwide, after breast cancer (11.7%). It is the first most frequent form of cancer in men and third for women (Sung et al., 2021). Compared to the developing nations, a higher rate of lung cancer -associated deaths in women is observed in developed nations (Barta et al., 2019). Increased incidence and mortality rates are observed in the Asian population (Bray et al., 2018). The five-year survival rate of lung cancer (19%) is significantly less than in other common cancers. The 5-year survival rate of lung cancer in the localized state is 57%, whereas it only 5% in metastatic forms. Early diagnosis increases the survival rate. 80% to 90% of lung cancer can directly be linked to tobacco use, which results in 1 million deaths worldwide each year and more than 160,000 deaths annually in the United States (Siegel et al., 2020). The remaining 10%-15% cases of lung cancer occur in never smokers, of younger age, mainly in African Americans and Asians, and more likely in women (Samet et al., 2009). Risk factors for Lung cancer in never smokers (LCIN) can be occupational and environmental exposure to carcinogens (radon, cooking fumes, asbestos, heavy metals, and second-hand smoke); infections (human papillomavirus infection (HPV), *Mycobacterium tuberculosis*); inherited genetic susceptibility, hormonal and dietary factors, and diabetes mellitus (Govindan et al., 2012; Pallis and Syrigos, 2013; Rivera and Wakelee, 2016).

### 1.3.1 Classification and mutation prediction of lung cancer

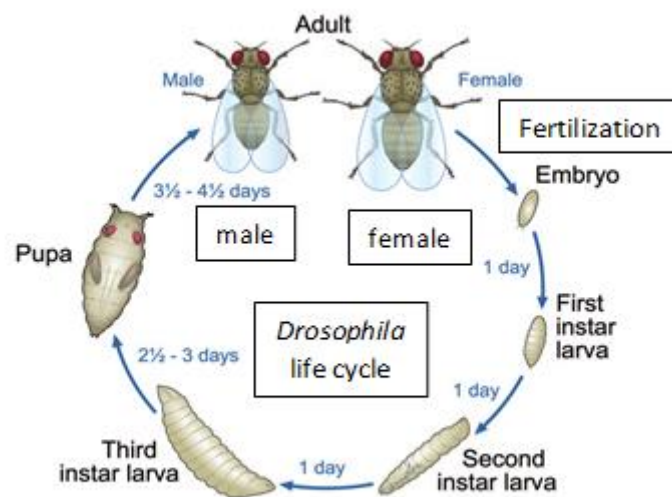
Conventional classification methodology classifies lung cancer as non-small cell lung cancer (NSCLC), which accounts for 85% of all cases of lung cancer, and small cell lung cancer (SCLC), which contributes 15% of all cases (Molina et al., 2008). WHO has further divided NSCLC as adenocarcinoma (LUAD) which is the most prevailing type of NSCLC (40%), squamous cell carcinoma (LUSC) (25-30%), and large cell carcinoma or non-small cell lung cancer not otherwise specified (NSCLC NOS) (10-15%). Adenocarcinoma (LUAD) is the most common type of cancer evident among never smokers and it is usually found in outer parts of the lung, while squamous cell carcinoma (LUSC) is found in the central part of the lung, bronchus and it is likely linked with tobacco use (American Cancer Society, 2016; Duma et al., 2019).

One of the recent advancements in the field of cancer is the approach of personalized treatment, which is based on the genetic and histologic components of the patient's tumor (Travis et al., 2015). Sequencing of different lung cancer types has showcased various molecular and mutational discrepancies. The heterogeneity of the tumor is the result of a combination of diverse mutations and genomic rearrangement. A high rate of somatic mutation (8.9 mutations per megabase) was evident in lung adenocarcinoma (LUADs) (Collisson et al., 2014; Govindan et al., 2012; Imielinski et al., 2012). The highest mutated pathway is the receptor tyrosine kinase RTK/RAS/RAF pathway (76%) which is followed by the TP53 pathway (63%) (Collisson et al., 2014). Smokers with the *TP53* gene are 3 times more likely to develop lung cancer than non-smokers with *TP53* (Hwang et al., 2003). Various other alterations were: cell cycle regulators (64%) PI3K-mTOR pathway activation (25%), oxidative stress pathways (22%), and mutation of various chromatin and RNA splicing factors (49%) (Collisson et al., 2014; Jamal-Hanjani et al., 2017). Oncogenes with the highest rate of mutation were *EGFR*, *KRAS*, ephrin receptor genes, *ERBB4*, *KDR*, *FGFR4*, *MET*, *BRAF*, and *NTRK* genes. The most commonly observed tumor suppressors were *TP53*, *STK11*, *NF1*, *RB1*, *KEAP1*, *ATM*, and *APC* (Ding et al., 2008). In the USA and Western Europe, 10-15% of patients with NSCLC are present with somatic mutations in *EGFR*, with a higher frequency in Asian patients (30-40%). *EGFR* mutations were more frequent (50%) both in females and in non-smokers with NSCLC (Duma et al., 2019; Rudin et al., 2009; Sharma et al., 2007). Somatic mutation of 8.1 mutations per megabase was evident in squamous cell carcinoma (LUSC). 34% of the LUSCs resulted due to mutation in oxidative stress response genes- *NFE2L2* and *KEAP1*, 44% due to alteration in squamous cell differentiation, 47% due to mutation of genes in the

phosphatidylinositol-3-OH kinase (PI3K) pathway. Tumor suppressor genes *CDKN2A* and *RB1* are inactivated in 72% of cases of lung SQCC. A low rate of mutation in *EGFR* and *KRAS* was observed in LUSCs (Hammerman et al., 2012).

#### 1.4 *Drosophila melanogaster* a unique model for research

*Drosophila melanogaster*, commonly known as the fruit fly, referred to as the “workhorse of genetics and developmental biology” is a tiny arthropod about 3 mm long. Its genetic diversity, brief generation time, low chromosome number, genome size, high fertility, easily manipulable genetic structure and low maintenance cost make it such an attractive model for research studies (Mirzoyan et al., 2019; Tenenbaum, 2003).



**Figure 3: Lifecycle of *Drosophila melanogaster*.** The life cycle of *Drosophila*, from embryogenesis to larval stage, a pupal stage, and finally the formation of adult fly, takes about 10 -12 days at 25°C (according to Ong et al., 2015).

The life cycle of *Drosophila* (Fig. 3) is very short. Its high reproducibility produces hundreds of genetically similar off-springs at 25°C from a single fertile mating pair. The breeding period is approximately 10-12 days from fertilized egg to an adult fly. The maximum life span of *Drosophila* is around 60-80 days based on its growing conditions. Due to the holometabolous property of *Drosophila*, its life cycle includes 4 developmental stages: Embryo, larva, pupa, and fly. Within the first 24 hours of fertilization, the egg gets developed into an embryo which later hatches as a first larval instar. The first larval instar feeds on the culture medium and molts into the second instar (after 24 hours) and third instar (after 48 hours). The third instar



larvae leave the medium for a place to pupariate (24-48 hours) and undergo metamorphosis, where all embryonic and larval tissues are degraded and adult tissues are developed from a set of cells known as imaginal discs. After pupation, an adult fly emerges from pupae after four to five day (Fernández-Moreno et al., 2007; Jennings, 2011).

*Drosophila* is one of the well-established and generally accepted model organisms. It was introduced first by Thomas Hunt Morgan and has been applied since then to a large number of human diseases (Bier, 2005; Pandey and Nichols, 2011). To be used as a model organism, *Drosophila* undergoes various genetic modifications. The Gal4 bipartite system introduced in 1993, which relies on the yeast transcription factor GAL4 and the corresponding DNA binding region, UAS (upstream activation sequence), were used for over expression or silencing of specific genes in the tissue of interest (Brand and Perrimon, 1993).

#### **1.4.1 *Drosophila melanogaster* as model organism to study cancer**

The *Drosophila* cancer models have given tremendous contributions to understand the origin and development of cancer, and the characteristics of cancer cells. Recently it played an important role in anti-cancer drug development and drug resistance research. In 2000 the whole genome of the *Drosophila melanogaster* was sequenced. It has around 13,600 genes arranged on four chromosomes with a total size of 180 Mb (Adams et al., 2000). The genome of the common fruit fly is 60% homologous to the genome of humans. Approximately 75% of the disease-causing genes have functional similarity in flies (Ugur et al., 2016). This information plays a key role in understanding the function of oncogenes and tumour suppressor genes. It also helps to understand the complex cellular mechanisms that promote tumour like cell-cell competition, cell polarity, EMT, apoptosis and metastasis (Papagiannouli and M., 2013). The simplicity of the *Drosophila* genome has the advantage, that the number of mutations needed to induce cancer is low. It helps to understand the particular gene functions easier and faster (Brumby and Richardson, 2005). Further studies in the common fruit fly will surely keep on delivering crucial information to understand more about the complex multistep disease Cancer.

#### **1.5 Tracheal system of *Drosophila* larvae**

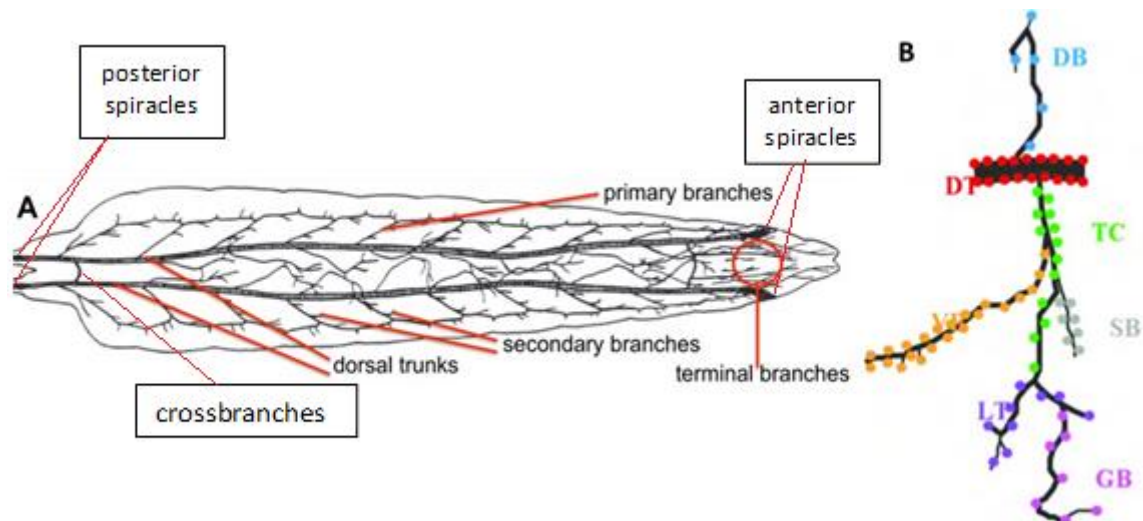
Tracheal system developments in *Drosophila* commence from mid-embryogenesis and are carried throughout the pupal stage of the fly (Loganathan et al., 2016). The larval trachea of

*Drosophila* is a single-layered epithelium of approximately 1600 cells. The *Drosophila* respiratory (tracheal) system (Fig. 4), which is a network of 10,000 interconnected epithelial tubules that spread all over the body cavity, enables the transport of oxygen from openings in the body wall known as spiracles into the primary and secondary tracheas to the terminal trachea known as tracheoles and eliminates carbon dioxide in the opposite direction (Wigglesworth, 1972). The entire tracheal system is bilaterally symmetrical and is covered by a chitin layer which provides mechanical strength to keep the airways open (Öztürk-Çolak et al., 2016). Even though the physiology of tracheal epithelium of *Drosophila* larvae is simpler in structure and its organization, it resembles the overall design of the human lung (Horowitz and Simons, 2008; Roeder et al., 2012).

The tracheal branching process can be divided into three phases. The first phase which is carried till the end of embryogenesis marks the formation of primary, secondary, and terminal branches; clears the liquid, and fills it with air for larval respiration. In the second phase, during the larval stage, tracheal cells undergo further growth, development, and remodelling and form tracheoles. During the third phase in the pupal stage, these larval tracheal cells undergo apoptotic cell death, and the remaining cells proliferate to form new branches thus remodelling the tracheal system into adult form.

Formation of 20 epithelial sacs, 10 on each side of the embryo (Tr1-Tr10) during the 10<sup>th</sup> stage of embryogenesis, which occurs 4-5 hours after egg laying, marks the beginning of the development of the *Drosophila* tracheal system. These sacs, with around 80 precursor cells structures the trunk of the tracheal system, and further these 80 precursor cells lead to sprouting of primary, secondary, and terminal branches cells in each body segment of the embryo via cell migration and rearrangement of tracheal cells and by cell elongation (Klowden, 2008). Beyond stage 11 of embryogenesis, there is no increase in the number of cell numbers (Samakovlis et al., 1996). In early stage 11, the placodes of precursor cells form tracheal pits. The beginning of embryo stage 11 marks the invagination, elongation, and molding of these pits to become a central stalk-like structure with six distinct buds. These six buds further form primary branches in stage 12 (Kadota et al., 2014; Loganathan et al., 2016; Rao et al., 2015). Later, during stage 15 secondary branches are formed which sprout into several terminal branches (tracheoles) in stage 16. Terminal branch formation initiated in late embryogenesis is continued in the larval period. Terminal branches are formed by a subset of secondary branch cells known as terminal cells. The branching patterns of the primary and secondary

branches are similar but not identical in different hemi segments. The cells in the primary branches are organized by intercellular junctions, whereas autocellular junction is formed within the same cell by the secondary branches by cell intercalation. A narrow lumen is formed in the cytoplasm which creates a 'seamless' tube without junction to transport oxygen from the secondary branches to the ends of the terminal cell (Samakovlis et al., 1996). Thus, a closed tubular network is formed when the dorsal trunk, primary and secondary branches fuse to the developed embryonic tracheal system (Uv et al., 2003).

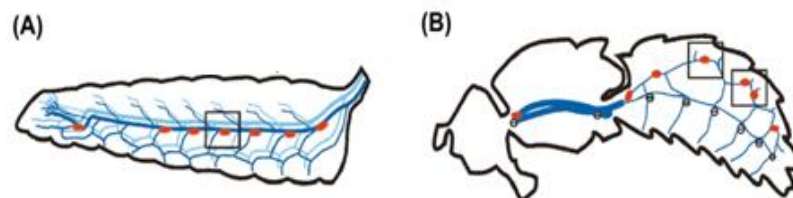


**Figure 4: Tracheal system in *Drosophila* larvae.** **A** The dorsal view of the tracheal system displays the dorsal trunks from which, the primary branches originate and which further leads to the secondary and terminal branch (Faisal et al., 2014). **B** Each tracheal metamere is composed of dorsal trunks, which is branched into primary, secondary and terminal branches. DB = Dorsal branch, DT = Dorsal trunk, TC = Transverse connectives, VB = Visceral branch, SB = Spiracular branch, LT = Lateral trunk, GB = Ganglionic branch (according to Tiklová et al., 2010).

All three (primary, secondary and terminal) levels of tracheal branching are induced by branchless (Bnl) and its receptor breathless (Btl). The Breathless (Btl) pathway is a homolog of the mammalian fibroblast growth factor receptor (FGFR) pathway, which is involved in triggering cell division, differentiation, and migration in humans. Epidermal growth factor receptor (EGFR)-dependent mechanism plays a predominant role to generate 10 pairs of tracheal sacs by tracheal invagination (Schottenfeld et al., 2010). This EGFR signaling is controlled by the EGF ligand activator molecule Rhomboid (Rho) and the intracellular signal transducers ERK and MEK and is regulated by Rho GTPase signaling (Hayashi and Kondo, 2018).

## 1.6 Inka cells and its role in ecdysis

Inka cells are few endocrine cells associated with the dorsal trunks of the larval tracheal system (Fig. 5). They are found at the branching points to the primary branches in dorsal trunks (Faisal et al., 2014). These cells have an important role to play in the process of ecdysis. Ecdysis is a cyclic replacement of the exoskeleton and it is a crucial feature for the survival and development of insects. It protects the body, lines the trachea and parts of the gut (Truman, 2005; White and Ewer, 2014; Zitnan and Adams, 2012). The process of ecdysis is highly hormonally mediated and there are two hormones, that play significant roles in initiating the ecdysis sequence, the eclosion hormone (EH) and the ecdysis triggering hormone (ETH) (Roller et al., 2010; Truman et al., 1981; Truman and Riddiford, 1970). The only known cells that release EH are two to four large neurosecretory cells (i.e.,  $V_m$ -neurons ( $V_m$ =ventromedial)) in the CNS (Horodyski et al., 1989; Truman and Copenhaver, 1989). There are recent studies shows that there are cells, that produce EH other than  $V_m$ -neurons (Scott et al., 2020). Inka cells are the only known cells that release ETH, which act on  $V_m$ -neurons to release EH (Diao et al., 2016; Kim et al., 2006 a, b). The increased EH concentration prevents further ETH release. The positive feedback system between EH and ETH signalling secure the hormonal action for the ecdysis (Ewer et al., 1997; Kingan et al., 1997).



**Figure 5: Illustration of Inka cells in *Drosophila*.** Diagram **A** illustrates the positions of Inka cells (red) in the tracheal system of the *Drosophila* larvae. Diagram **B** shows the positions of Inka cells (red) in the tracheal system of the adult *Drosophila* (according to Zitnan and Adams, 2012).

## 1.7 EGFR pathway

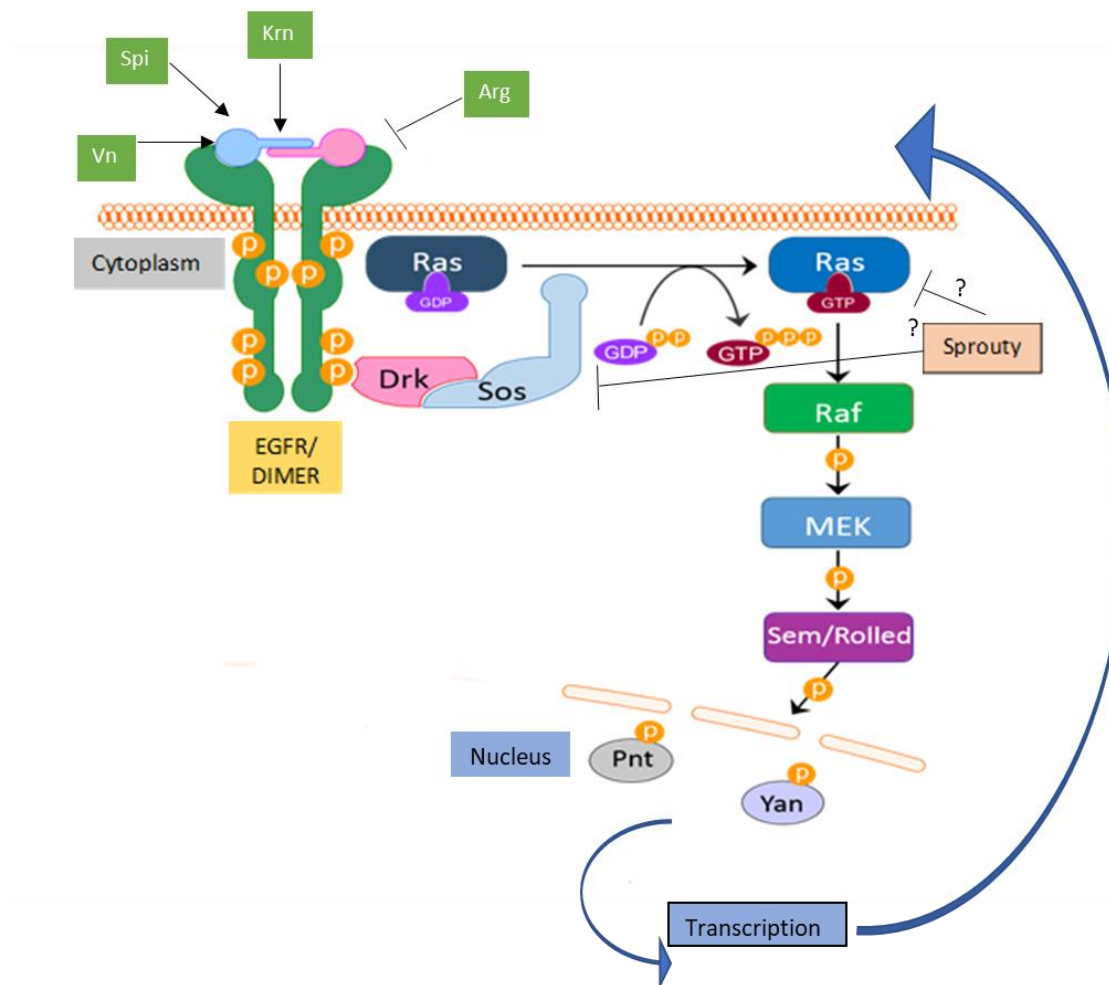
The EGFR signalling pathway (Fig. 6) is one of the most important pathways for cell proliferation, survival, migration, differentiation, and apoptosis in mammals (Oda et al., 2005). Any type of mutation in this pathway can result in cancer. The transmembrane protein, EGFR (Epidermal growth factor receptor), which belongs to the epidermal growth factor receptor (HER/ErbB) protein family of receptor tyrosine kinases (RTKs) act as a receptor for the ligands

from the EGF family. The *EGFR* gene is found on chromosome 7p12 - 13. The ErbB family comprises four receptors, which include EGFR (ErbB-1/HER1), ErbB-2 (Neu, HER2), ErbB-3 (HER3), and ErbB-4 (HER4). All ErbB proteins have an extracellular cysteine-rich ligand-binding domain, an alpha-helical transmembrane domain, an intracellular tyrosine kinase domain, and a carboxy - terminal regulatory domain (Mitsudomi and Yatabe, 2010). The EGFR receptor of humans has seven ligands (Harris et al., 2003). Each ligand binds to the cysteine - rich extracellular domain of the ErbB receptor leading to the formation of homo or heterodimers. This dimerization is followed by autophosphorylation of specific tyrosine residues within the cytoplasmic regulatory domain, which activates the receptor and serves as docking sites for several adaptor proteins such as phospholipase C $\beta$ , CBL, GRB2, SHC, and p85. Several downstream pathways, such as Ras/Raf/mitogen-activated protein kinase (MAPK) pathway, Phosphatidylinositol 3-kinase (PI $_3$ K)/Akt pathway, Phospholipase C (PLC)/ protein kinase (PKC) signalling cascade, and the signal transducer and activator of transcription STAT3 and STAT5 pathways, were activated by the ligand - dependent activation of EGFR pathway leading to cell proliferation, migration, adhesion, differentiation, survival, and apoptosis (Mitsudomi and Yatabe, 2010; Scaltriti and Baselga, 2006; Yarden and Sliwkowski, 2001). EGFR signalling pathways develop malignancy by regulating the cell cycle progression, anti- apoptotic property, activating angiogenesis and by boosting tumor cell motility and metastasis (Carcereny et al., 2015).

Like vertebrate EGFR, the *Drosophila* epidermal growth factor receptor (EGFR) is involved in normal embryonic and adult development and signaling activity (Ghiglione et al., 2002). It is synonymised as, faint little ball (flb) for embryonic phenotype, torpedo remarking an oogenesis defect observed in eggs, DER, and Ellipse for imaginal disc and adult eye phenotypes. It shares 40.5% of identity with the vertebral EGFR (Lusk et al., 2017). *Drosophila's* genome has only one member of the EGFR/ErbB family (Shilo, 2003). Different ligands in the *Drosophila* interact with the epidermal growth factor receptor (EGFR) thus leading to signal activation. These ligands are gurken (*grk*), spitz (*spi*), vein (*vn*), and keren (*krn*). Three of the four activating ligands *grk*, *spi*, and *krn* are homologous to TGF- $\alpha$ , a known ligand of the vertebrate EGFR, whereas, *vn* is homologous to mammalian neuregulin ligand. The major ligand Spitz (*spi*) is involved in many developmental processes. It is activated by two proteins Rhomboid-1 (Rho-1) and Star (S). Keren (*krn*) is a functional homologue to *spi* (Lusk, 2017). Gurken (*grk*) is restricted to oogenesis (Ghiglione et al., 2002). The secreted ligand Vein

(*vn*) acts in tissues where low activation levels are required (Schnepp et al., 1996). Other than these four extracellular ligands, EGFR of *Drosophila* has two other ligands, argos (*arg*) and kekkon (KEK). Argos (*arg*) acts as a ligand antagonist which acts by competing with other activating ligands thus forming a clamp-like structure around *spi*. The transmembrane protein Kekkone (KEK), is a part of negative feedbacks and it forms a non-functional heterodimer with the receptor (Ghiglione et al., 1999). Spitz, Keren, and Gurken produced as inactive transmembrane precursors, are retained in the endoplasmic reticulum and processed by the protein Star(S). It is further transported to the Golgi compartment where it is cleaved by Rhomboid (RHO) serine proteases to release the active secreted ligand form (Shilo, 2003). Ligand binding promotes EGFR dimerization which is further followed by autophosphorylation thus triggering the canonical DRK/SOS/RAS/RAF/DSOR1/Rolled (RI) pathway. The Downstream of Receptor Kinase (DRK), a homolog of mammalian Grb2 binds to phosphorylated tyrosines on EGFR through its SH2 domain. DRK adheres to SOS (Son of sevenless) which activates RAS by GDP to GTP conversion. RAS is positively regulated by EGF and negatively regulated by Sprouty (*sty*), which is an extracellular inhibitor of EGF signalling. RAS promotes activation of RAF which phosphorylates mitogen-activated Protein kinase (MAPK) / extracellular signal-regulated kinase (ERK) kinase (MEK). Activated MEK phosphorylates Rolled, the *Drosophila* homolog of MAP kinase (MAPK)/extracellular signal-regulated kinase (ERK), which further phosphorylates the transcriptional factors Pointed (PNT) and Yan. Once the EGF signaling pathway is activated, Yan gets repressed and PNT get activated leading to activation of the EGF target gene (Gabay et al., 1996; Lusk et al., 2017; Shilo, 2003).

Overexpression and somatic mutations of the EGFR account for lung cancer. More than 90% of EGFR mutations occur as short in-frame deletions in exon 19 or as point mutations in exon 21 (Bethune et al., 2010). DNA mutations in EGFR as detected by polymerase chain reaction (PCR) can occur in regions corresponding to the extracellular or intracellular portions of the protein. In non-small cell lung cancer, overexpression of EGFR or mutations in intracellular EGFR has been observed in 43-89% of cases. The EGFR gene may also undergo amplification (Bethune et al., 2010).



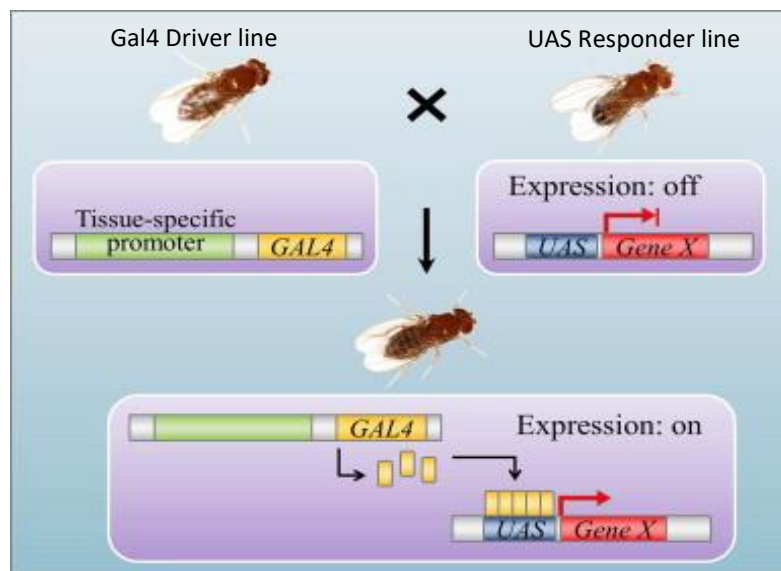
**Figure 6: Epidermal growth factor receptor (EGFR) pathway in *D. melanogaster*.** Vein, Spitz and Keren are the activating ligands of Egfr whereas, Argos inhibits the signalling. These ligands bind to the receptor leading to dimerization which is followed by autophosphorylation and activation of Drk, Sos, Ras, Raf, MEK, and Rolled resulting in activation of Pointed and Yan thus, causing target gene transcription. Sprouty is an extracellular inhibitor of EGF signalling. Question marks refer to the still unknown mechanism of Sprouty action. Vn= Vein, Spi= Spitz, Krn= Keren, Arg= Argos, Drk= Downstream of receptor, Sos= Son of sevenless, GDP= Guanosine diphosphate, GTP= Guanosine triphosphate, Ras= Rat sarcoma, Raf= Rapidly accelerated fibrosarcoma, MEK= Mitogen Activated Protein Kinase (MAPK)/ Extracellular Signal-regulated Kinase (ERK) kinase, MAPKK= MAPK kinase, Dsor1= Downstream of raf1 (according to Lusk et al., 2017).

## 1.8 UAS-Gal4 system

The binary UAS-Gal4 system (Fig. 7), the “workhorse” of fly transgenic models uses an activator from yeast, Gal4, which activates transcription in flies. This protein, extensively known for its DNA binding and transcriptional activation is placed downstream of a promoter/enhancer. The UAS-Gal4 system also uses a Gal4 target sequences, UAS (upstream activation sequence) (Brand and Perrimon, 1993; Brumby and Richardson, 2005). Both, Gal4 and UAS are divided into different fly line as driver (Gal4) and responder lines, respectively.

The location of expression is determined by the Gal4 line and the transcriptional control of UAS elements is done by the gene of choice (Duffy, 2002; Roman et al., 2001). By crossing the flies expressing Gal4 (driver) with the flies carrying UAS (responder), the promoter activates Gal4 expression, which binds to UAS thus enabling transgene expression in a temporal and spatial manner (Brand and Perrimon, 1993; Brumby and Richardson, 2005).

A significant number of Gal4 and UAS lines are accessible and Barry Dickson's group have initiated a RNAi library for all *Drosophila* genes, which allows conditional gene inactivation (Dietzl et al., 2007). *Btl*-Gal4 and *ppk4*-Gal4 are namely the common Gal4 lines available to address lung cancer in the airway epithelium of the *Drosophila* larvae. Gal4 activity in *Drosophila* is temperature dependent. Thus, temperature regulation can help to achieve wide expression levels of any responder (Duffy, 2002). The UAS-Gal4 system lack temporal control



**Figure 7: The binary UAS-Gal4 system of *Drosophila melanogaster*.** Fly with tissue specific enhancer/promoter fused with transcriptional Gal4 when crossed with the fly carrying upstream activating sequence (UAS) fused with gene of interest, the progeny will display the expression of the gene of interest, X, at the specific tissue of interest. (according to Cho et al., 2014).

over the target gene expression. This issue was addressed by the TARGET (temporal and regional gene expression targeting) system, which uses temperature sensitive repressor of Gal4, Gal80 molecule, which upon a shift to lower temperature (19°C) inhibit the expression of Gal4. Increase in the temperature (29°C), inhibits the Gal80 molecule thereby, leading to expression of the target gene (McGuire et al., 2004).



## 1.9 Aim of the study

Lung cancer is the second most diagnosed cancer worldwide. It is still associated with a poor prognosis because of the late diagnosis and insufficient therapy strategies. The poor clinical outcome points out the importance and necessity of new therapeutic approaches and development of the new drugs, that improve the prognosis even in late tumour stages. The traditional small molecule drug screening approaches with *in vitro* cell culture systems did not progress the clinical outcome over the last two decades. But many of the *in vitro* developed small molecules fail to meet the requirements or show higher toxicity *in vivo* (Pandey and Nichols, 2011). Though the *in vivo* screening models in mice or pig have an advantage over the traditional *in vitro* models, a large number of animals are needed to reach the expected result. Invertebrates like the *Drosophila melanogaster* offers a suitable model, where the orthologues of most cancer genes are conserved.

As mutations in the EGFR signalling pathway are one of the most frequent lung cancer inducers, the major aim of this study is to develop tailored lung cancer models in the fruit fly *Drosophila melanogaster* through the targeted expression of constitutively active EGFR (EGFR<sup>CA</sup>). The phenotypes caused by the expression of EGFR<sup>CA</sup> in pan-tracheal and Inka cell driver lines will be studied in detail so that these *Drosophila* lung cancer models become a suitable choice for drug-screening approaches and drug-resistance experiments.

## 2. Materials and Methods

### 2.1 Devices

Analytical balance (ABS)	Kern & Sohn GmbH (Balingen, Germany)
Axio Imager	Zeiss (Oberkochen, Germany)
Balance (MXX-412)	Denver Instrument GmbH (New York, USA)
Centrifuge (5415 D)	Eppendorf (Hamburg, Germany)
Centrifuge (5417 R)	Eppendorf (Hamburg, Germany)
Incubator (TH30)	Edmund Bühler GmbH (Tübingen, Germany)
Incubators (WB250K)	Heinrich Eimecke GmbH (Kiel, Germany)
LabGard (IBS)	INTEGRA biosciences GmbH (Biebertal, Germany)
Light source (U-RFL-T)	Olympus (Hamburg, Germany)
Magnetic stirrer (RET)	IKA® (Staufen, Germany)
Magnetic stirrer (MR3001)	Heidolph (Schwabach, Germany)
Multipette (Xstream)	Eppendorf (Hamburg, Germany)
Power supply (EV245)	ConsortNT (Nürnberg, Germany)
Stereo microscope (MZ10F)	Leica Microsystems (Wetzlar, Germany)
Stereo microscope (S6E)	Leica (Wetzlar, Germany)
Stereo microscope (Stemi 506)	Zeiss (Oberkochen, Germany)
Stereo microscope, fluorescence (SZX12)	Olympus (Hamburg, Germany)
Spectrophotometer (DS-11)	DeNovix® (Wilmington, USA)
Thermocycler (Labcycler)	SensoQuest GmbH (Göttingen, Germany)
Thermomixer comfort	Eppendorf (Hamburg, Germany)
Water bath	Memmert (Schwabach, Germany)

### 2.2 General materials

Air-permeable membrane	nerbe plus GmbH (Winsen, Germany)
Ceapren stopper (Ø 50 mm)	Greiner Bio-One (Kremsmünster, Austria)
Cellulose stopper (Ø 29 mm)	nerbe plus GmbH (Winsen, Germany)
Cell spreader	nerbe plus GmbH (Winsen, Germany)

Combitips (advanced, 10 ml)	Eppendorf (Hamburg, Germany)
Cover slips (24 x 50 mm)	Carl Roth (Karlsruhe, Germany)
Deep well plates, 96-well	nerbe plus GmbH (Winsen, Germany)
<i>Drosophila</i> food vial (50 ml)	nerbe plus GmbH (Winsen, Germany)
<i>Drosophila</i> food vial (16 ml, 175 ml)	Greiner Bio-One (Kremsmünster, Austria)
Falcon tubes (15 ml, 50 ml)	nerbe plus GmbH (Winsen, Germany)
Filtropour BT50	Sarstedt (Nümbrecht, Germany)
Forceps	neoLab® (Heidelberg, Germany)
Petri dishes, plastic	nerbe plus GmbH (Winsen, Germany)
Reaction tubes, low binding (0.2, 0.5, 1.5, 2 ml)	nerbe plus GmbH (Winsen, Germany)
Reaction tube, screw, 2 ml	Sarstedt (Nümbrecht, Germany)
Serological pipettes (5 ml, 10 ml, 25 ml)	nerbe plus GmbH (Winsen, Germany)
Slides	Carl Roth (Karlsruhe, Germany)
Tips, low-binding (10, 100, 1000 µl)	nerbe plus GmbH (Winsen, Germany)

### 2.3 General chemicals

Apple juice	Stute (Paderborn, Germany)
brewer`s yeast	Leiber (Bramsche, Germany)
Cornmeal	Biohof Heidelicht (Gerdau, Germany)
Molasses	Sigma Aldrich (Munich, Germany)
Methyl 4-Hydroxybenzoate	Sigma-Aldrich (Munich, Germany)
reagentplus® (nipagin)	
Normal goat serum	Polysciences Inc. (Warrington, USA)
PFA	Kanne Brottrunk (Selm-Bork, Germany)
Sugar beet syrup	BD (Franklin Lakes, USA)
Tryptone	BD (Franklin Lakes, USA)
Yeast extract	Stute (Paderborn, Germany)

Unless not otherwise stated, all other chemicals were purchased from Carl Roth (Karlsruhe, Germany).

## 2.4 Fly Husbandry

Normal Medium (NM), 500ml:

31.25 g	Cornmeal
31.25 g	Brewer's yeast
10 g	Glucose monohydrate
15 g	Molasses
15 g	Sugar beet syrup
5 g	Agar-agar

- Add 500 ml H<sub>2</sub>O
- Cook for 10-15 min in the water bath
- Autoclave for 15-20 min
- Cool down to 60°C
- Add: 5 ml 10% (v/v) propionic acid  
15 ml 10% (w/v) nipagin (in 70% Ethanol)
- Transfer directly to appropriate fly vials
- Store at 4°C after cooling

Fly stocks were kept in the normal medium in *Drosophila* food vials at 18°C at a 12h light/dark cycle. Flies required for the experiments were collected from the stock and maintained at room temperature (RT). The culture was changed every two to three weeks. Flies can be inspected and handled once they are anesthetized using nitrogen and placed on ice.

## 2.5 *Drosophila* fly lines and crossings

Concentrated Medium (CM):

43 g	Cornmeal
25 g	Yeast extract
25 g	Sucrose
5 g	Agar-agar

- Add 500 ml H<sub>2</sub>O
- Cook for 10-15 min in the water bath
- Autoclave for 15-20 min

- Cool down to 60°C
- Add: 5 ml 10% (v/v) propionic acid  
15 ml 10% (w/v) nipagin (in 70% Ethanol)
- Transfer directly to appropriate fly vials
- Store at 4°C after cooling

The desired genotypes were produced by crossing of fly lines to be able to utilize the Gal4/UAS system. Adult males and virgin females were collected. They were mated in a ratio of 1:3. Gal4/UAS lines crossed with  $w^{1118}$  served as controls. These populations were maintained in a vial culture at 25 °C. At least every four days the parental flies were transferred to fresh food vials. L3 larvae of the F1 generation were collected to perform all experiments. All the fly lines present were either generated in the same laboratory, gifts from other research groups or from the Bloomington stock center. The name of the donors and Bloomington stock numbers are given in Tab. 1.

**Table 1: *Drosophila* fly lines**

Name	Genotype	Vendor
<i>ppk4-Gal4</i>	<i>yw67c23; pkk4- gal4; +/+</i>	Provided by Michael Welsh, University of Iowa, USA
<i>btl-Gal4</i>	<i>w-; btl-GAL4, UAS-GFP/Sp; TM2/TM6b</i>	Provided by Maria Leptin Group Heidelberg, Germany
<i>UAS-Egfr<sup>CA</sup></i>	<i>w [*]; P{w[+mC] =Egfr.2.A887T.UAS}8-2</i>	Bloomington (9533)
<i>w<sup>1118</sup></i>	<i>w [1118]</i>	Bloomington (5905)
<i>c929-Gal4</i>	<i>P{GawB}crc929</i>	Provided by Kim Kaiser, Glasgow, UK

## 2.6 Microscopy

Microscopic analysis was performed with dissected tracheae or the whole larvae. Various specific staining procedures were performed with the dissected trachea for analysis of the epithelial structure and evidence of distinct proteins (2.6.2). Based on the microscopic analysis, the epithelial thickness, the nuclei number and the nuclei size of the dissected

tracheal trunks were evaluated (2.6.3). The quantification of the photos was done using the microscope AxioVision software (AxioVision SE64 Rel. 4.8).

### 2.6.1 Dissection of trachea, intestine and filet preparation from L3 larvae

➤ Phosphate buffered saline (PBS):

136 mM	NaCl
2.7 mM	KCl
1.5 mM	KH <sub>2</sub> PO <sub>4</sub>
7 mM	Na <sub>2</sub> HPO <sub>4</sub>

- Add H<sub>2</sub>O
- Adjust pH to 7.3 with HCl
- Autoclave

➤ Hemolymph-like solution (HL<sub>3</sub>):

5 mM	KCl
70 mM	NaCl
1.5 mM	CaCl x 2H <sub>2</sub> O
10 mM	NaHCO <sub>3</sub>
20 mM	MgCl <sub>2</sub> x 6H <sub>2</sub> O
5 mM	trehalose
115 mM	saccharose
5 mM	HEPES

- Add H<sub>2</sub>O
- Adjust pH to 7.1 with NaOH
- Filter sterilize
- Store at 4°C

L3 larvae were used widely for this experiment. The required tissue has to be dissected to quantify the nuclei size and to perform various immunohistochemical analyses. The larvae were washed to get rid of remaining medium using PBS. The tracheal dissection was done by removing the head region with the dark mouth hooks at first. With a forceps the larvae were held near the posterior spiracles. Another forceps was used to grab the cuticle near the first one and pulled over the larval body until total removal. The branches of the trachea and dorsal

trunks stayed at the posterior part on the spiracles. Remaining organs around the trachea were removed carefully. With two needles in the anterior head and the posterior region the larvae were fixed on the ventral side. With the help of two sharp forceps the larval body was opened along the full length. From the body cavity, the main parts of the fat body and the intestines were removed. The cuticle was stretched outwards towards both sides and fixed with needles. The muscle layer on the inner part of the cuticle and remaining body parts were removed.

### 2.6.2 Immunohistochemistry

- Fixation solution:
  - 4 % (w/v) paraformaldehyde (PFA)
    - Add PFA with PBS
    - Heat to 50-60 °C until complete dissolution
    - Storage at -20 °C
- Washing buffer (PBST):
  - 0.1 % (w/v) triton X-100
  - Add with PB
- Blocking buffer:
  - 10 % (v/v) normal goat serum
  - Add with PBST

A dissection dish was taken; on which 5- 10 larval tissues were dissected using cold HL<sub>3</sub> buffer (2.6.1). So obtained tissues were collected in a 1.5 ml low-binding reaction-tube lid. After removing the HL<sub>3</sub> buffer solution, the tissue was fixed 50 µl fixation solution for 30 min. Later, these tissues underwent three consecutive washing steps with 100 µl washing buffer, 10 min each. Then using 100 µl blocking buffer for 1 hour the blocking step was performed. First antibody was incubated overnight at 4°C (Tab. 2). Before incubation of the second antibody, three additional washing steps with 100 µl washing buffer for 10 min were carried out next day (Tab. 2). At least 3 hours at RT or overnight at 4 °C in the darkness the second antibody was incubated. It was followed by three successive washing steps with 100 µl washing buffer

20 min each. Before microscopically analyzing the tissue, a drop of Roti®-Mount was used to arrange the tissue on a slide. To avoid photo bleaching this slide preparation was enclosed by a cover slip and kept in darkness until the analysis. All steps were carried out in the lid of a 1.5 ml reaction tube except, the fixation process with the filet preparation, which was conducted in the dissection dish with a drop of fixation buffer.

**Table 2: Antibodies and dilutions**

Primary antibodies	Dilution	Vendor
$\alpha$ -Coracle, mouse (C615.16)	1:500	DSHB (Iowa City, USA)
$\alpha$ -phospho-Histone H3 (Ser10) AF 488 conjugate, rabbit	1:100	Cell Signaling Technology (Danvers, USA)
$\alpha$ -GFP, mouse (12A6-b)	1:100	DSHB (Iowa City, USA)
<b>Fluorescent dyes/ conjugated proteins</b>		
Roti®-Mount FluorCare DAPI (4,6-diamidino-2-phenylindole)	-	Carl Roth (Karlsruhe, Germany)

All antibodies were stored in 50 % glycerol at -20 °C. Before being used, these antibodies were diluted in blocking buffer.

### 2.6.3 Quantification of nucleus number and size

For the proper quantification, it was necessary to ensure that the same segment of the trachea was obtained in all larvae during dissection. This enabled the use of the same segment for every measurement, as it was feasible to count the segments from the posterior end, the first or second segment from the posterior end used for the quantification. As mentioned above, before being analyzed the dissected tracheae were arranged on a slide with a drop of Roti®-Mount (Tab. 2). Mainly DAPI channel was used to take the pictures in 10x or in 20x magnification. Based on the size of the larvae and tracheae the magnification was adjusted. One to five slices from the top to the bottom of the tracheal tube were made using the Z-stack unity. Based on the requirement the DAPI channel was adjusted. AxioVision was used for measuring the nucleus number, whereas, Image J software was used to quantify the nucleus size.



## **2.7 Collection of L1 and L2 larvae**

Adult males and virgin females were mated in a ratio of 1:3. After 12 hours of the mating time, the adult flies were transferred to new vials. The vials with embryos were kept at RT. After 24 hours the L1 larvae and 48-56 hours, the L2 larvae were collected by transferring the food medium with larvae to a glass dish. The larvae were collected carefully using forceps under the microscope and transferred to new vials, each vial containing around 20 larvae and kept at RT. The developments were observed on everyday basis.

## **2.8 Development of pupae and larvae**

The conversion from the embryos to adult flies was monitored over a period of two to three weeks. The main differences observed in pupae and adult formation was determined. On an everyday basis, newly developed pupae and adult flies were counted.

## **2.9 Statistics**

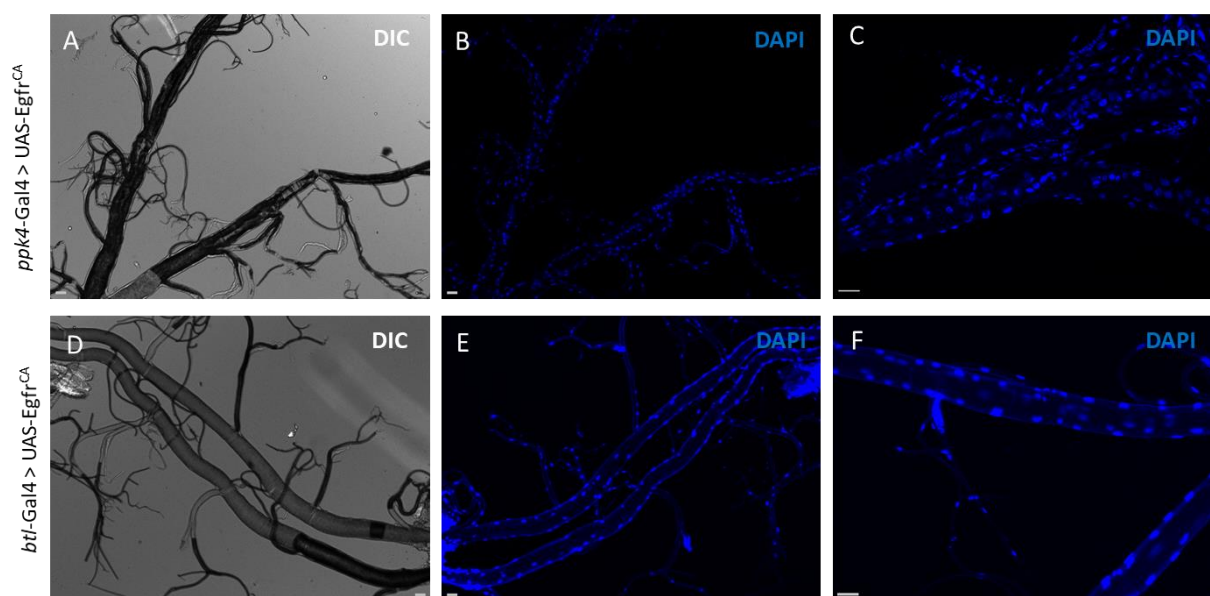
For statistical analysis and generating graphs, Microsoft Excel was used. As data showed a normal distribution of the data an unpaired t-test was performed.

### 3. Results

To study cancer induced changes in the *Drosophila* tracheal system, as the first step, screening of trachea driven Gal4 lines was done, with a focus on tumour phenotypes including meta and hyperplasia of the corresponding cells. Ectopic expression of a constitutively active *Drosophila* epidermal growth factor receptor isoform (Egfr<sup>CA</sup>) was induced in the airway epithelium of larvae by using the responder line UAS-Egfr<sup>CA</sup>. Initially, to achieve Egfr over expression in the entire trachea, Egfr<sup>CA</sup> responder lines were crossed with two different trachea-specific Gal4 driver lines, *ppk4*-Gal4 and *btl*-Gal4 (Fig. 8). In contrast, *c929*-Gal4 was used to express Egfr<sup>CA</sup> only in the dorsal trunks of the tracheal system.

#### 3.1. Targeted expression of Egfr<sup>CA</sup> throughout the larval tracheal system

A more prominent pan tracheal cancer-like phenotype was observed with *ppk4*-Gal4 than with *btl*-Gal4, and hence all the further experiments were conducted using *ppk4*-Gal4 as the driver line to upregulate Egfr<sup>CA</sup> throughout the trachea. *ppk4*-Gal4 crossed with the wildtype *w*<sup>1118</sup> was used as controls. The larval tracheae were dissected carefully, stained and examined under the microscope to study in detail the morphological and structural difference caused by expression of Egfr<sup>CA</sup> (*ppk4*-Gal4 > UAS- Egfr<sup>CA</sup>) against the control group (*ppk4*-Gal4 > *w*<sup>1118</sup>). A consistent pattern of tumour like phenotypes was evident from the early larval stage, throughout all larval stage confirming constant ectopic expression of Egfr<sup>CA</sup> in the trachea.

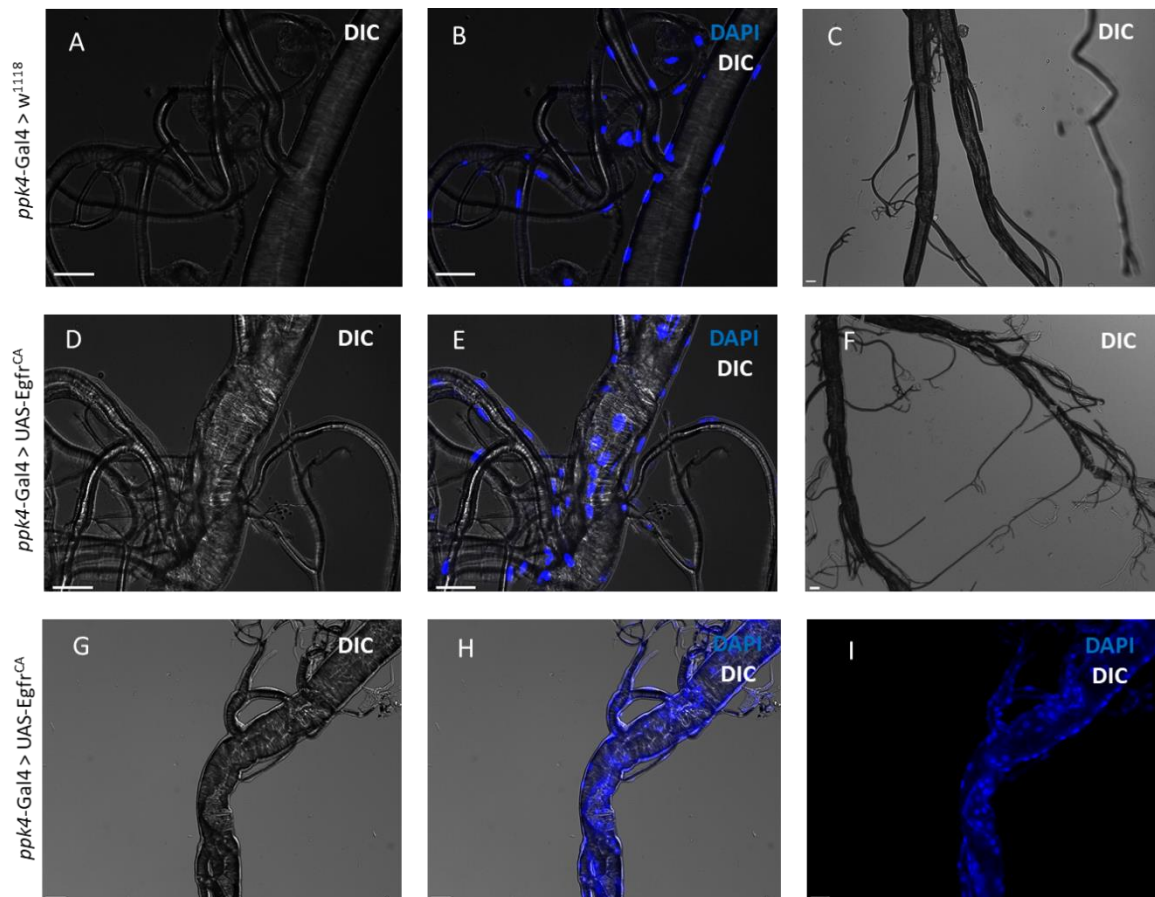


**Figure 8: Tracheal phenotype caused by Egfr<sup>CA</sup> expression induced by *ppk4*-Gal4 and *btl*-Gal4, respectively.** The pan tracheal driver lines *ppk4*-Gal4 and *btl*-Gal4 crossed with the responder line UAS-

$Egfr^{CA}$ . The first row displays dissected trachea of *ppk4*-Gal4 larvae (**A-C**) and the second row show the trachea of *btl*-Gal4. Both larval tracheas were examined with DIC (**A-D**) and analysed following DAPI staining (**B, C, E, F**) under the microscope. Scale = 50  $\mu$ m

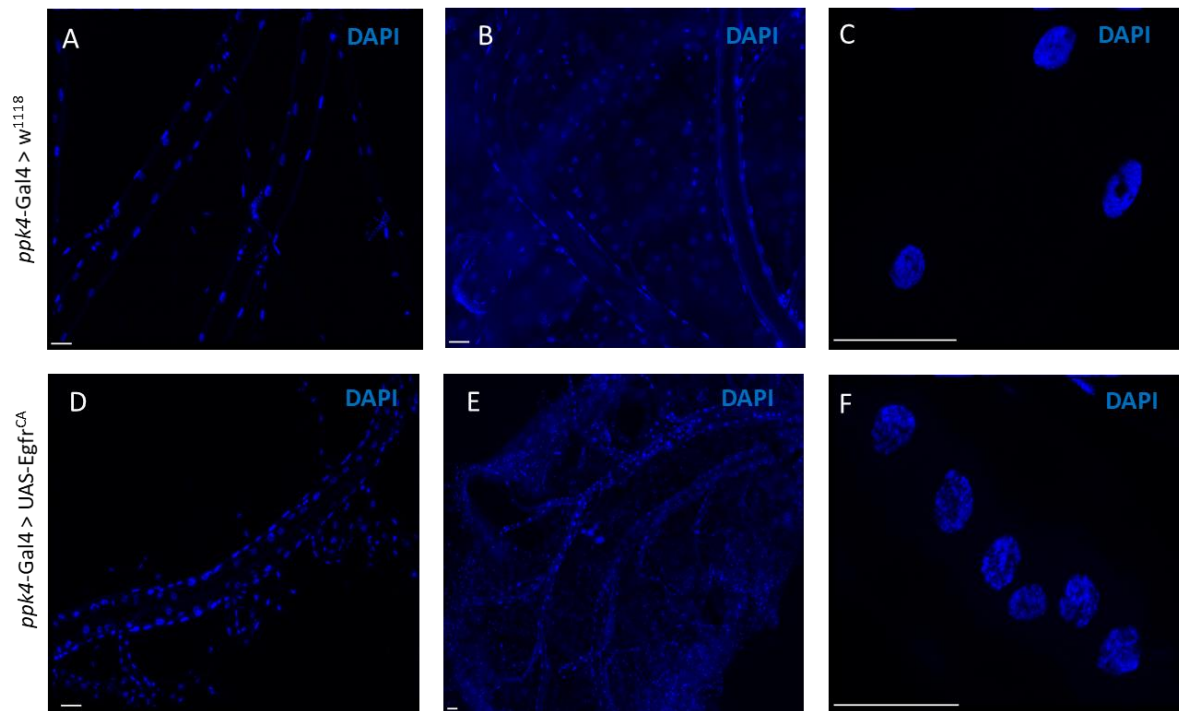
### 3.1.1 Morphological changes in trachea expressing $Egfr^{CA}$

The morphological changes observed included increased epithelial thickness (Fig. 9 D, E), tracheal malformation (Fig. 9 D-I), and increase in number and size of nuclei (Fig. 9 E, H, I); (Fig. 9 B). Moreover, the corresponding animals show increased lethality in initial larval stages (Fig. 13, 14), and significantly low numbers of newly hatched male flies were observed (Fig. 16). All the above-mentioned pathological changes were observed in different degrees in all dissected trachea with upregulated  $Egfr^{CA}$ . It seemed that the onset of increase in thickening of the airway epithelium and the malformation of the trachea occurred very early in larval developmental stages and they have an important role when it comes to the survival chance of a larvae. A higher number of nuclei were observed on those areas where the trachea malformation is severe suggesting signs of increased cell division.

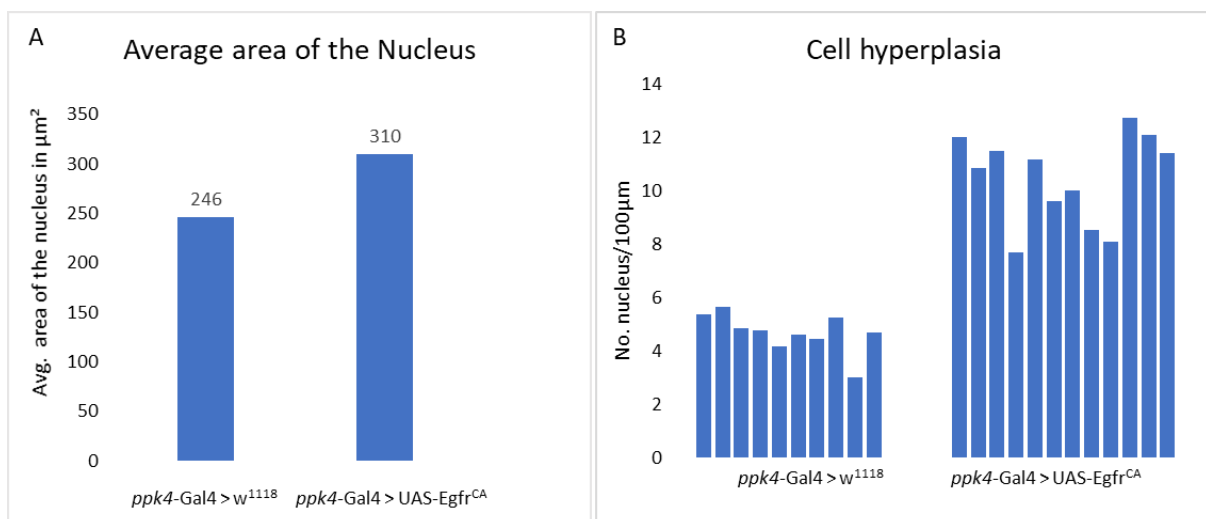


**Figure 9: Morphological and structural changes caused by ectopic expression of  $Egfr^{CA}$  in the trachea.** The driver line *ppk4*-Gal4 crossed with the responder line *UAS-Egfr<sup>CA</sup>* was compared with the same driver line crossed with *w<sup>1118</sup>*(control). The first row displays dissected trachea of control larvae (**A-C**).

The second and third rows show the dissected trachea of larvae with over expressed  $Egfr^{CA}$  (**D-I**). Both larval tracheas were examined with DIC (**A-H**) and DAPI (**B, E, H, I**) under the microscope. Scale = 50  $\mu m$ .



**Figure 10:  $Egfr^{CA}$  upregulation leads to changes in number and size of the nucleus of the tracheal cells.** The first row displays dissected trachea of control larvae (**A-C**) and the second row illustrates dissected trachea of the upregulated  $Egfr^{CA}$  larvae (**D-F**). All the larval tracheae were stained with DAPI and examined under the microscope. **B** and **E** illustrate larval fillet preparation. **D** and **E** show a significant increase in the number of nuclei whereas, **F** shows an increase in the size of the nuclei. Scale = 50  $\mu m$ .



**Figure 11: Comparison of number and size of nucleus in control and upregulated  $Egfr^{CA}$ .** 10-12 L3 larvae from both *ppk4-Gal4 > w<sup>1118</sup>* and *ppk4-Gal4 > UAS-Egfr<sup>CA</sup>* were dissected and stained with DAPI to visualise the nucleus. Area of 20 nucleus from both groups were measured and its average was calculated (**A**). The number of nucleus between 2 primary branches (S1- S2, S2- S3) were counted in both groups (**B**). Statistical significance in both cases were tested with an unpaired t-test. (**A**)  $p = 0.027$ , (**B**)  $p = 2.65 \times 10^{-9}$ .

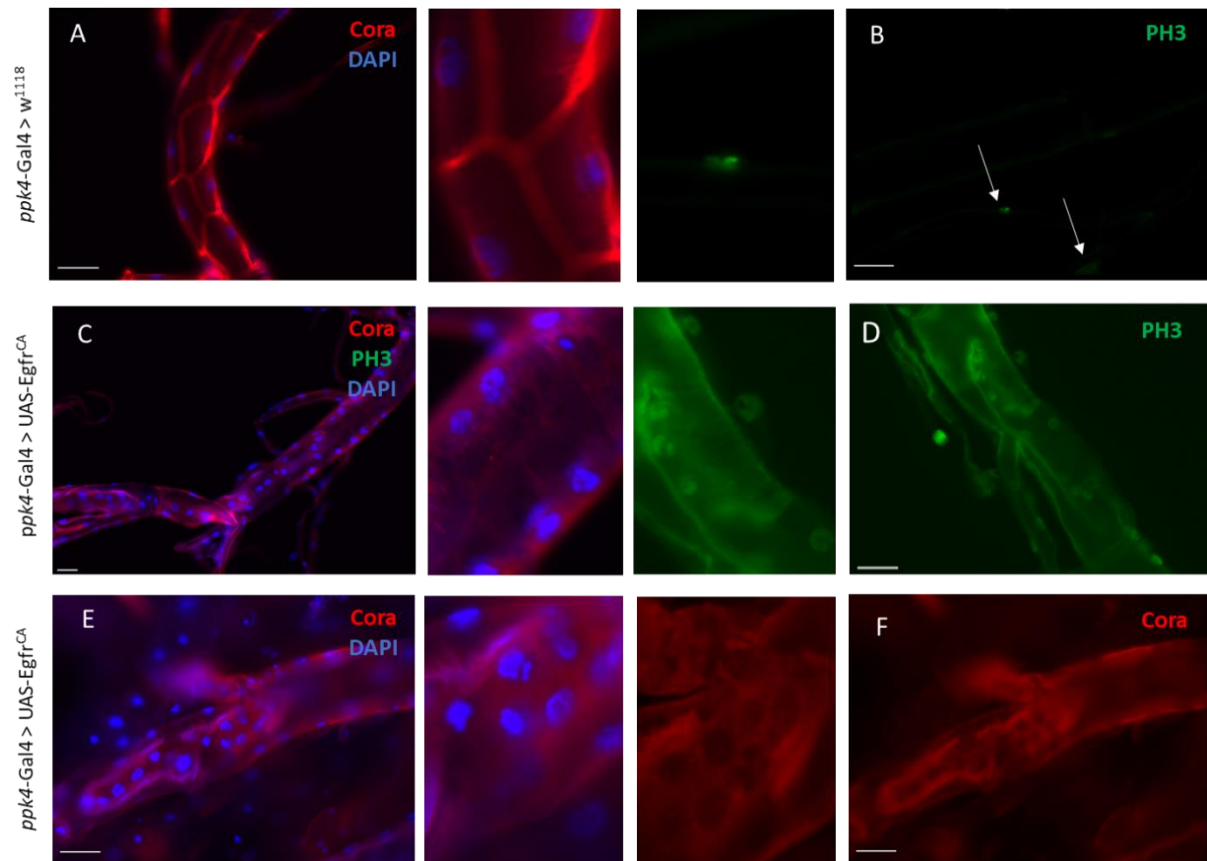
To quantify the number and sizes of the nuclei, 10-12 larvae of each cross were dissected and the trachea were stained. The nuclei between two main branches of the trachea were counted and the areas of the nuclei were measured. It was observed that the number of nuclei in *ppk4-Gal4 > UAS-Egfr<sup>CA</sup>* were significantly higher than the control group (Fig. 10, 11 B). The average number of nucleus between 2 main branches in *ppk4-Gal4 > w<sup>1118</sup>* were 4,6/100µm and that of *ppk4-Gal4 > UAS-Egfr<sup>CA</sup>* were 12,3/100µm. The average area of the nucleus in *ppk4-Gal4 > UAS-Egfr<sup>CA</sup>* was significantly higher than the control group, which is an indicator of upregulated cell division.

### 3.1.2. Analysis of the trachea of *Egfr<sup>CA</sup>* overexpressing animals

A cellular level analysis using different staining reagents and immunohistochemical methods was performed to understand the structural changes in trachea of with *Egfr<sup>CA</sup>* expression. Antibodies directed against Coracle (Cora), a septate junction related protein, were used in the immunohistochemical staining to visualize the cell border. DAPI was used further to stain the cell nucleus.

An organised cell pattern with typical cell shapes were observed in the tracheal epithelium of the control larvae stained with anti-Coracle (Fig. 12 A). While, an irregular cellular pattern with an indefinite cellular shape was noticed in the *Egfr<sup>CA</sup>* upregulated tracheal epithelium (Fig. 12 C, E, F). Cell nucleus enlargement with an increase in the number of nuclei were again noticed. The overall structure of the tracheal epithelial seems to be pseudo-stratified in *Egfr<sup>CA</sup>* upregulated larvae stained with Coracle and DAPI.

The DNA structure in the enlarged nucleus was decentralized and relaxed whereas the smaller and condensed nuclei were observed in the control (Fig. 10 C, F). A staining with phospho-histone H3 (pH3), the mitosis marker was performed to confirm the mitotic activity. Most of the nuclei in *Egfr<sup>CA</sup>* tracheae were positive for pH3 in contrast to a few of the nuclei in the control were positive (Fig. 12 B, D). It clearly indicates an enhanced mitotic activity in *Egfr<sup>CA</sup>* trachea.



**Figure 12: Cellular level analysis of upregulated  $Egfr^{CA}$ .** The first row displays trachea of control (A, B). The second and third rows displays trachea of the upregulated  $Egfr^{CA}$  larvae (D-F). The middle columns show the region of interest in magnified version. Coracle antibody staining (Cora) (A, C, E, F), DAPI (A, C, E) and phospho-histone H3 (pH3) (B, D) Scale = 50  $\mu$ m.

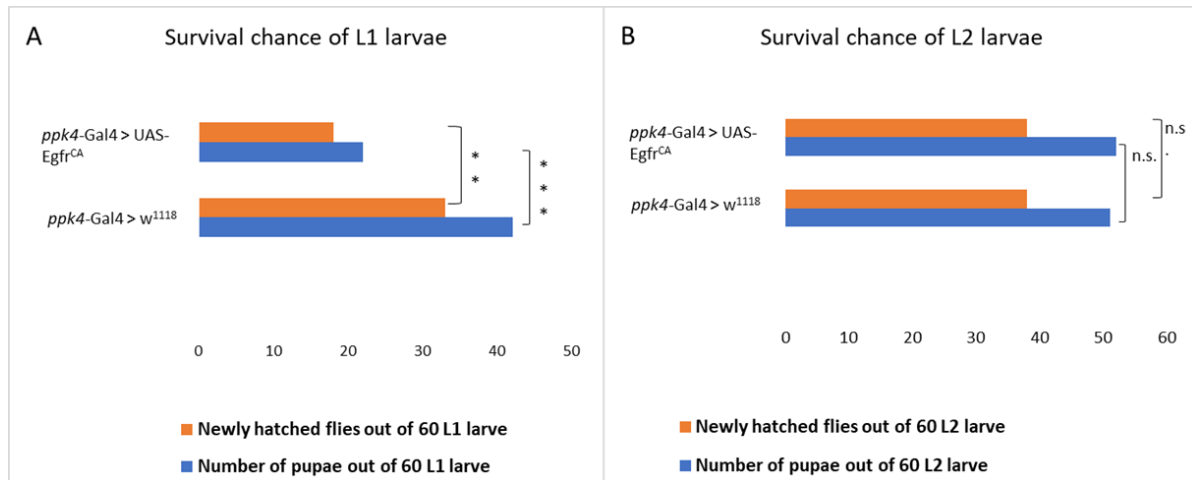
### 3.1.3. Increased lethality in early larval stages

An ectopic expression of  $Egfr^{CA}$  by crossing with the *ppk4*-Gal4 driver line led to an increased mortality rate in early larval stage. It was observed that 38 out of 60 L1 larvae died either in the food-medium or they crawled out from the food and died on the wall of the vial. In the control group only 18 of the 60 L1 larvae died (Fig. 13, 14). The increased lethality of L1 larvae with upregulated  $Egfr^{CA}$  was statistically highly significant ( $p < 0.0005$ ). The survived  $Egfr^{CA}$  larvae could develop further to pupae and adult flies without any significantly increased lethality. Out of 22 pupae, 18 could develop into adult flies. Fig. 13 (A).

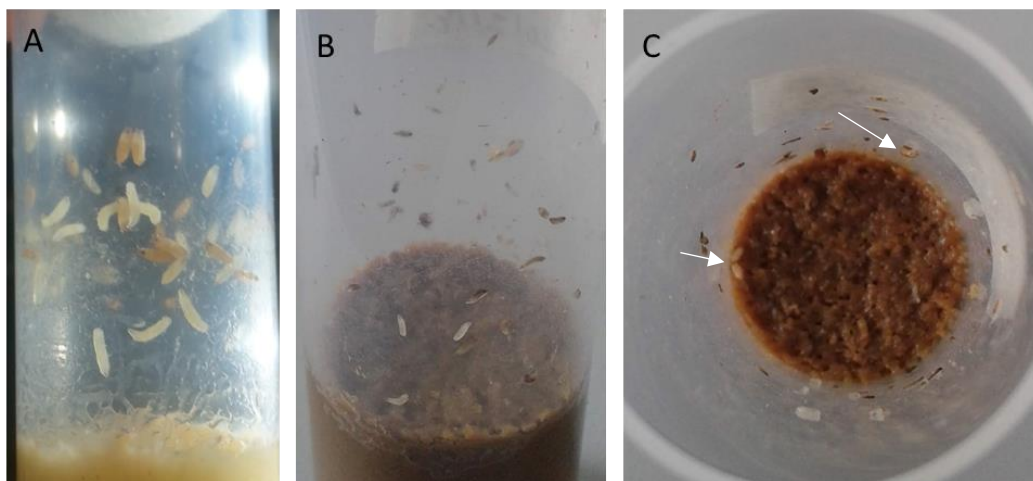
To quantify the survival chance of L2 larvae with over expressed  $Egfr^{CA}$  a similar experiment was initiated. The findings suggested that there was no increased risk of death for L2 larvae which are older than 36 hours in comparison with the control larvae. 52 out of 60 L2 larvae from *ppk4*-Gal4 > UAS- $Egfr^{CA}$  could reach the pupae stage and 38 of them developed to adult



fly. In the control larvae the numbers were almost similar. As expected, there was no statistical significance observed between the two groups.  $p=1$  for both the groups. Fig. 13 (B).

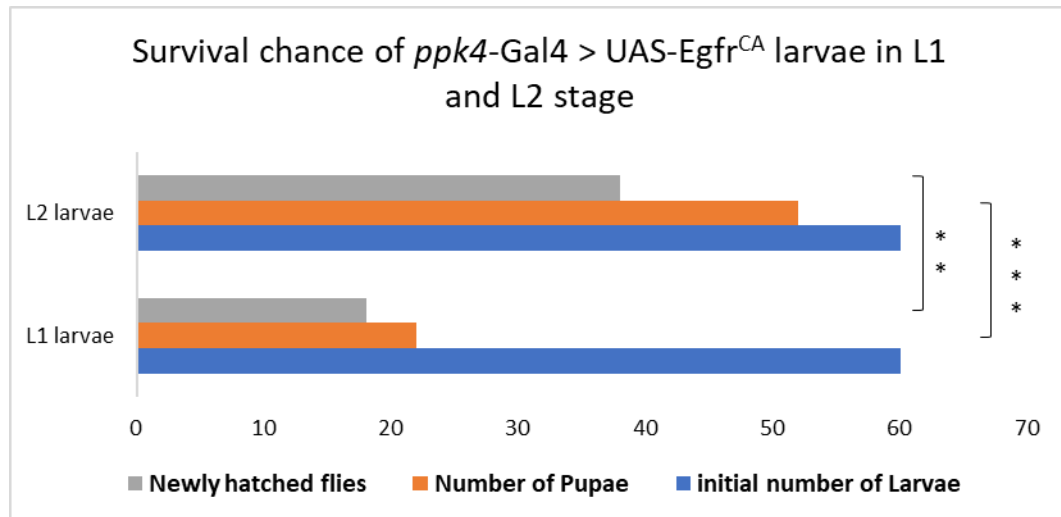


**Figure 13: Effect of deregulated  $Egfr^{CA}$  expression on larval lethality in L1 and L2 stages.** 60 L1 larvae from both, *ppk4-Gal4 > w<sup>1118</sup>* and *ppk4-Gal4 > UAS-Egfr<sup>CA</sup>* which were not older than 24 hours after hatching the eggs, were collected carefully and transferred to new vials with 20 larvae each. Further developments were observed for another 14 days (A). The same procedure was performed with the L2 larvae (chosen L2 larvae were 36-48 hours old) (B). The statistical significance was tested with unpaired t-test, \* =  $p < 0.05$ , \*\* =  $p < 0.005$ , \*\*\* =  $p < 0.0005$ , n.s. = not significant.



**Figure 14: Incidence of death during the life stages of  $Egfr^{CA}$  upregulated larvae.** Larval development after 6 Days of *ppk4-Gal4 > w<sup>1118</sup>* (A). The control larvae went through a normal development into pupae whereas  $Egfr^{CA}$  upregulated larvae, after 6 days showed an increased incidence of death in the early larval stage (B, C). The survived larvae could reach the pupation (C arrow) without any significant time lag.

An increased incidence of larval lethality was observed till the early stage of the L2 larvae. A comparison of the results of upregulated  $Egfr^{CA}$  L1 and L2 larvae was performed. This implies that the L1 larvae experienced extremely high lethality when compared to the L2 larvae. This result was statistically significant ( $p = 0.0004$ ).



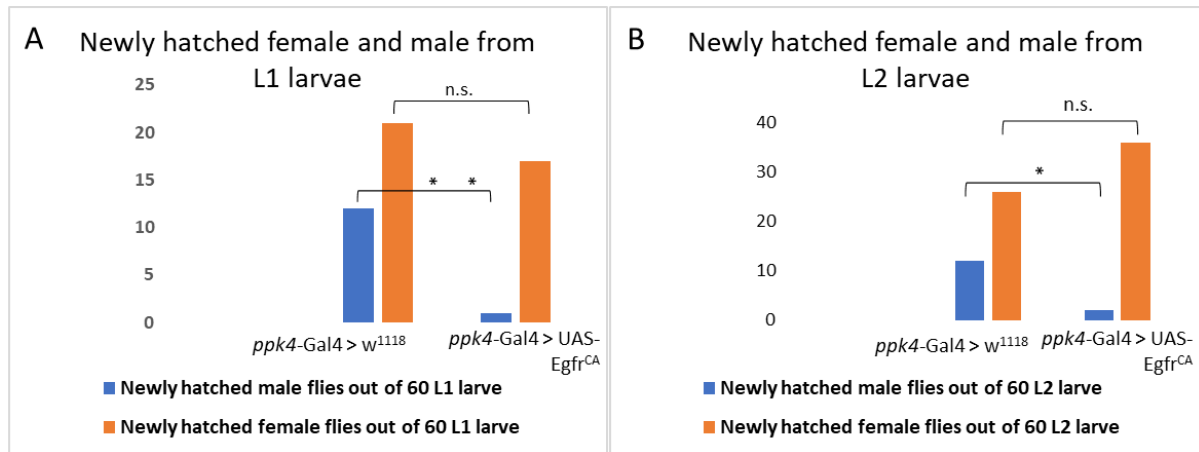
**Figure 15: Effect of overexpressed  $Egfr^{CA}$  on larval lethality in L1 and L2 stages.** Larval development of 60 L1 and L2 larvae of  $ppk4-Gal4 > UAS-Egfr^{CA}$  was observed over 15 days. Statistical significance was tested with unpaired t-test, \* =  $p < 0.05$ , \*\* =  $p < 0.005$ , \*\*\* =  $p < 0.0005$ , n.s. = not significant

### 3.1.4. Female biased sex ratio in $Egfr^{CA}$ expressing pupae

One of the observations of the above experiment was that the number of newly hatched male flies were much lesser than the female flies when  $Egfr^{CA}$  was expressed in the trachea. To quantify this observation, the newly hatched flies from L1 and L2 larvae of both  $ppk4-Gal4 > w^{1118}$  and  $ppk4-Gal4 > UAS-Egfr^{CA}$  were segregated based on their sex. Among the 18 survived flies from 60 upregulated  $Egfr^{CA}$  L1 larvae, only 1 turned out to be male. In contrast, in control L1 larvae, among the 33 survived flies 12 were male (Fig. 16 A). This difference in number of male flies was statistically highly significant ( $p=0.005$ ). No significant difference was evident in the number of female hatched flies ( $p=0.27$ ) (Fig. 16 A).

This same pattern was repeated within L2 larvae of both  $ppk4-Gal4 > w^{1118}$  and  $ppk4-Gal4 > UAS-Egfr^{CA}$ . From 60  $Egfr^{CA}$  L2 larvae, 2 were males and 36 were females. While in the control cross 12 were male and 26 were female. The statistical difference in the number male hatched flies was significant ( $p=0.007$ ) (Fig. 16 B). An insignificant statistical value was depicted by the difference in number of female hatched flies ( $p= 0.066$ ) (Fig. 16 B).





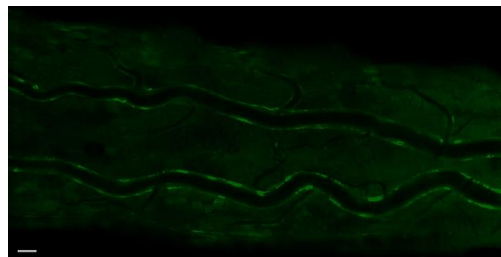
**Figure 16: Disproportional distribution of the sexes in upregulated  $Egfr^{CA}$  survivors.** Based on the gender newly enclosed flies from 60 larvae of both control and overexpressed  $Egfr^{CA}$  in L1 and L2 larval stage were separated. Statistical significance was tested with unpaired t-test, \* =  $p < 0.05$ , \*\* =  $p < 0.005$ , \*\*\* =  $p < 0.0005$ , n.s. = not significant.

### 3.2 Targeted expression of $Egfr^{CA}$ in tracheal endocrine cells of *Drosophila*

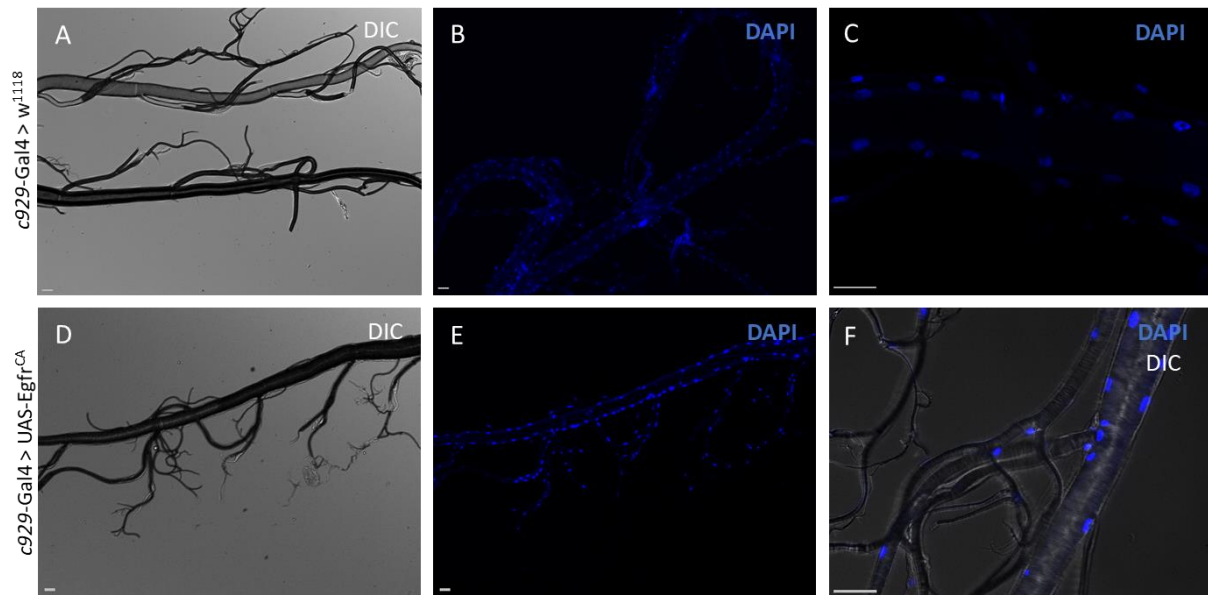
#### Larvae

*c929-Gal4* (*amontillado*- or *Amon-Gal4*) was used to restrict the ectopic expression of  $Egfr^{CA}$  to a smaller group of cells called Inka cells, the ecdysis triggering hormone (ETH) producing cells, associated with the dorsal trunks of the larval tracheal system. They are found at the branching points to the primary branches of the trachea (Fig. 17).

*c929-Gal4* driver line crossed with the wildtype  $w^{1118}$  served as control. The dissected and stained trachea of both upregulated  $Egfr^{CA}$  and control were examined under a microscope. There were no significant morphological changes noticed in the airway system in the larvae with overexpressed  $Egfr^{CA}$  compared to the control trachea. The nuclei showed no relevant changes in their numbers and sizes. Malformations and thickening of cells were not present in either group (Fig. 18).

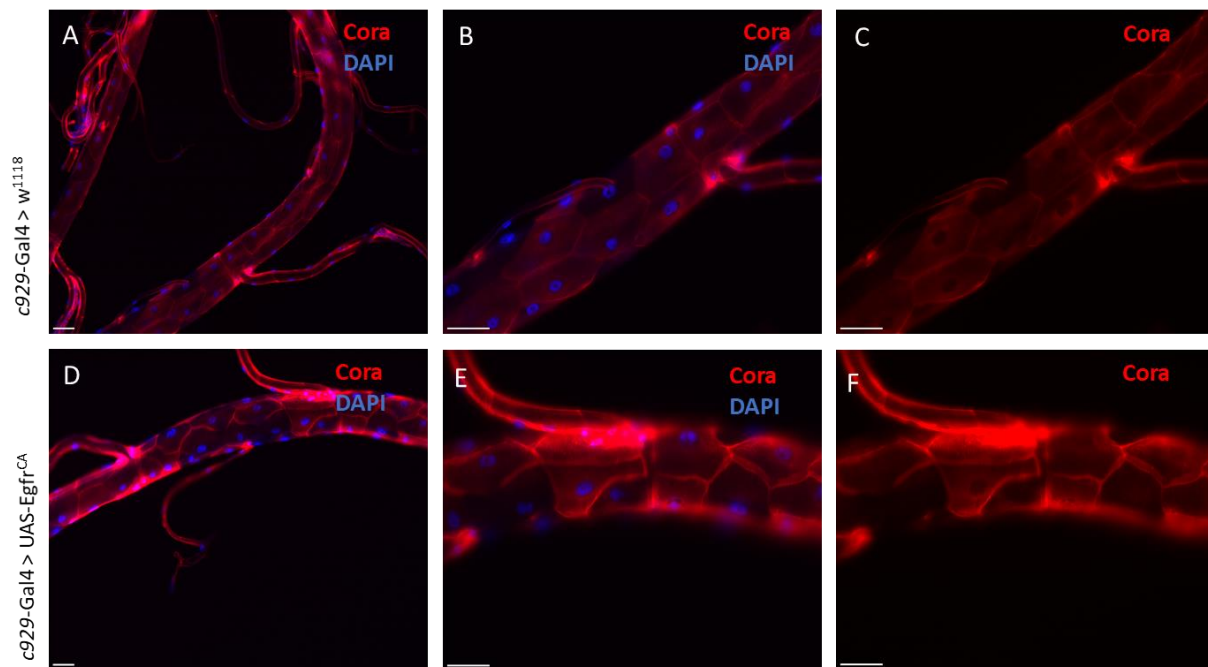


**Figure 17: The airway epithelium of *Drosophila* larvae with Inka cells.** These cells are found at the branching points to the primary branches of the trachea. Scale = 50  $\mu$ m.



**Figure 18: Illustration of the *c929* cells (Inka cells) in the trachea expressing *Egfr<sup>CA</sup>* with DIC and DAPI.** The UAS responder line *c929-Gal4* crossed with *Egfr<sup>CA</sup>* was compared with the same responder line crossed with *w<sup>1118</sup>* (control). The first row shows dissected trachea of control larvae (A-C). The second row displays the trachea of the upregulated *Egfr<sup>CA</sup>* larvae (D-F). Both larval tracheas were examined with DIC (A, D, F) and DAPI (B, C, E, F). Scale = 50  $\mu$ m.

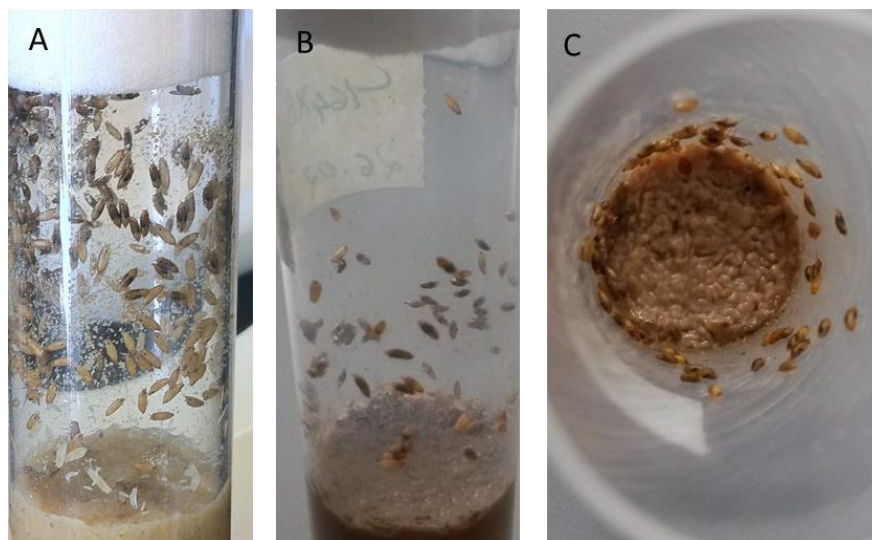
To confirm, that no relevant changes occurred at the cellular level an Immunohistochemical staining method with Coracle was performed. There were no significant changes observed from immunohistochemical analysis (Fig. 19).



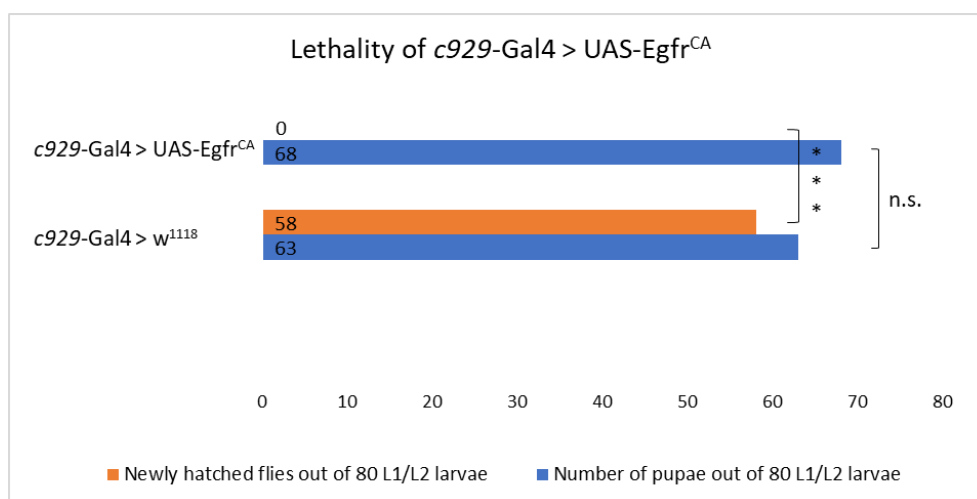
**Figure 19: Cellular level analysis of upregulated *Egfr<sup>CA</sup>* at the Inka cells of the trachea.** The first row displays the trachea of control (A-C) and the second row shows the trachea of the upregulated *Egfr<sup>CA</sup>* larvae (D-F). Coracle antibody staining (Cora) (A-F), DAPI (A, B, D, E) Scale = 50  $\mu$ m.

### 3.2.1. Lethality in larval and pupal stage caused by the $Egfr^{CA}$ expression at the Inka cells.

As there were no significant morphological changes observed at the cellular level, the focus was kept on the development of the larvae to adult flies. 80 larvae of L1/L2 stage from each control and expressed  $Egfr^{CA}$  were observed over 20 days. 63 of the larvae in the control group underwent a normal development to pupae and 58 of them made it to adult flies (Fig. 20 A). In contrast, 68 of the larvae with expressed  $Egfr^{CA}$  in Inka calls died at the pupal stage after making it to the pupal stage without any time lag (Fig. 20 B, C). The statistical difference in the number of hatched flies in control and in  $Egfr^{CA}$  was statistically highly significant ( $p=4.17E-09$ ) (Fig.21).



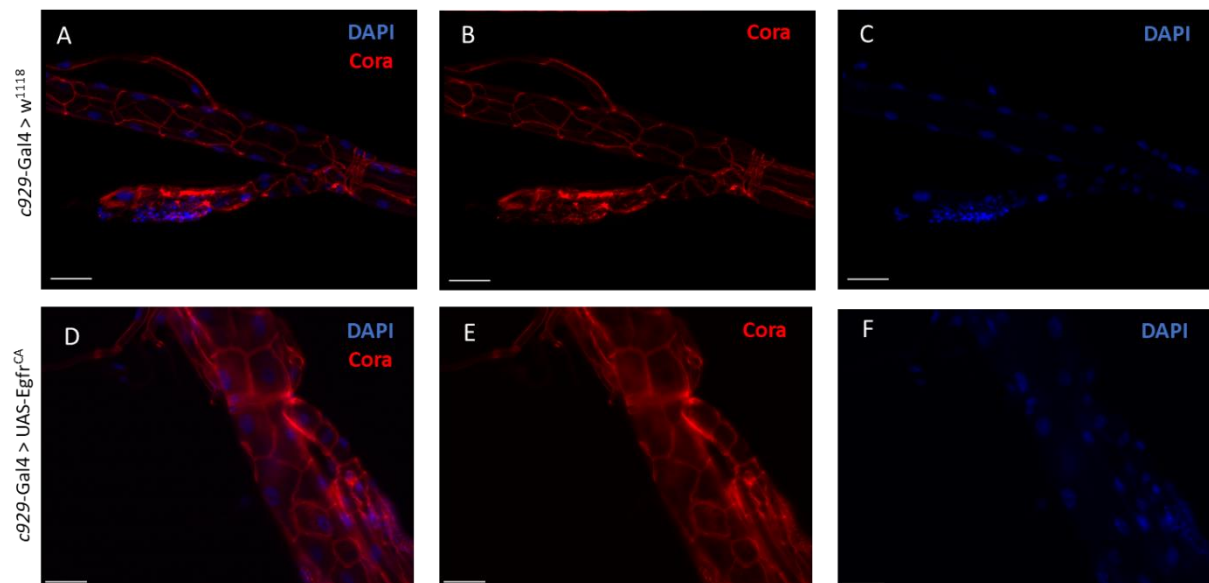
**Figure 20: Larval development of  $Egfr^{CA}$  expressing animals.** Larval development of  $c929-Gal4 > w^{1118}$  after 12 Days (A). It underwent a normal development into pupae and pupae hatched to new flies. In contrast, after 20 days  $c929-Gal4 > UAS-Egfr^{CA}$  illustrates dead Pupae (B, C). Larvae could reach the pupation without any significant time lag but they never enclosed to adult flies.



**Figure 21: Larval development and lethality of the pupae in  $Egfr^{CA}$  expression in Inka cells of the trachea.** 80 L1/L2 larvae from control and  $c929-Gal4 > UAS-Egfr^{CA}$  were collected and relocated to new vials. Further developments were observed for another 20 days. Statistical significance was tested with unpaired t-test, \* =  $p < 0.05$ , \*\* =  $p < 0.005$ , \*\*\* =  $p < 0.0005$ , n.s. = not significant.

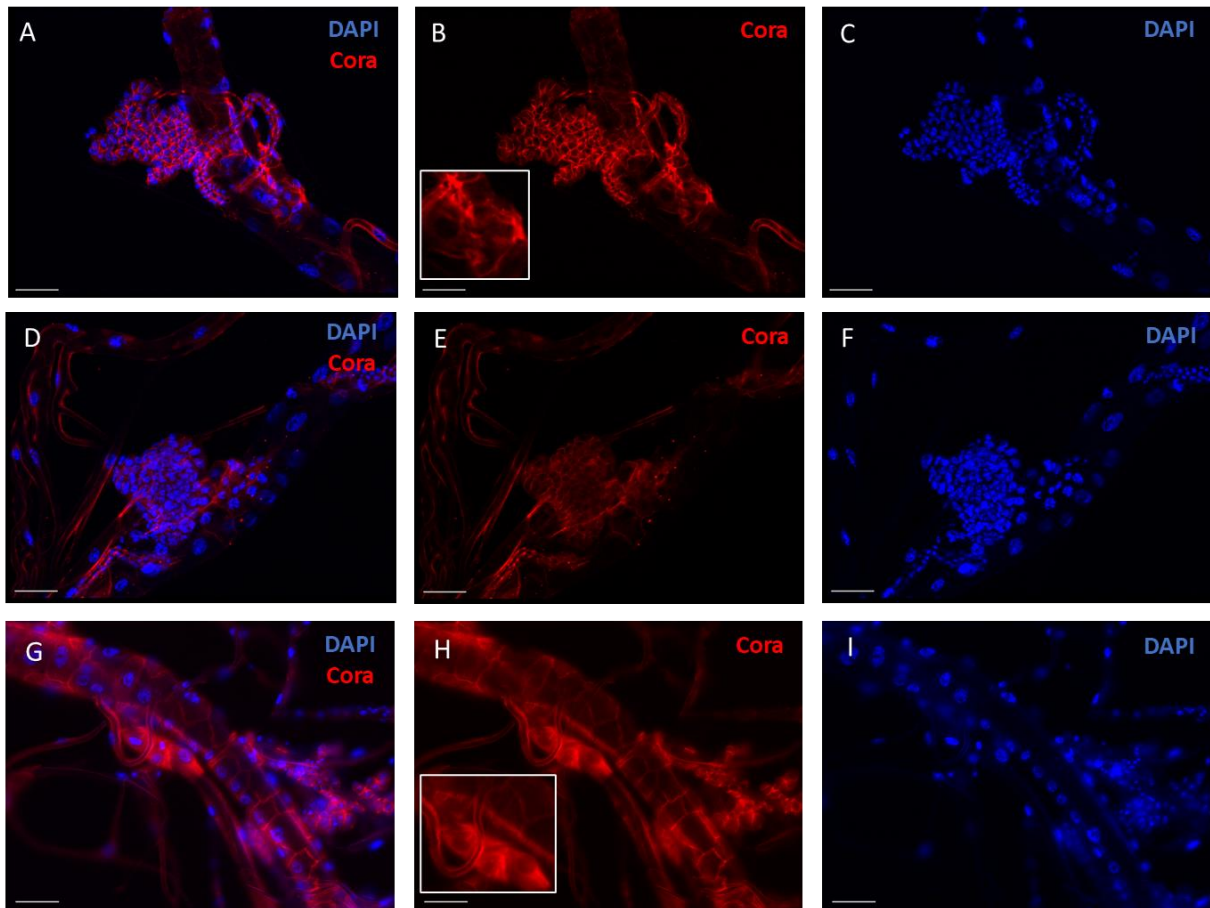
### 3.2.2. Analysis of the pupal trachea at the cellular level

To understand the pathological developments during the pupal stage, which led to 100% lethality of the pupae, a detailed analysis at the cellular level was performed. The organism undergoes a self-degeneration of its tissue during their transformation from pupae to adults. This made the dissection of the trachea in the pupal stage extremely challenging. Since in the late pupae stage, the trachea undergoes a self-degeneration as a part of metamorphosis, only pupae younger than 48 hours were chosen for dissection. The trachea of the pupae was dissected carefully and stained with DAPI and Coracle and analysed under a microscope. The trachea of the  $Egfr^{CA}$  did not show any remarkable pan tracheal morphological or structural changes. The cell borders were not disturbed, the number and the size of the nuclei were similar to that of the control.



**Figure 22: Cellular level analysis of pupal trachea of upregulated  $Egfr^{CA}$ .** The first row displays the trachea of control pupae (A-C) and the second row shows the pupal trachea of the upregulated  $Egfr^{CA}$  at Inka cells (D-F). Coracle antibody staining (Cora) (A, B, D, E), DAPI (A, C, D, F) Scale = 50  $\mu$ m.

Questionable pathological changes were observed at the dorsal trunks of the tracheal system. The branching points to the primary branches displayed an irregular cellular pattern with enlarged cells with an oversized nucleus. The cell borders were thickened. Furthermore, cell masses with numerous small nuclei were also noticed.



**Figure 23: Cancer like phenotype in the pupal trachea of upregulated  $Egfr^{CA}$ .** The first and the second rows display a call mass with numerous small nuclei possibly originate from the dorsal trunk of the trachea of the upregulated  $Egfr^{CA}$  pupae (A-F). The third row shows cells with enlarged nucleus and thickened cell wall starting from the branching points to the primary branches, where Inka cells are found. In B and H, the region of interest displayed in magnified version. Coracle antibody staining (Cora) (first two columns), DAPI (first and third column) Scale = 50  $\mu m$ .

Though it cannot be concluded that these cell masses are cancerous and originate from the Inka cells, the location and characters like hyperplasia, hypertropia, thickened cell walls and the fact that the expression of  $Egfr^{CA}$  in Inka cells led to 100% lethality in the pupal stage make the observations more meaningful and emphasizes more on typical characteristics of cancer phenotypes.

## 4. Discussion

In the last two decades, *Drosophila melanogaster* has been used to understand the molecular mechanisms of human diseases as it is a well-studied and highly tractable genetic model organism. Mammals and *D. melanogaster* share many basic biological, physiological, and neurological properties. Nearly 75% of disease-causing genes in humans are expected to have functional homologs in *Drosophila* (Pandey and Nichols, 2011). Different organs of *Drosophila* share substantial similarities with the corresponding human organs. This is also true for the tracheal system of *Drosophila*, which shows significant similarities with the human lung in its structure, function, the pattern of the immune response and gene expression profile.

Lung cancer is one of the most relevant cancer entities in humans. Here, different *Egfr* mutations play an important role for lung cancer, the effect of the ectopic expression of *Egfr*<sup>CA</sup> in the larval tracheal system of the common fruit fly, *Drosophila melanogaster* was investigated in this experiment. By using *ppk4*-Gal4 and *btl*-Gal4 as driver lines a gene expression of the whole tracheal system was reached. Whereas the *c929*-Gal4 driver line was used to express *Egfr*<sup>CA</sup> in the Inka cells, the ecdysis-triggering hormone (ETH) producing endocrine cells of the larval tracheal system.

### 4.1 Impacts of upregulated *Egfr*<sup>CA</sup> in the whole tracheal system of *Drosophila* larvae

The respiratory system of *Drosophila* larvae consists of a single-layered epithelial tubule that spread all over the body to transport oxygen from spiracles into the primary and secondary tracheas to the tracheoles and eliminates carbon dioxide in the opposite direction (Wigglesworth, 1972). The ectopic expression of *Egfr*<sup>CA</sup> crossed with a pan-tracheal driver line (*ppk4*-Gal4) showed severe impacts on the respiratory system of *Drosophila* larvae. As shown in figures 9-12, the impacts observed include an increase in number and size of the nucleus, thickened epithelium, malformation of the trachea, and significantly Increased lethality in the initial larval stage.

The histological features of human lung cancer differ from the observed tumour-like phenotypes like hyper- and metaplasia. In some areas, enlarged cells with a large single nucleus or more than one nucleus were observed. These observed phenotypes exhibit a very



early stage of tumour development in the *Drosophila* tracheal system. Therefore, a direct comparison between the observed tumour-like phenotypes and human lung cancer is unreasonable (Bossen et al., 2019). Instead, the observed phenotypes can help us to understand the initiation of tumour development, and they facilitate us to realize the basic characteristics and behaviour of lung cancer at early stages of its development.

The histological characteristics of the tumor tissues have a very important role to play in the classification and staging of human lung cancer. In lung cancers like adenocarcinoma in situ and minimally invasive adenocarcinoma, the size of the nucleus and increased mitotic activity is considered as a prognostic factor for an unfavourable outcome (Kadota et al., 2014). On the other hand, the increased number and size of the nucleus show increased mitotic activity in the trachea of the upregulated *Egfr<sup>CA</sup>* larvae. The observed phenotype with a significant number of nuclei, that were positive for the mitotic marker anti-pH3 strengthens this statement. In normal tracheal development, the cell division is expected to end at stage 11 of embryogenesis (Samakovlis et al., 1996) and pH3 as a mitotic marker should not be positive like it was observed in the control trachea. This increased cell division represents one of the hallmarks of cancer, the cell proliferation of tissues that does usually not occur under the respective conditions.

The phenotypes observed in the *Drosophila* model that include thickened epithelium and severe malformation of the dorsal trunks in the upregulated *Egfr<sup>CA</sup>* disturbs the normal physiological function of the larval tracheal system of *Drosophila*. The areas with severe tracheal malformation were observed to have significantly increased nucleus sizes and densities, which is a suggesting sign of increased cell proliferation as compared to the other parts of the same trachea. Hyper- and metaplasia and phenotypes like severe tracheal malformation interrupts the oxygen supply to the organs. As a result of persisting hypoxia, phenotypes like thickened epithelium can be observed (Arquier et al., 2006). If this was the case in survived L3 larvae of upregulated *Egfr<sup>CA</sup>*, it can be presumed that the larval death in the early stages of the development was caused by more severe structural changes, which were capable of suspending the oxygen supply to the downstream parts of the respiratory system.

To get a better picture of the above-mentioned phenotypes, an immunohistochemical analysis with anti-Coracle antibodies was performed. This analysis was conducted to visualise the cell

boundaries which would be stained by the anti-coracle antiserum. A diffuse appearance of the cell boundaries was observed, which may indicate the loss of the septate junction structure. Septate junctions are involved in maintaining the epithelial polarity. This situation of disturbed cell polarity can be compared with an activated epithelial-mesenchymal transition (EMT)-like process in the epithelial cells. EMT is characterised by the absence of cellular apical-basal polarity and the tight-junctions between the cells. Through EMT, the cells acquire the characteristics of mesenchymal cells and will be equipped with invasive and metastatic properties (Nieto et al., 2016). The epithelial cells of upregulated *Egfr<sup>CA</sup>* lose their polarity and probably gains the characteristics of mesenchymal-like cells, thus potentially exhibiting metastatic and invasive properties. The invasive and metastatic properties displayed in for example human breast cancer is attributed to Polyploid giant cancer cells (PGCCs) and epithelial-mesenchymal transition (EMT) (Fei et al., 2015). The upregulation or higher activity of EGFR in epithelial cells of the human lung results in an EMT-like phenotype, squamous metaplasia, goblet cell hyperplasia and decreased epithelial junctional barrier integrity (Shaykhiev et al., 2013).

#### **4.2 Reason for the increased larval lethality of expressed *Egfr<sup>CA</sup>***

The ectopic expression of the *Egfr<sup>CA</sup>* in the pan-tracheal driver line (*ppk4-Gal4*) caused a significantly higher lethality in the initial larval stages. More than 60% of the L1 larvae with *Egfr<sup>CA</sup>* expressed in the trachea were either found dead in the food medium or they crept out of the food and died on the vial-wall (Fig. 14). The increased lethality in the L1 larval stage was statistically highly significant and it was the most prominent among all the phenotype that could be observed.

Referring to the observed phenotypes in L3-larvae that comprised thickened epithelium, malformation of the trachea and increased number and size of the nuclei indicate the early phase of the tumour formation, implied that the organ-wide expression with a pan-tracheal driver line should have played an important role in larval death. Secondly, the dead L1 larvae are expected to have a more severe expression of the phenotypes than in the survived L3 larvae. This discrepancy is otherwise hard to explain. This highly significant phenotype will enable us to set up straight-forward screening approaches and at the same time, it allows us to do a more refined tumour-characterization. The lifecycle of *Drosophila* is fast and the life



span is short, after genetical crossing, it can bring out a big progeny which help to provide a quick, effective and economic approach to drug discovery (Bossen et al., 2019). For instance, a drug that reduces the lethality in the L1-stage or during any other developmental stage can be considered as an effective treatment option.

On the other hand, there was no increased lethality found from the late L2 larval stage of upregulated *Egfr<sup>CA</sup>* as compared with the control group. The survived larvae could develop further to pupae and adult flies without showing any significant delay or increased lethality in any of these developmental stages. The initial larval stage (L1) seems to be the most vulnerable phase of the upregulated *Egfr<sup>CA</sup>* larvae. More than half of the dead L1 and early L2 larvae of upregulated *Egfr<sup>CA</sup>* left the food medium prior to death (Fig.14), a clear indication of oxygen deficiency (Bossen et al., 2019). This explanation supports the conclusion that the larval death in the initial larval stage was due to the undersupply of oxygen caused by severe phenotypes.

#### **4.3 Explanations for a female-biased survival of upregulated *Egfr<sup>CA</sup>***

One of the observations of the conducted study was a female-biased sex ratio in survival in response to ectopic *Egfr<sup>CA</sup>* expression in the trachea. The number of males in newly hatched flies of upregulated *Egfr<sup>CA</sup>* was significantly less than in the control group. The sex-ratio moved in favour of the female flies (Fig.16). There is no sufficient evidence, which proves that the *Egfr*-pathway in the airway-epithelium of *Drosophila* has any role in sex determination or other sex-related traits. The studies show that in *Drosophila* insulin/insulin-like growth factor-1 (IGF-1) signalling (IIS) plays a central role in growth, development, reproduction and the process of ageing (Garofalo, 2002) (Magwere et al., 2004). Another study indicates that even in mammals and possibly also in humans, the IGF-1 receptor plays a key role in regulating the lifespan (Holzenberger et al., 2003). *Drosophila* females are larger in size than males and they have an increased Insulin/IGF signalling. Possibly, the males show a comparatively higher number of mutations than females that reduce insulin/IGF function during development, which possibly leads to growth-retardation and pupation delay (Burger and Promislow, 2004). The increased Insulin/IGF signalling, which plays a key role in growth, development and ageing possibly equip the female fly to survive these challenges.

A possible relation between EGFR signalling and IGF-1 signalling was observed in a study about head and neck squamous cell carcinoma (HNSCC) in humans. If EGFR signalling is inhibited, the activation of insulin-like growth factor type 1 receptor (IGF1R) can have a stronger influence on cell development (Jameson et al., 2011). Upregulation of EGFR can reduce the activity of IGF-1 and thus the cell growth. Trastuzumab, a monoclonal antibody regulates IGFBP-2 (IGF binding proteins-2) and IGFBP-3 expression in HER 2 positive breast cancers, which influences IGF1R downstream signalling (Dokmanovic et al., 2011). These studies show the ability of EGFR to influence the IGF signalling in humans.

As mentioned earlier, a correlation between EGFR in the airway epithelium of *Drosophila* and IGF signalling has not yet been proved. Until it is proven otherwise, a female-biased survival of the upregulated *Egfr<sup>CA</sup>* larvae can be explained through the robust nature and developmental advantages through the increased Insulin/IGF signalling of the female *Drosophila*.

#### **4.4 Targeted expression of *Egfr<sup>CA</sup>* in Inka cells of larval tracheal system does not provoke any morphological or structural changes.**

The ectopic expression of the *Egfr<sup>CA</sup>* in Inka cells of the larval respiratory system showed no significant morphological changes compared with the control group. The number and size of the nuclei showed no significant changes (Fig. 18). The immunohistochemical analysis with anti-Coracle antiserum did not reveal any substantial changes. Especially, the branching points to the primary branches in the dorsal trunks of the trachea, where the Inka cells are located, did not show any remarkable changes (Fig. 19). The larval development in both overexpressed *Egfr<sup>CA</sup>* and the control group were normal till the beginning of the pupal stage. Developmental delay and increased larval death were also not observed.

As compared with the pan-tracheal driver lines like *ppk4*-Gal4 and *btl*-Gal4 the number of cells that are affected by the ectopic expression using *c929*-Gal4 as the driver line is much less in number. Though the Inka cells are very few in number, they have an important function throughout the larval and pupal development stages. Through a positive feedback loop, the eclosion hormone (EH) from the centrally located neurosecretory neurones (ventromedial (*V<sub>m</sub>*) neurons) and the ecdysis-triggering hormone (ETH) from the Inka cells simultaneously stimulate the release of each other. These two hormones play a central role in the stimulation

of ecdysis and the physiological preparations, that are related to moulting (Shen et al., 2021). A recent study demonstrates that there are other neurons (i.e.,  $D_I$  (dorsolateral) and  $D_m$  (dorsomedial) neurons) and other somatic cells that express EH in addition to the  $V_m$ -neurons.  $D_I$ - and  $D_m$  neurons express EH only in the adult stage and some tracheal cells and other somatic cells in all developmental stages. The  $D_I$ -neurons do not have an ETH-receptor or its isoform, hence can't be regulated by ETH. The  $D_I$ -neurons are the only cell, that express EH and that are not regulated by ETH. The regulatory mechanism of the  $D_m$ -neurons and other somatic cells are yet to be studied. The non- $V_m$  cells that express EH do exist and have a significant role to play in ecdysis. It shows that the control of the onset of ecdysis is more widely distributed in terms of relevant cells and probably more diversely regulated as formerly expected (Scott et al., 2020).

There are two possible explanations for the normal phenotype observed in the larval tracheal system. Firstly, the changes caused by the upregulation of  $Egfr^{CA}$  in Inka cells are not sufficient enough to interrupt the morphology and the function of Inka cells at any of the larval stages. The normal larval development and normal tracheal phenotype strengthen these findings. The second scenario, which seems to be unlikely here; the upregulation of  $Egfr^{CA}$  does disturb the secretion of the ETH, but as observed in the recent studies, the non- $V_m$ -EH expressing cells (tracheal and other somatic cells) in the larval stages, can compensate this disturbance by releasing sufficient EH, if they are not regulated by ETH. There are other important observations in this study, explained below, that might be sufficient to rule out this possibility by showing that the EH expression in tracheal and other somatic cells are more likely to be governed by ETH.

#### **4.5 Impact of the upregulated $Egfr^{CA}$ in Inka cells in pupae**

Unlike in larval stages, the severe impact was observed through the upregulation of  $Egfr^{CA}$  in Inka cells of the pupae. It caused 100% lethality in the pupal stage, eclosion of the adult fly never took place. The cellular level analysis of the trachea did not show any pan-tracheal morphological changes. But possible tumour-like morphological changes such as severe regional hyperplasia and hypertropia and thickened cell membranes with irregular cellular pattern were observed in the dorsal trunks at the branching points of the primary branches, where Inka cells are normally located (Fig. 23).

The observed phenotype and the 100% lethality in the pupal stage point towards a disfunction of Inka cells, where the ETH production is drastically suppressed or even totally terminated. As it is known that the strong positive feedback between ETH and EH is extremely important for the ecdysis and thereby for eclosion (Ewer et al., 1997), the strong suppression or absence of the ETH will cause the suppression of the EH. As mentioned earlier, there are cells other than non- $V_m$ -neurons that are capable of expressing EH and it was observed that the EH regulation in one of these cells namely  $D_I$ -neurons are possibly not controlled by ETH. But they are present only in the adult fly. The regulatory mechanism of the EH secretion in cells other than  $V_m$ -neurons and  $D_I$ -neurons are yet to be studied (Scott et al., 2020). Considering all these pieces of information, it can be concluded that the ETH is necessary and unreplaceable for the EH secretion in a pupal stage and that the  $EGFR^{CA}$ -induced changes in these cells interfere substantially with their normal role during the process of pupation.

On the other hand, a larval experiment, where  $V_m$ -neurons were ablated, led to an aberrant release of ETH from the Inka cells (Clark et al., 2004), and caused the death of almost two-thirds of such larvae by tracheal air filling due to the lack of EH. Tracheal air filling is a physiological mechanism in each larval stage, which normally leads to cuticle shedding, as a part of ecdysis. The survived one-third developed to the adult fly showing a non-lethal eclosion deficits (McNabb et al., 1997). In contrast, the knock-out of the EH-gene led to the larval death of 90% of EH-null mutants and none survived to adulthood and the Inka cells in the EH-null mutants did not release any ETH (Krüger et al., 2015). These studies show that EH is essential for larval development and eclosion and there are cells other than non- $V_m$ -neurons that express EH and unlike in the adult fly, the regulation of EH in the larval and pupal stage governed probably only through ETH.

There are further experiments required to clarify the authenticity of the tumour-like phenotypes observed in the pupal trachea. Further observation and examination of the pupal development of the upregulated  $Egfr^{CA}$  in Inka cells will help to understand the development stage, where the pupal development interrupted and terminated. It is possible that an Injection of ETH during the pupal stage might lead to the eclosion of flies, which are less affected by the tumour-like phenotype. This will help to strengthen the above-mentioned conclusions. The extreme phenotype with 100% pupal lethality caused by the upregulation  $Egfr^{CA}$  in Inka cells can be used to set up straight-forward drug-screening approaches.

## 5. Zusammenfassung

Krebs ist eine der führenden Todesursachen weltweit. Dem Robert Koch Institut zu Folge ist es in Deutschland, nach Herz-Kreislauf-Erkrankungen, die zweithäufigste Todesursache. Dabei sind den unterschiedlichen Formen des Lungenkrebses, die meisten Todesfälle aller Krebserkrankungen zuzuordnen. Hauptgründe dafür sind eine verspätete Diagnosestellung sowie der Mangel an effektiven therapeutischen Möglichkeiten. Diese Situation erfordert effektive Strategien in der Medikamenten-Entwicklung sowie Forschung zum Thema Arzneimittel-Resistenzen. In dieser Arbeit wurde das respiratorische System von genetisch veränderten Taufliegen (*Drosophila melanogaster*) als Modell für Lungenkrebs entwickelt.

Der Epidermalen Wachstumsfaktor Rezeptors (EGFR) Signalweg spielt eine Schlüsselrolle in der Proliferation, dem Überleben, der Migration, der Differenzierung sowie der Apoptose von Zellen. Mutationen im EGFR-Signalweg gelten sogar als eine der häufigsten Auslöser von Lungenkrebs, insbesondere der nicht-kleinzelligen Form dieser Erkrankung (NSCLC). In dieser Studie wurde eine gezielte Expression einer konstitutiv aktiven Isoform des Epidermalen Wachstumsfaktor Rezeptors (Egfr<sup>CA</sup>) im Trachealsystem von *Drosophila* durchgeführt. Die ektopische Expression von EGFR<sup>CA</sup> im gesamten Atemwegssystem mit Hilfe eines spezifischen, binären Expressionskontrollsystems induzierte dabei Phänotypen mit schwerwiegenden Folgen wie Atemwegs-Malformationen, Verdickung der Epithelien und Hyper- sowie Metaplasien, welche heufig bereits im frühen Larvenstadium zum Tod führten.

Andererseits führte die gezielte Expression von EGFR<sup>CA</sup> in sogenannten Inka-Zellen, den *ecdysis-triggering hormone* (ETH) produzierende Trachealzellen, nicht zu signifikanten phänotypischen Veränderungen im Larvenstadium. Im Puppenstadium dagegen zeigte sich eine Letalität von 100%. Die Analyse der Trachea im Puppenstadium ergab auf Zellebene Tumor-ähnliche morphologische Veränderungen, wie schwere regionale Hyperplasie und Hypertrophie sowie Verdickungen der Epithelien an den Verzweigungen aus den Hauptästen des Atemwegsystems (*dorsal trunks*), dort, wo normalerweise die Inka-Zellen sitzen. Die durchgängige Letalität im Puppenstadium deutet auf die Fehlfunktion der Inka-Zellen hin, in denen die *ecdysis-triggering hormone* Produktion und damit die Sekretion von *eclosion hormone* (EH) deutlich supprimiert oder sogar komplett eingestellt wurde. Es stellt sich heraus, dass die EGFR<sup>CA</sup>-induzierten Veränderungen dieser Zellen wesentlich die Funktion der Zellen im Prozess des Puppenstadiums beeinflussen.

Da die beobachteten Ausprägungen in den beiden unterschiedlichen Expressionssystemen hoch signifikant und leicht zu quantifizieren sind, können sie effektiv für weiterführende Medikamenten-Entwicklung sowie Forschung zum Thema Arzneimittel-Resistenzen genutzt werden.

## Abstract

Cancer is one of the leading causes of death worldwide. According to the Robert Koch Institute (RKI), it is the 2nd leading cause of death after heart diseases in Germany. The various forms of lung cancer account for the highest number of deaths of all cancers. Delayed diagnosis and lack of effective therapeutic approaches are the main reasons for this. This situation demands more functional strategies in drug development and drug-resistance research. In this thesis, the respiratory system of the genetically modified common fruit flies *Drosophila melanogaster* was used to develop specific lung cancer models.

Epidermal Growth Factor Receptor (EGFR) signalling pathway plays a key role in cell proliferation, survival, migration, differentiation, and apoptosis. Mutations in the EGFR pathway are one of the most frequent inducers of Lung cancer, especially in non-small cell lung cancer (NSCLC). In this study, the targeted expression of the constitutively active EGFR (EGFR<sup>CA</sup>) in the tracheal system of *Drosophila* was performed. Ectopic expression of EGFR<sup>CA</sup> throughout the airway system using a specific binary expression control system induced phenotypes with severe consequences such as airway malformations, epithelial thickening, hyperplasia and metaplasia, which often led to death in the early larval stage.

On the other hand, the targeted expression of EGFR<sup>CA</sup> in Inka cells, ecdysis-triggering hormone (ETH) producing cells in the trachea, did not show any significant phenotypes in the larval stage. But in the pupal stage, it led to 100% lethality. The cellular level analysis of the pupal trachea displayed possible tumour-like morphological changes like severe regional hyperplasia and hypertropia and thickened epithelium at the branching points of the primary branches in dorsal trunks, where Inka cells are normally located. The complete lethality in the pupal stage points towards the disfunction of Inka cells, where the ecdysis-triggering hormone production, and thereby the eclosion hormone (EH) secretion, is drastically suppressed or even totally terminated. It points out that the EGFR<sup>CA</sup>-induced changes in these cells interfere substantially with their normal role during the process of pupation.

As the phenotypes observed in both pan-tracheal and Inka cell driver lines are highly significant and easily monitorable, they can be efficiently used for straight-forward drug-screening approaches and drug-resistance experiments.

## 6. References

- Adams, M. D. et al. 2000. "The Genome Sequence of *Drosophila Melanogaster*." *Science* Vol. 287 pages 2185-2195.
- American Cancer Society. 2016. "Lung Cancer (Non-Small Cell) What Is Non-Small Cell Lung Cancer?" American Cancer Society: 1–75.
- Arquier, Nathalie et al. 2006. "Analysis of the Hypoxia-Sensing Pathway in *Drosophila Melanogaster*." *Biochemical Journal*.
- Bade, Brett C., and Charles S. Dela Cruz. 2020. "Lung Cancer 2020: Epidemiology, Etiology, and Prevention." *Clinics in Chest Medicine*.
- Barta, Julie A., Charles A. Powell, and Juan P. Wisnivesky. 2019. "Global Epidemiology of Lung Cancer." *Annals of Global Health*.
- Bellen, Hugo J. et al. 2004. "The BDGP Gene Disruption Project: Single Transposon Insertions Associated with 40% of *Drosophila* Genes." *Genetics*.
- Bethune, Gillian, Drew Bethune, Neale Ridgway, and Zhaolin Xu. 2010. "Epidermal Growth Factor Receptor (EGFR) in Lung Cancer: An Overview and Update." *Journal of Thoracic Disease*.
- Bier, Ethan. 2005. "*Drosophila*, the Golden Bug, Emerges as a Tool for Human Genetics." *Nature Reviews Genetics*.
- Bossen, Judith et al. 2019. "An EGFR-Induced *Drosophila* Lung Tumor Model Identifies Alternative Combination Treatments." *Molecular Cancer Therapeutics*.
- Brand, A. H., and N. Perrimon. 1993. "Targeted Gene Expression as a Means of Altering Cell Fates and Generating Dominant Phenotypes." *Development* 118 (2) pages 401–415.
- Bray, Freddie et al. 2018. "Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries." *CA: A Cancer Journal for Clinicians*.
- Brumby, Anthony M., and Helena E. Richardson. 2005. "Using *Drosophila Melanogaster* to Map Human Cancer Pathways." *Nature Reviews Cancer* 5, pages 626–639.
- Burger, Joep M.S., and Daniel E.L. Promislow. 2004. "Sex-Specific Effects of Interventions That Extend Fly Life Span." *Science of aging knowledge environment: SAGE KE*.
- Burgio, Ernesto, and Lucia Migliore. 2015. "Towards a Systemic Paradigm in Carcinogenesis: Linking Epigenetics and Genetics." *Molecular biology reports*.
- Caldwell, Philip E., Magdalena Walkiewicz, and Michael Stern. 2005. "Ras Activity in the *Drosophila* Prothoracic Gland Regulates Body Size and Developmental Rate via Ecdysone Release." *Current Biology*.



- Carcereny, Enric et al. 2015. "The Epidermal Growth Factor Receptor (EGFR) in Lung Cancer." *Translational Respiratory Medicine*.
- Cho, Kyoung Sang, Se Min Bang, and Amanda Toh. 2014. "Lipids and Lipid Signaling in *Drosophila* Models of Neurodegenerative Diseases." In *Omega-3 Fatty Acids in Brain and Neurological Health*.
- Clark, Anthony C., Marta L. Del Campo, and John Ewer. 2004. "Neuroendocrine Control of Larval Ecdysis Behavior in *Drosophila*: Complex Regulation by Partially Redundant Neuropeptides." *Journal of Neuroscience*.
- Collisson, Eric A. et al. 2014. "Comprehensive Molecular Profiling of Lung Adenocarcinoma: The Cancer Genome Atlas Research Network." *Nature*.
- Cruz, Josefa, David Martín, and Xavier Franch-Marro. 2020. "Egfr Signaling Is a Major Regulator of Ecdysone Biosynthesis in the *Drosophila* Prothoracic Gland." *Current Biology*.
- Diao, Feici et al. 2016. "The Splice Isoforms of the *Drosophila* Ecdysis Triggering Hormone Receptor Have Developmentally Distinct Roles." *Genetics*.
- Dietzl, Georg et al. 2007. "A Genome-Wide Transgenic RNAi Library for Conditional Gene Inactivation in *Drosophila*." *Nature*.
- Ding, Li et al. 2008. "Somatic Mutations Affect Key Pathways in Lung Adenocarcinoma." *Nature*.
- Dokmanovic, Milos et al. 2011. "Trastuzumab Regulates IGFBP-2 and IGFBP-3 to Mediate Growth Inhibition: Implications for the Development of Predictive Biomarkers for Trastuzumab Resistance." *Molecular Cancer Therapeutics*.
- Duffy, Joseph B. 2002. "GAL4 System in *Drosophila*: A Fly Geneticist's Swiss Army Knife." *Genesis*.
- Duma, Narjust, Rafael Santana-Davila, and Julian R. Molina. 2019. "Non-Small Cell Lung Cancer: Epidemiology, Screening, Diagnosis, and Treatment." *Mayo Clinic Proceedings*.
- Eguchi, Takashi et al. 2014. "The New IASLC-ATS-ERS Lung Adenocarcinoma Classification: What the Surgeon Should Know." *Seminars in Thoracic and Cardiovascular Surgery*.
- Ewer, John, Stephen C. Gammie, and James W. Truman. 1997. "Control of Insect Ecdysis by a Positive-Feedback Endocrine System: Roles of Eclosion Hormone and Ecdysis Triggering Hormone." *Journal of Experimental Biology*.
- Faisal, Muhammad N. et al. 2014. "Transcriptional Regionalization of the Fruit Fly's Airway Epithelium." *PLoS ONE*.
- Fei, Fei et al. 2015. "The Number of Polyploid Giant Cancer Cells and Epithelial-Mesenchymal Transition-Related Proteins Are Associated with Invasion and Metastasis in Human Breast Cancer." *Journal of Experimental and Clinical Cancer Research*.

- Fernández-Moreno, Miguel Angel, Carol L. Farr, Laurie S. Kaguni, and Rafael Garesse. 2007. "*Drosophila Melanogaster* as a Model System to Study Mitochondrial Biology." Methods in molecular biology (Clifton, N.J.).
- Fouad, Yousef Ahmed, and Carmen Aanei. 2017. "Revisiting the Hallmarks of Cancer." American Journal of Cancer Research.
- Gabay, Limor et al. 1996. "EGF Receptor Signaling Induces Pointed P1 Transcription and Inactivates Yan Protein in the *Drosophila* Embryonic Ventral Ectoderm." Development.
- Garofalo, Robert S. 2002. "Genetic Analysis of Insulin Signaling in *Drosophila*." Trends in Endocrinology and Metabolism.
- Ghiglione, Christian et al. 2002. "Mechanism of Activation of the *Drosophila* EGF Receptor by the TGF $\alpha$  Ligand Gurken during Oogenesis." Development.
- Ghiglione, Christian et al. 1999. "The Transmembrane Molecule Kekk1 Acts in a Feedback Loop to Negatively Regulate the Activity of the *Drosophila* EGF Receptor during Oogenesis." Cell.
- Globocan. 2019. "Lung Fact Sheet." International Agency for Research on Cancer, WHO.
- Govindan, Ramaswamy et al. 2012. "Genomic Landscape of Non-Small Cell Lung Cancer in Smokers and Never-Smokers." Cell.
- Hammerman, Peter S. et al. 2012. "Comprehensive Genomic Characterization of Squamous Cell Lung Cancers." Nature.
- Hanahan, Douglas, and Robert A. Weinberg. 2011. "Hallmarks of Cancer: The next Generation." Cell.
- Hanahan, Douglas, and Robert A. Weinberg. 2017. "Biological Hallmarks of Cancer." In Holland-Frei Cancer Medicine,.
- Harris, Raymond C., Eunkyung Chung, and Robert J. Coffey. 2003. "EGF Receptor Ligands." Experimental Cell Research.
- Hayashi, Shigeo, and Takefumi Kondo. 2018. "Development and Function of the *Drosophila* Tracheal System." Genetics.
- Holzenberger, Martin et al. 2003. "IGF-1 Receptor Regulates Lifespan and Resistance to Oxidative Stress in Mice." Nature.
- Horodyski, F. M., L. M. Riddiford, and J. W. Truman. 1989. "Isolation and Expression of the Eclosion Hormone Gene from the Tobacco Hornworm, *Manduca sexta*." Proceedings of the National Academy of Sciences of the United States of America.
- Horowitz, Arie, and Michael Simons. 2008. "Branching Morphogenesis." Circulation Research.

- Hwang, Shih Jen et al. 2003. "Lung Cancer Risk in Germline P53 Mutation Carriers: Association between an Inherited Cancer Predisposition, Cigarette Smoking, and Cancer Risk." *Human Genetics*.
- Imielinski, Marcin et al. 2012. "Mapping the Hallmarks of Lung Adenocarcinoma with Massively Parallel Sequencing." *Cell*.
- Inamura, Kentaro. 2017. "Lung Cancer: Understanding Its Molecular Pathology and the 2015 WHO Classification." *Frontiers in Oncology*.
- Inamura, Kentaro. 2017. "Lung Cancer: Understanding Its Molecular Pathology and the 2015 WHO Classification." *Frontiers in Oncology* 7(AUG).
- International Agency for Research on Cancer (IARC). 2018. "GLOBOCAN 2018: Latest Global Cancer Data." *CA: A Cancer Journal for Clinicians*.
- Jamal-Hanjani, Mariam et al. 2017. "Tracking the Evolution of Non-Small-Cell Lung Cancer." *New England Journal of Medicine*.
- Jameson, Mark J. et al. 2011. "Activation of the Insulin-like Growth Factor-1 Receptor Induces Resistance to Epidermal Growth Factor Receptor Antagonism in Head and Neck Squamous Carcinoma Cells." *Molecular Cancer Therapeutics*.
- Jennings, Barbara H. 2011. "*Drosophila*- a Versatile Model in Biology & Medicine." *Materials Today*.
- Kadota, Kyuichi et al. 2014. "Prognostic Significance of Adenocarcinoma in Situ, Minimally Invasive Adenocarcinoma, and Nonmucinous Lepidic Predominant Invasive Adenocarcinoma of the Lung in Patients with Stage I Disease." *American Journal of Surgical Pathology*.
- Kim, Young Joon et al. 2006 a. "Central Peptidergic Ensembles Associated with Organization of an Innate Behavior." *Proceedings of the National Academy of Sciences of the United States of America*.
- Kim, Young Joon et al. 2006 b. "A Command Chemical Triggers an Innate Behavior by Sequential Activation of Multiple Peptidergic Ensembles." *Current Biology*.
- Kingan, Timothy G., William Gray, Dušan Žitňan, and Michael E. Adams. 1997. "Regulation of Ecdysis-Triggering Hormone Release by Eclosion Hormone." *Journal of Experimental Biology*.
- Klein, Christoph, Helmut G. Kallenborn, and Christiane Radlicki. 1999. "The 'Inka Cell' and Its Associated Cells: Ultrastructure of the Epitracheal Glands in the Gypsy Moth, *Lymantria dispar*." *Journal of Insect Physiology*.
- Klowden, Marc J. 2008. "Physiological Systems in Insects." In *Physiological Systems in Insects*.

- Kramer, Susanne et al. 1999. "Sprouty: A Common Antagonist of FGF and EGF Signaling Pathways in *Drosophila*." *Development*.
- Krüger, Eileen et al. 2015. "Genetic Analysis of Eclosion Hormone Action during *Drosophila* Larval Ecdysis." *Development (Cambridge)*.
- Lhomond, Stéphanie et al. 2015. "Adaptation of the Secretory Pathway in Cancer through IRE1 Signaling." *Methods in Molecular Biology*.
- Loganathan, Rajprasad, Yim Ling Cheng, and Deborah J. Andrew. 2016. "Organogenesis of the *Drosophila* Respiratory System." In *Organogenetic Gene Networks: Genetic Control of Organ Formation*.
- Lusk, Jay B., Vanessa Y.M. Lam, and Nicholas S. Tolwinski. 2017. "Epidermal Growth Factor Pathway Signaling in *Drosophila* Embryogenesis: Tools for Understanding Cancer." *Cancers*.
- Magwere, Tapiwanashe, Tracey Chapman, and Linda Partridge. 2004. "Sex Differences in the Effect of Dietary Restriction on Life Span and Mortality Rates in Female and Male *Drosophila Melanogaster*." *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*.
- McGuire, Sean E., Zhengmei Mao, and Ronald L. Davis. 2004. "Spatiotemporal Gene Expression Targeting with the TARGET and Gene-Switch Systems in *Drosophila*." *Science's STKE: signal transduction knowledge environment*.
- McNabb, Susan L. et al. 1997. "Disruption of a Behavioral Sequence by Targeted Death of Peptidergic Neurons in *Drosophila*." *Neuron*.
- Mirzoyan, Zhasmine et al. 2019. "*Drosophila Melanogaster*: A Model Organism to Study Cancer." *Frontiers in Genetics*.
- Mitsudomi, Tetsuya, and Yasushi Yatabe. 2010. "Epidermal Growth Factor Receptor in Relation to Tumor Development: EGFR Gene and Cancer." *FEBS Journal*.
- Molina, Julian R. et al. 2008. "Non-Small Cell Lung Cancer: Epidemiology, Risk Factors, Treatment, and Survivorship." *Mayo Clinic Proceedings*.
- Nieto, M. Angela, Ruby Yun Y.J. Huang, Rebecca A A. Jackson, and Jean Paul P. Thiery. 2016. "EMT: 2016." *Cell*.
- NIH, National Cancer Institute. 2019. "Definition of Drug - NCI Dictionary of Cancer Terms." *National Cancer Institute*.
- Oda, Kanae, Yukiko Matsuoka, Akira Funahashi, and Hiroaki Kitano. 2005. "A Comprehensive Pathway Map of Epidermal Growth Factor Receptor Signaling." *Molecular systems biology*.
- Ong, Cynthia et al. 2015. "*Drosophila Melanogaster* as a Model Organism to Study Nanotoxicity." *Nanotoxicology*.

Organization, World Health. 2017. "WHO | Cancer Key Facts." WHO.

Öztürk-Çolak, Arzu, Bernard Moussian, Sofia J. Araújo, and Jordi Casanova. 2016. "A Feedback Mechanism Converts Individual Cell Features into a Supracellular ECM Structure in *Drosophila* Trachea." eLife.

Pallis, Athanasios G., and Konstantinos N. Syrigos. 2013. "Lung Cancer in Never Smokers: Disease Characteristics and Risk Factors." Critical Reviews in Oncology/Hematology.

Pandey, Udai Bhan, and Charles D. Nichols. 2011. "Human Disease Models in *Drosophila Melanogaster* and the Role of the Fly in Therapeutic Drug Discovery." Pharmacological Reviews.

Papagiannouli, Fani, and Bernard M. 2013. "Modeling Tumorigenesis in *Drosophila*: Current Advances and Future Perspectives." In Future Aspects of Tumor Suppressor Gene.

Park, Yoonseong, Valery Filippov, Sarjeet S. Gill, and Michael E. Adams. 2002. "Deletion of the Ecdysis-Triggering Hormone Gene Leads to Lethal Ecdysis Deficiency." Development.

Pitot, Henry C. 1993. "The Molecular Biology of Carcinogenesis." Cancer.

Rao, Prashanth R et al. 2015. "Developmental Compartments in the Larval Trachea of *Drosophila*." eLife.

Ray, Robert P., and Trudi Schüpbach. 1996. "Intercellular Signaling and the Polarization of Body Axes during *Drosophila* Oogenesis." Genes and Development.

Rivera, Gabriel Alberto, and Heather Wakelee. 2016. "Lung Cancer in Never Smokers." Advances in Experimental Medicine and Biology 893: 43–57.

Roeder, Thomas et al. 2012. "A *Drosophila* Asthma Model - What the Fly Tells Us about Inflammatory Diseases of the Lung." Advances in Experimental Medicine and Biology.

Roller, Ladislav et al. 2010. "Ecdysis Triggering Hormone Signaling in Arthropods." Peptides.

Roman, Gregg, Keita Endo, Lin Zong, and Ronald L. Davis. 2001. "P{switch}, a System for Spatial and Temporal Control of Gene Expression in *Drosophila Melanogaster*." Proceedings of the National Academy of Sciences of the United States of America.

Rudin, Charles M. et al. 2009. "Lung Cancer in Never Smokers: Molecular Profiles and Therapeutic Implications." Clinical Cancer Research.

Ryder, Edward et al. 2007. "The DrosDel Deletion Collection: A *Drosophila* Genomewide Chromosomal Deficiency Resource." Genetics.

- Samakovlis, Christos et al. 1996. "Development of the *Drosophila* Tracheal System Occurs by a Series of Morphologically Distinct but Genetically Coupled Branching Events." Development.
- Samet, Jonathan M. et al. 2009. "Lung Cancer in Never Smokers: Clinical Epidemiology and Environmental Risk Factors." Clinical Cancer Research.
- Scaltriti, Maurizio, and José Baselga. 2006. "The Epidermal Growth Factor Receptor Pathway: A Model for Targeted Therapy." Clinical Cancer Research.
- Schnepp, Brace, Gary Grumblin, Timothy Donaldson, and Amanda Simcox. 1996. "Vein Is a Novel Component in the *Drosophila* Epidermal Growth Factor Receptor Pathway with Similarity to the Neuregulins." Genes and Development.
- Schottenfeld, Jodi, Yanjun Song, and Amin S. Ghabrial. 2010. "Tube Continued: Morphogenesis of the *Drosophila* Tracheal System." Current Opinion in Cell Biology.
- Scott, Robert L. et al. 2020. "Non-Canonical Eclosion Hormone-Expressing Cells Regulate *Drosophila* Ecdysis." iScience.
- Seyfried, Thomas N., and Leanne C. Huysentruyt. 2013. "On the Origin of Cancer Metastasis." Critical Reviews in Oncogenesis.
- Sharma, Sreenath V., Daphne W. Bell, Jeffrey Settleman, and Daniel A. Haber. 2007. "Epidermal Growth Factor Receptor Mutations in Lung Cancer." Nature Reviews Cancer.
- Shaykhiev, Renat et al. 2013. "EGF Shifts Human Airway Basal Cell Fate Towarda Smoking-Associated Airway Epithelial Phenotype." Proceedings of the National Academy of Sciences of the United States of America.
- Shen, Chen Hui et al. 2021. "Eclosion Hormone Functions in Larva-Pupa-Adult Ecdysis in *Leptinotarsa decemlineata*." Journal of Asia-Pacific Entomology.
- Shilo, Ben Zion. 2003. "Signaling by the *Drosophila* Epidermal Growth Factor Receptor Pathway during Development." Experimental Cell Research.
- Siddiqui, Imtiaz A. et al. 2015. "Resveratrol Nanoformulation for Cancer Prevention and Therapy." Annals of the New York Academy of Sciences.
- Siegel, Rebecca L., Kimberly D. Miller, Hannah E. Fuchs, and Ahmedin Jemal. 2021. "Cancer Statistics, 2021." CA: A Cancer Journal for Clinicians.
- Siegel, Rebecca L., Kimberly D. Miller, and Ahmedin Jemal. 2020. "Cancer Statistics, 2020." CA: A Cancer Journal for Clinicians.
- Sung, Hyuna et al. 2021. "Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries." CA: A Cancer Journal for Clinicians.

- Tenenbaum, David. 2003. "What's All the Buzz? Fruit Flies Provide Unique Model for Cancer Research." Journal of the National Cancer Institute.
- Tiklová, Katarína et al. 2010. "Epithelial Septate Junction Assembly Relies on Melanotransferrin Iron Binding and Endocytosis in *Drosophila*." Nature Cell Biology.
- Travis, William D. et al. 2015. "The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances since the 2004 Classification." Journal of Thoracic Oncology.
- Truman, J. W., and P. F. Copenhaver. 1989. "The Larval Eclosion Hormone Neurones in *Manduca sexta*: Identification of the Brain-Proctodeal Neurosecretory System." Journal of Experimental Biology.
- Truman, James W. 2005. "Hormonal Control of Insect Ecdysis: Endocrine Cascades for Coordinating Behavior with Physiology." Vitamins and Hormones.
- Truman, James W., and Lynn M. Riddiford. 1970. "Neuroendocrine Control of Ecdysis in Silkworms." Science.
- Truman, James W. et al. 1981. "Eclosion Hormone May Control All Ecdyses in Insects." Nature.
- Tu, Meng Ping, Diane Epstein, and Marc Tatar. 2002. "The Demography of Slow Aging in Male and Female *Drosophila* Mutant for the Insulin-Receptor Substrate Homologue Chico." Aging cell.
- Ugur, Berrak, Kuchuan Chen, and Hugo J. Bellen. 2016. "*Drosophila* Tools and Assays for the Study of Human Diseases." DMM Disease Models and Mechanisms.
- Uv, Anne, Rafael Cantera, and Christos Samakovlis. 2003. "*Drosophila* Tracheal Morphogenesis: Intricate Cellular Solutions to Basic Plumbing Problems." Trends in Cell Biology.
- White, Benjamin H., and John Ewer. 2014. "Neural and Hormonal Control of Postecdysial Behaviors in Insects." Annual Review of Entomology.
- Wigglesworth, V. B. 1972. The Principles of Insect Physiology.
- Wu, Song, Wei Zhu, Patricia Thompson, and Yusuf A. Hannun. 2018. "Evaluating Intrinsic and Non-Intrinsic Cancer Risk Factors." Nature Communications.
- Yarden, Yosef, and Mark X. Sliwkowski. 2001. "Untangling the ErbB Signalling Network." Nature Reviews Molecular Cell Biology.
- Zitnan, D., and M. E. Adams. 2012. "Neuroendocrine Regulation of Ecdysis." In Insect Endocrinology.

## **7. Eidesstattliche Erklärung**

Hiermit erkläre ich, Lijo Jose Vattoly, dass diese Dissertation mit dem Titel: „Targeted expression of the Epidermal growth factor receptor in the respiratory system of *Drosophila melanogaster* to develop a lung cancer model“, sowie die darin beschriebenen Arbeiten, abgesehen von der Beratung durch meinen akademischen Betreuer, nach Inhalt und Form meine eigene Arbeit sind. Zudem versichere ich, dass die Arbeit noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegt wurde, dass sie noch nicht veröffentlicht ist und ich eine Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Sie wurde nach den Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft angefertigt.

Heide, 25.03.2021

---

Lijo Jose Vattoly



## 8. Acknowledgment

First and foremost, I am extremely indebted to Prof. Dr Thomas Roeder for making this interesting thesis possible at his lab and for his thorough eye, scientific support and supervision throughout my work. I sincerely appreciate his patience and positive attitude throughout my work. It was a new but great experience for me to work with *Drosophila melanogaster*.

I owe a huge debt of gratitude to Reshmi who was always there to comment on my views and help me understand and enrich my ideas. I want to express my special thanks to all members of the Molecular Physiology department for a pleasant working environment. I also extend my sincere thanks to Dr Christine Fink for her supervision and organization of the lab. I am very much grateful to Judith Bossen for her collegial and helping mentality. Many thanks go to Jakob and Stella, it has been a pleasure to work alongside you. Special thanks go to the technical assistants Britta Laubenstein and Christiane Sandberg for the lab maintenance that helped in the smooth running of our lab. Thanks also to Heidi and Heidrun.

My heartfelt thanks to my dear friends Jerrin Antony, Jesny Antony, Julia Otto and Dr Lisanne Matuschka for all the support and helping hand offered.

Most importantly, I would like to express my deep gratitude and love to my parents, siblings and other family members, without their love and support I would not be the person I am today.

Above all, I would like to thank my wife Alina for her love, unwavering belief and constant support, for all the late nights and early mornings, and for being there for me in my ups and downs. Thank you for being my muse, guide, editor, and proof-reader. But most of all, thank you for being my best friend. Thank you, my dear little son, Adam, for being so cute, naughty and co-operative. Dada loves you a lot!!

Last but not least I would fail in all aspects if I do not express my gratitude to God almighty, for the wisdom and perseverance that he has bestowed upon me throughout my life.

THANK YOU